Funktionen der alternsrelevanten Proteine
Thioredoxin-1 und Telomerase Reverse Transkriptase in umweltinduzierten Prozessen

Inaugural-Dissertation

der Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

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Düsseldorf, Mai 2010
aus dem Leibniz-Institut für umweltmedizinische Forschung
an der Heinrich-Heine Universität Düsseldorf

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

Referent: PD. Dr. Judith Haendeler
Koreferent: Prof. Dr. William Martin

Tag der mündlichen Prüfung:
Erklärung

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(Nicole Büchner)

Düsseldorf, 26. Mai 2010
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1. Altern und Alternstheorien


Eine einheitliche Theorie des Alterns gibt es nicht. Die verschiedenen Alternstheorien, die stetig erweitert und verändert werden, stammen aus unterschiedlichen Forschungsbereichen und verstehen sich nicht als Gegenmodelle, sondern als unterschiedliche Erklärungsansätze, die jeweils einen Aspekt des Alterns besonders betonen.


Zellzyklusarrest und damit zu replikativer Seneszenz (Hayflick und Moorhead 1961; Vaziri et al. 1993). Die Telomere sind demzufolge zentrale Regulatoren des Alternsprozesses.


Ein Großteil der freien Radikale bilden die reaktiven Sauerstoffspezies (ROS), die vorwiegend in Geweben mit hohem Sauerstoffumsatz, wie zum Beispiel Gehirn, Herz, Muskel und Leber gebildet werden. Zu den ROS zählen das Superoxidanion (O$_2^-$), das Hydroxylradikal (OH) und Wasserstoffperoxid (H$_2$O$_2$). Außerdem reagiert das O$_2^-$-Radikal direkt mit Stickstoffmonoxid (NO), wodurch Peroxynitrit (ONOO$^-$) gebildet wird, welches Zellmembranen leicht passieren kann. Durch die Bildung von Peroxynitrit, kommt es zusätzlich zum oxidativen Stress auch zu nitrosativem Stress, der ebenfalls zu zellschädigenden Modifikationen von Makromolekülen führt.

ROS haben verschiedene Ursprünge in der Zelle. Die wichtigsten endogenen ROS Quellen sind die NADPH Oxidasen, die Mitochondrien und die entkoppelte NO-Synthase (NOS), die mit vorschreitendem Alter statt NO verstärkt O$_2^-$ produziert (Higashi et al. 2006). Im Allgemeinen sind


Die antioxidativen Systeme unterscheiden sich in den einzelnen Zellkompartimenten. Im Zytosol stehen die Kupfer-Zink-Superoxiddismutase (SOD 1), die Katalase, Glutathionperoxidasen, Glutaredoxin und das Thioredoxinsystem im Vordergrund, während im Nukleus besonders Trx-1 und die APEX nuklease 1 (apurinic/apyrimidinic endonuclease 1) hervorzuheben sind (Lukosz, ... , Bächinger et al.). In den Mitochondrien können die O$_2$^{-}-Radikale durch die Mangan-Superoxiddismutase (SOD 2) zu H$_2$O$_2$ dismutiert werden. Zudem verfügen Mitochondrien über ein eigenes Thioredoxinsystem, das aus Thioredoxin-2 und der zugehörigen Thioredoxin-2-Reduktase (TR2) besteht. Das aus der Dismutation entstandene H$_2$O$_2$ wird von Glutathionperoxidasen oder vom Peroxiredoxin 3/Thioredoxin-2-System zu H$_2$O reduziert (Thomas et al. 2008).

Lange Zeit glaubte man dass die Bildung von ROS generell schädlich für die Zelle ist. Heute weiß man, dass ROS in der Zelle für den Organismus lebensnotwendig sind und eine wichtige Rolle bei Signaltransduktionsprozessen in der Zelle spielen (Ristow und Zarse 2010). So ist eine Vielzahl von Proteinen bekannt, deren Funktionen redoxreguliert sind (Gutierrez et al. 2006; Lopez-Mirabal und Winther 2008; Lukosz, ..., Bächinger et al. 2010).


zu einer erhöhten Produktion von ROS und einer metabolischen Ineffizienz, was allgemein als mitochondriale Dysfunktion beschrieben wird (Allen et al. 1999; Hutter et al. 2002; Zwierschke et al. 2003; Hutter et al. 2004; Passos et al. 2007).


2. Hautalterung


Veränderungen in der Extrazellulären Matrix, die hauptsächlich aus Kollagen I\textalpha\textsubscript{1} (Kol I\textalpha\textsubscript{1}) besteht (Jenkins 2002). Im Vergleich zu junger Haut wurden in gealterter Haut vermindertes Kol I\textalpha\textsubscript{1}, erhöhtes Elastin und höhere Level des Kollagens abbauenden Enzyms Matrixmetalloproteinase-1 (MMP-1) gezeigt (Jenkins 2002). Vorrangige Aufgabe der Matrixmetalloproteinase (MMPs) ist es, dermale Matrixproteine proteolytisch zu degradieren, die Hauptsubstrate der MMPs sind Strukturproteine der Dermis, wie Kollagene und Gelatine. Dieser Abbau führt letztendlich zu den klinisch und histologisch sichtbaren Veränderungen, die charakteristisch für gealterte Haut sind.


Von UV-Strahlung ist bekannt, dass sie mehrere biologische Effekte über unterschiedliche Mechanismen erzeugt, in denen ROS eine wichtige Rolle spielen. Sowohl für UVA als auch für UVB wurde gezeigt, dass sie zu oxidativen Schäden und zur ROS Produktion führt (Herrling et al. 2002; Herrling et al. 2003). Im allgemeinen beinhaltet die Wirkung von UV-Strahlung folgende Mechanismen (Gonzalez und Pathak 1996): (a) direkte Interaktion von Photonen mit Zielmolekülen wie DNA, (b) Generierung von reaktiven freien Radikalen und/oder ROS und (c) Induktion von proinflammatorischen Molekülen, welche die physiologische Produktion von ROS induzieren. Für IRA zeigte sich ebenfalls eine Erhöhung von intrazellulären ROS.

Für alle drei Strahlungsarten (UVA, UVB, IRA) wurde nachgewiesen, dass sie die Expression und Aktivität von MMP-1 steigern, und es dadurch zu einem Ungleichgewicht zwischen Kollagenneubildung und Kollagenabbau kommt, was Veränderungen der extrazellulären Matrix nach sich zieht. (Krutmann 2003).

2.1 Effekte von Thioredoxin-1 bei strahlungsinduzierter Hautalterung

Dermale Fibroblasten können vor den negativen Effekten von UVA, UVB und IRA durch nicht-enzymatische und enzymatische Antioxidantien geschützt werden (Yan et al. 2005; Russo und Halliday 2006; Schroeder et al. 2007). Es war daher von Interesse, ob auch Trx-1 in der Lage ist, die Haut vor Lichtalterung zu schützen. Zu diesem Zweck habe ich den Einfluss von Trx-1 auf
strahlungsinduzierte Veränderungen der Expression von Kollagen Iα1 (Kol Iα1) und MMP-1 sowie die zugrunde liegenden zellulären Signalwege untersucht (Büchner et al. 2008).

Eine Bestrahlung der Fibroblasten mit UVA, UVB und IRA führte, wie erwartet zu einer Erhöhung der MMP-1 RNA- und Proteinlevel. Überraschenderweise inhibierte Trx-1 nur die durch UVA nicht aber die durch UVB induzierte Expression von MMP-1. Der Befund, dass Trx-1 der UVA-induzierten Heraufregulation der MMP-1 Expression entgegenwirkt, deckt sich mit Untersuchungen von Yan et al., in denen gezeigt wurde, dass das Antioxidans Tempol den gleichen Effekt hervorruft (Yan, Hong et al. 2005). Alle drei Strahlungsarten reduzierten die Kol Iα1 RNA Expression signifikant. Interessanterweise wirkte die Inkubation der dermalen Fibroblasten mit Trx-1 den durch UVB und IRA induzierten negativen Effekten auf die Kol Iα1 Expression entgegen, während sich kein Einfluss auf die UVA-Regulation zeigte (Abbildung 1). Diese Befunde weisen eindeutig darauf hin, dass die Regulation beider Gene durch die verschiedenen spektralen Anteile des Sonnenlichts über unterschiedliche Signalwege verläuft.

Es ist bekannt, dass die UVB induzierte Heraufregulation von MMP-1 in dermalen Fibroblasten von der Phosphoinositol-3-Kinase abhängig ist (Brenneisen et al. 2002). Im Gegensatz dazu wurde für Trx-1 gezeigt, dass es den Phosphoinositol-3 Kinase/Akt-Signalweg aktiviert (Haendeler et al. 2004). Daher ist es nicht verwunderlich, dass die exogene Gabe von Trx-1 die Steigerung der UVB induzierten MMP-1-mRNA-Expression nicht verhindern kann.

Es wurde bereits gezeigt, dass Trx-1 an die p50-Untereinheit des Transkriptionsfaktors NFκB binden und dessen DNA-Bindeaktivität erhöhen kann (Matthews, Wakasugi et al. 1992). Ebenso wurde gezeigt, dass UVA die Translokation einer anderen NFκB-Untereinheit, p65, in den Zellkern induziert.

Interessanterweise konnte Trx-1 die Heraufregulation von MMP-1 sowie die Herabregulation von Kol Iα1 durch IRA blockieren, was auf einen gemeinsamen Signalweg in der Regulation beider Gene hinweist. Es ist bekannt, dass die IRA induzierte MMP-1-Heraufregulation funktionell von der Bildung mitochondrialer ROS abhängt (Schroeder et al. 2007) und dass die Überexpression von Trx-1 die mitochondrialen ROS Bildung inhibiert (Zhou et al. 2007). Demzufolge kann man vermuten, dass die inhibitorischen Effekte von Trx-1, im Fall von IRA-regulierter Genaktivität, auf die antioxidative Kapazität von Trx-1 zurückzuführen ist. Es wäre deshalb denkbar Trx-1 in Kombination mit bereits genutzten Therapeutika, die andere Signalwege beeinflussen, als "anti-Photoaging-Cocktail" zu verwenden.

Es ist nicht erstaunlich, dass Trx-1 sich derart unterschiedlich auf die durch UVA, UVB und IRA induzierten Effekte, bezüglich der Regulation von MMP-1 und Kol Iα1, auswirkt. Dies liegt in der Multifunktionalität des Proteins begründet, welches sowohl als Oxidoreduktase fungiert, aber auch über seine Cysteine an Position 32 und 35 mit anderen Proteinen wechselwirken, und damit deren Funktion regulieren kann. Im Zytoplasma steht höchstwahrscheinlich die Oxidoreduktasefunktion im Vordergrund. Nach Translokation in den Zellkern ist wahrscheinlich die Reduktion von Transkriptionsfaktoren entscheidend, da viele Transkriptionsfaktoren nur an DNA binden und damit genregulatorisch wirken können wenn kritische Cysteinreste reduziert vorliegen (Lukosz, ..., Büchner et al. 2010). Zur Unterscheidung, ob die zytoplasmatische oder die nukleäre Funktion zur Verhinderung von strahlungsinduzierten Hautalterungsphänomenen notwendig ist, könnten spezifische Trx-1-Mutanten eingesetzt werden, welche die Translokation in den Zellkern durch Mutation mehrerer Lysinreste im Carboyterminus von Trx-1 verhindern (Schroeder et al. 2007).
3. Gefäßalterung

Gefäßalterung ist durch eine beeinträchtigte Endothelfunktion und eine Versteifung der Arterien charakterisiert (O’Rourke und Hashimoto 2007). Im Prozess der Gefäßalterung spielen unter anderem auch die durch oxidativen Stress induzierten Veränderungen von biologischen Molekülen eine Rolle, so dass sich die von Harman postulierte Alternstheorie der freien Radikale auch in der Entwicklung von altersassozierten Gefäßveränderungen wiederspiegelt.


Weitere Mechanismen, die als wesentlich für die Gefäßalterung diskutiert werden sind: (a) Eine gesteigerte Arginase I-Expression und Aktivität (Santhanam et al. 2007), die dazu führt, dass die Enzyme Arginase I und eNOS um das gleiche Substrat, L-Arginin kompetitiven und so die NO Bioverfügbarkeit weiter reduzieren (Kim et al. 2009). (b) Eine gestörte Redox-Balance, die sich in gealterten Gefäßen durch eine gesteigerte Aktivität der NADPH Oxidase, einer Anhäufung von dysfunktionalen Mitochondrien, sowie einer reduzierten Trx-1-Proteinmenge und Aktivität. Die Folge ist eine daraus folgende erhöhte Sensitivität der Endothelzellen gegenüber apoptotischen Prozessen (Hoffmann et al. 2001).


4. Regulation von TERT


TERT unter physiologischen Bedingungen vor dem Src-induzierten Kernexport. Für die Retention im Zellkern ist die katalytische Aktivität von Shp-2 notwendig, was darauf hindeutet, dass Shp-2 entweder das phosphorylierte Tyrosin 707 in TERT dephosphoryliert oder dessen Phosphorylierung durch Inaktivierung der Src-Kinasen Src oder Yes verhindert (Büchner et al. 2010). In Endothelzellen wird die Menge an Shp-2 sowie die Shp-2-Phosphataseaktivität durch oxidativen Stress verringert, was letztendlich zum Export von TERT aus dem Zellkern führt.


Zusammenfassend zeigen diese neuen Befunde, dass sowohl die mitochondriale TERT als auch die nukleäre TERT wesentliche Zellfunktionen aufweisen (Abbildung 2).

### Abb. 2: Kompartiment-spezifische Funktionen von TERT

TERT schützt die Telomere im Nukleus vor Degradation. Über einen TOM/TIM abhängigen Mechanismus wird TERT in die Mitochondrien importiert. Im Mitochondrium bindet TERT an mitochondriale DNA (mtDNA) und beeinflusst über Komplex I die Atmungskettenaktivität. Die einzelnen Atmungskettenkomplexe sind nach allgemeiner Konvention mit römischen Zahlen benannt.

Wie bereits erwähnt sind Mitochondrien extrem sensitiv gegenüber oxidativen Stress. Daher untersuchte ich auch, ob TERT einen Einfluss auf die Bildung mitochondrialer ROS hat. Die Herabregulation von TERT mittels shRNA führte zu einer Erhöhung der Menge an mitochondrialen

5. Regulation von mitochondrialer TERT

Nachdem zuvor gezeigt wurde, dass TERT wichtige Funktionen in der Aufrechterhaltung der Mitochondrienfunktion und dem Schutz der mtDNA vor exogenen Noxen hat und unter dem Gesichtspunkt, dass Mitochondrien sensitiv gegenüber oxidativem Stress sind, stellte sich die Frage, ob mitochondriale TERT durch oxidativen Stress reguliert wird und wenn dies der Fall wäre, wie diese Regulation erfolgt. Interessanterweise konnte ich in Mitochondrien aus HEK293 Zellen und aus primären humanen Endothelzellen genau die Proteine nachweisen, welche im Zellkern an der Regulation von TERT beteiligt sind, nämlich Akt und Src (Büchner et al. 2010). Unter oxidativem Stress konnte ich eine Inaktivierung der Kinase Akt, eine Aktivierung der Src-Kinase und eine Reduktion der Menge an mitochondrialen TERT-Protein feststellen, die hier allerdings nicht auf einem Export beruhen kann, da ein Export von Proteinen aus dem Mitochondrium nicht möglich ist. Daher muss die Abnahme an TERT auf einem Abbau des Proteins beruhen. Eine TERT-Mutante, die nicht mehr durch Src am Tyrosin 707 phosphorylierbar ist, war hiervon nicht betroffen (Abbildung 3).

Abb. 3: TERT Regulation im Mitochondrium. Im Mitochondrium liegen die Kinasen Src und Akt sowohl in der aktiven Form als auch in der inaktiven Form vor. Unter Normalbedingungen (A) liegt Src vermehrt in der inaktiven und Akt vermehrt in der aktiven Form vor. Das TERT Protein ist unter diesen Bedingungen nicht an Tyrosin 707 phosphoryliert. (B) Unter oxidativem Stress liegt Akt vermehrt in der inaktiven und Src überwiegend in der aktiven Form vor. Infolgedessen wird TERT an Tyrosin 707 phosphoryliert und abgebaut. TERT-Y707F symbolisiert eine Mutante, die von dieser Regulation nicht betroffen ist.

Diese Beobachtungen führen zu dem Schluss, dass in Mitochondrien die gleichen Mechanismen zur Regulation von TERT genutzt werden wie im Zellkern. Untermauert wird dies durch Experimente in unserer Arbeitsgruppe, in denen auch Shp-2 in Mitochondrien nachgewiesen wurde (persönliche Kommunikation S. Jakob)

Es wurde bereits gezeigt, dass der durch oxidativen Stress induzierte Export von TERT aus dem Zellkern zu einer Erhöhung der TERT-Menge im Mitochondrium führt (Ahmed et al. 2008), was im ersten Moment konträr zu den von mir erhobene Daten erscheint. Allerdings kann man aus der Summe

6. Effekt von TERT auf die durch körperliche Ertüchtigung reduzierten Alternsmerkmale in vivo


Die molekularen Mechanismen, welche der verlangsamen Gefäßalterung zugrundeliegen, sind jedoch noch weitgehend unbekannt. Bezugnehmend auf die protektiven Effekte von TERT und eNOS auf die Endothelzellalterung, wurden in Kooperation mit der Arbeitsgruppe von Prof. Dr. Laufs in Homburg/Saar, die Effekte von körperlicher Ertüchtigung in Abhängigkeit von TERT und eNOS untersucht. Zu diesem Zweck wurden TERT−/−- bzw. eNOS defiziente (eNOS−/−) Mäuse einem freiwilligen Lauftraining unterzogen. Im Anschluss an diese Form von körperlicher Ertüchtigung, wurden die vaskulären Veränderungen der TERT−/−-Mäuse im Vergleich zu den Wildtyp-Geschwistern analysiert (Werner, ... , Büchner et al. 2009). Wildtyp-Mäuse zeigten nach dem freiwilligen Lauftraining in der Aorta eine erhöhte Expression und Aktivität von TERT und eine Heraufregulation der telomerassozierten Proteine TRF2 (Telomeric Repeat Binding Factor 2) und Ku70 (70 kDa Untereinheit des Ku Proteins). Da replikative Seneszenz und Apoptose im Endothel miteinander assoziiert sind (Hoffmann et al. 2001), wurde in den Aorten ebenfalls die Expression von


7. Direkte Effekte von ultrafeinen Partikeln auf Endothelzellen


Jedoch sind bis heute die molekularen Vorgänge, die von nicht-toxischen-, in vivo-relevanten Konzentrationen ultrafeiner Partikel im Endothel ausgelöst werden, vollständig unbekannt. Daher wurde in unserer Arbeitsgruppe eine Pilotstudie in Endothelzellen gestartet, um aufzuklären, ob nicht-

Abb. 5.: Carbon black (CB) induziert zelluläre Seneszenz. (A) HUVECs reagieren auf CB uf-Behandlung mit einer erhöhten seneszenzassozierten (SA) β–Galaktosidaseaktivität. (B) Nach CB uf-Behandlung lässt sich eine erhöhte β–Galaktosidase-Proteinnenge im Immunoblot nachweisen. (C) CB uf wirkt sich als exogene Noxe negativ auf die Endothelfunktion aus. Eine Erhöhte ROS Menge, deformierte Mitochondrien und eine reduzierte TERT-Expression und Aktivität führen als Folge zu zellulärer Seneszenz.

8. Ausblick

In meinen Untersuchungen konnte ich zeigen, dass Trx-1 die strahlungsabhängige Regulation von MMP-1 und Kol \( \alpha \)1 über unterschiedliche Signalwege beeinflusst. Bei der Signaltransduktion spielen unterschiedliche Transkriptionsfaktoren aber möglicherweise auch die Oxidoreduktasefunktion von Trx-1 eine Rolle. Durch den Einsatz verschiedener Trx-1-Mutanten, die in unserem Labor erzeugt wurden, soll in weiteren Arbeiten untersucht werden, welche Trx-1-Funktion für die Regulation des jeweiligen strahlungsinduzierten Signalweg relevant ist. Die unterschiedlichen Trx-1-Mutanten sollen transient in Zellen exprimiert werden. Eine Applikation der veränderten Proteine in rekombinanter Form wird hierbei nicht in Betracht gezogen, da deren Herstellung relativ aufwändig wäre. Um die Rolle der anti-oxidativen Kapazität von Trx-1 zu untersuchen, stehen Trx-1-Mutanten zur Verfügung in denen zum einen beide redoxaktiven Cysteine 32 und 35 zu Serin mutiert sind oder zum anderen nur eines der beiden Cysteine mutiert ist. In Endothelzellen wurde bereits gezeigt dass die Trx-1-Mutante in der beide redoxaktiven Cysteine mutiert sind, keine Oxidoreduktasefunktion mehr hat (Haendeler et al. 2002). Eine Interaktion von Trx-1 mit Transkriptionsfaktoren würde man im Zellkern


Abb. 7: Unterscheidung nukleärer und mitochondrialer Funktionen von TERT in vitro und in vivo. (A) Um TERT Funktionen mit subzellulären Lokalisationen des Proteins korrelieren zu können, sollen Zelllinien, die keine endogene TERT enthalten mit Transgenen für nukleäre bzw. mitochondriale TERT erzeugt werden. (B) Entsprechende Tiermodelle erhält man durch Kreuzung TERT defizienter Mäuse mit Tieren, die diese Transgene exprimierenin der F2-Generation, hier gezeigt für mitochondriale TERT.
TERT-Defizienz führt aufgrund der großen Länge von Telomeren in Maus-Laborstämmen erst nach mehreren Generationen zu einem durch Telomererosion hervorgerufenen Alterungsphänotyp, so dass dieser in den geschilderten Kreuzungen nicht auftritt. Mit Hilfe der so erhaltenen Mäuse können dann die spezifischen, telomerunabhängigen nukleären und mitochondrialen Funktionen von TERT im gesamten Tier und allen Organen untersucht werden.
9. Zusammenfassung


Es existiert eine Vielzahl von Schlüsselproteinen, die in Alternsprozessen eine wesentliche Rolle spielen. In dieser Arbeit sind zwei Proteine in den Vordergrund gestellt deren Wirkung sich in umweltinduzierten Prozessen bemerkbar macht. Das reoxaktive Protein Thioredoxin-1 (Trx-1) und die katalytische Untereinheit der Telomerase, die Telomerase Reverse Transkriptase (TERT).

Trx-1 ist ein 12 kDa großes, reoxaktives Protein, das sowohl in einem im antioxidativen Thioredoxinsystem eine Rolle spielt, als auch durch Interaktion mit anderen Proteinen, wie zum Beispiel Transkriptionsfaktoren, zelluläre Prozesse beeinflussen kann. Im Prozess der Lichtalterung kann Trx-1 durch Einflussnahme auf mehr als einen Signalweg dem Prozess des Kollagenabbaus in Folge von strahlungsinduzierter Lichtalterung, entgegenwirken.


Zusammen mit unseren Kooperationspartnern konnte ich in meinen Untersuchungen zudem zeigen, dass positive Effekte von körperlichem Training auf das kardiovaskuläre System, die Alternsprozessen entgegen wirken, über TERT vermittelt werden. In der hierbei beeinflussten Signalkaskade wirkt eine, durch körperliches Training erzielte, höhere Enzymaktivität der endothelialen Stickstoffoxidsynthase (eNOS) auf die Stickstoffmonoxid (NO)-Bioverfügbarkeit ein und bewirkt über einen TERT-
regulierten Prozess eine veränderte Expression von antiapoptotischen und zellzyklusregulatorischen Proteinen.


Zusammenfassend zeigen die Arbeiten dieser Doktorarbeit, dass sowohl die Mitochondrienfunktion als auch die nicht-mitochondriale Redoxregulation für das Verständnis der zellulären Alterungsprozesse wichtig sind. Im Hinblick auf eine „Healthy-Aging“-Therapie ist es daher von entscheidender Bedeutung bei einer pharmakologischen Intervention die zelluläre Kompartimentierung spezifischer Proteine, insbesondere von Trx-1 und TERT, zu berücksichtigen.
10. Summary

The basic mechanisms of aging have been explained by three main theories: the theory of telomere shortening, the oxidative stress theory and the mitochondrial theory of aging. The respective theories state that the telomeres of chromosomes, endogenously generated reactive oxygen species (ROS) or dysfunctional mitochondria play a critical role in the aging process. Telomere shortening below a critical length results in replicative senescence. A similar consequence has been proposed for the dysbalance of oxidative and antioxidative systems during aging leading to an increased ROS production. Impairment of mitochondrial function also results in elevated, harmful ROS levels.

Numerous molecules play key roles in the aging process. In this thesis two proteins have been highlighted, the impact of which is noticeable in environmentally induced processes: Thioredoxin-1 (Trx-1) and the catalytic subunit of telomerase, Telomerase Reverse Transcriptase (TERT). Trx-1 is a 12 kDa redox active protein, which can also modify cellular functions by direct protein-protein interactions, e.g. with transcription factors. In the process of photoaging Trx-1 has protective properties by influencing different signaling pathways involved in the light-induced disturbance of the collagen equilibrium of the extracellular matrix in the skin. Telomerase was long known to avert premature aging by preventing telomere shortening. Recent reports have revealed that its catalytic subunit TERT is also present in mitochondria. In my investigations I could demonstrate mitochondrial localization and new telomere independent functions of TERT. It protects mitochondrial DNA, improves respiratory chain function through complex I, counteracts mitochondrial ROS production and prevents apoptosis. Under conditions of oxidative stress mitochondrial TERT is regulated by the kinases Src and Akt, which determine the fate of nuclear TERT and are also present in the mitochondria.

Together with our cooperation partners I could show that the anti-aging effects of physical exercise on the cardiovascular system are mediated by TERT. Increased activity of the endothelial nitric oxide (NO) synthase and elevated NO-bioavailability result in a TERT-dependent inhibition of apoptotic and cell cycle regulatory processes.

In another line of experiments I could show that ultra fine carbon particles present in printer toner downregulate TERT activity and induce senescence in endothelial cells providing a potential explanation for the hazardous influence of nanoparticles on the cardiovascular system.

Taken together, my work demonstrates that mitochondrial functions and non-mitochondrial redox regulation have to be considered when trying to understand cellular aging. Therefore, any pharmacological intervention aimed at a "healthy-aging" therapy has to take into account the subcellular compartmentalization of specific proteins, in particular of Trx-1 and TERT.
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Changes of MMP-1 and collagen type Iα1 by UVA, UVB and IRA are differentially regulated by Trx-1

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A R T I C L E   I N F O

Article history:
Received 8 January 2008
Received in revised form 3 April 2008
Accepted 14 April 2008
Available online 22 April 2008

Keywords:
Aging
Thioredoxin-1
UV light
Infrared
Metalloproteinase-1
Collagen

A B S T R A C T

Exposure of human skin to solar radiation, which includes ultraviolet (UV) radiation (UVA and UVB) visible light and infrared radiation, induces skin aging. The effects of light have been attributed to irradiation-induced reactive oxygen species (ROS) formation, but the specific signaling pathways are not well understood. Detrimental effects of solar radiation are dermal diseases and photoaging. Exposure of cultured human dermal fibroblasts to UVA, UVB or IRA increased ROS formation in vitro. One important redox regulator is the oxidoreductase thioredoxin-1 (Trx). Trx is ubiquitously expressed and has anti-oxidative and anti-apoptotic properties. Besides its function to reduce H₂O₂, Trx binds to and regulates transcription factors. The aim of this study was to investigate whether Trx influences the regulation of MMP-1 and collagen Iα1 by UVA, UVB and IRA. We irradiated human dermal fibroblasts with UVA, UVB and IRA. UVA, UVB and IRA upregulated MMP-1 expression. Trx inhibited UVA-induced MMP-1 upregulation in a B dependent manner. UVA, UVB and IRA reduced collagen Iα1 expression. Incubation with Trx inhibited the effects of UVB and IRA on collagen Iα1 expression. In conclusion, MMP-1 and collagen Iα1, which play important roles in aging processes, seems to be regulated by different transcriptional mechanisms and Trx can only influence distinct signaling pathways induced by UVA, UVB and probably IRA. Thus, Trx may serve as an important contributor to an “anti-aging therapeutic cocktail”.

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1. Introduction

The skin acts as a physiological barrier protecting the organism against pathogens and chemical or physical damage. Aside from this important function, the skin shows age spots or wrinkles and signs of photoaging at the sun-exposed sites. It has become apparent that chronic sun exposure not only accelerates the formation of age spots and wrinkles, but also has various effects on skin function. Photoaging as a result of chronic sun exposure is characterized by dermal connective tissue changes, which give the skin a yellowish hue and leathery consistency. Histopathological studies of photodamaged skin have revealed reduced amounts of collagen and increased expression and activity of matrix metalloproteinases (MMPs). Further studies on the degradation system of collagen have shown that aging and irradiation accelerate the degradation of extracellular matrix, demonstrating a decrease in dermal collagen and an increase in the matrix metalloproteinase-1 (MMP-1) expression level, which cleaves interstitial collagen, associated with aging (Fligiel et al., 2003; Jenkins, 2002). One major signaling pathway, which contributes to photoaging, is the formation of reactive oxygen species (ROS) (Jenkins, 2002). UV (UVA and UVB) irradiation and infrared A (IRA) induce ROS formation in dermal fibroblasts (Russo and Halliday, 2006; Schroeder et al., 2007a). Dermal fibroblasts can be protected against the adverse effects of UVA, UVB and IRA irradiation by a number of both enzymatic and non-enzymatic anti-oxidants (Yan et al., 2005; Russo and Halliday, 2006; Schroeder et al., 2007a). The endogenous anti-oxidant capacity of the skin is a major determinant in its response to oxidative stress-mediated damage. Thus, anti-oxidants constitute an important group probably capable of preventing the occurrence and reducing the severity of irradiation-induced skin diseases and photoaging. One of the molecules, which is highly regulated by changes of the redox status in cells, is the oxidoreductase thioredoxin-1 (Trx). Trx is a 12-kDa protein, which is ubiquitously expressed in mammalian cells (Holmgren, 1989) and exerts its enzymatic activity as an oxidoreductase via cysteines 32 and 35 in the active site (Holmgren, 1989; Martin, 1995). Besides its well described function as an oxidoreductase, Trx exerts several other functions. By binding to different proteins, it modulates their function: Inhibition of binding to the apoptosis signaling kinase 1 and to the transcription factors AP1, NFκB and IRF1 and NFκB and IRF1 modulates the ability of Trx to regulate cellular functions (Liu et al., 2000; Tanaka...
et al., 2001; Malik et al., 2006; Schroeder et al., 2007b). Therefore, we investigated the effects of Trx on UVA, UVB and IRA irradiation induced changes in MMP-1 and collagen I expression.

2. Materials and methods

2.1. Cell culture

Human dermal fibroblasts were isolated from foreskin of three different donors. Cells were cultivated in Eagle’s minimal essential medium with Earle’s salts (MEM, PAA Laboratories Pasching, Austria) supplemented with 10% fetal bovine serum (Gibco, Karlsruhe, Germany), 1% anti-biotics/anti-mycotics (penicillin, streptomycin, and amphotericin B), 1% glucose and were cultivated on 100 mm plastic culture dishes (Greiner, Solingen, Germany) at 37 °C in humidified air with 5% CO₂. Cells were used between passages 5 and 10, grown to 100% confluence before treatment. Media was changed to serum-free MEM 24 h before irradiation with UVA, UVB and IRA respectively.

2.2. Irradiation

For irradiations, medium was replaced by phosphate buffered saline (PBS), lids were removed, and cells were exposed to radiation from a Hydrosun 500 H IRA device (Hydrosun Medizintechnik GmbH, Müllheim, Germany), or a UVA SUN 5000 Biomed UV1 metal halogenide irradiation device, or a bank of four Philips TL20W/12RS UVB fluorescent bulbs (Philips, Hamburg, Germany). The IRA device was water-filtered and equipped with a black filter and emits wavelengths between 760 and 1400 nm leading to an irradiance of 360 mW/cm² at a distance of 20 cm measured through Hydrosun HBM1 irradiance measuring device (Hydrosun). The TL20W/12RS lamp emits most of its energy in the UVB range (290–320 nm) with an emission peak at 310 nm. The UV1 output was determined with a UV-Dosimeter (290–320 nm) with an emission peak at 310 nm. The UVA1 output was measured with a UV-Dosimeter (290–320 nm) with an emission peak at 310 nm. The UVA1 lamp was set to deliver a dose of 33 J/m² UVB (represents a duration of 15 min). The IRA output was measured with a UV-Dosimeter (290–320 nm) with an emission peak at 310 nm. The IRA lamp was set to deliver a dose of 100 J/m² UVB (represents a duration of 15 min).

2.3. Real-time PCR measurements of relative mRNA levels

RNA was extracted using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instruction. The gene-specific mRNA expression of the isolated total RNA was determined by semi-quantitative differential real-time PCR. The RNA concentration was determined via photometric measurement at 260 nm. Aliquots of total RNA (100 ng) were applied for cDNA-Synthesis using the Superscript™ III First-Strand synthesis system for RT-PCR (Invitrogen, Karlsruhe, Germany). The PCR reactions were carried out on an ABI Prism 7000 (Applied Biosystems, Foster City, CA, USA) using SYBR® Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany). For relative expression comparison between control cells and treated cells the 2-ΔΔCT method was used, utilizing the18S RNA as housekeeping gene. Three independent experiments were performed for each of three different dermal fibroblast cells with duplicate determinations each and the mean value of these was calculated. Primer was designed with the help of the online-software Primer3. The following primer pairs were used (5’–3’): 18S RNA: GCC CTT AGA GGT GAA ATT CTT G; CAT TCT TGGC AAA TGC TTT CG; MMP-1: CAT GAA AGG TGG ACC AAC AAT TT; CCA AGA GAA TGG CCG AGT TC Col1a1: CGC TAC TAC CGG GCT GAT GAT; GTC TTT GGG TCC TTC CTT ATG TA.

2.4. Immunoblot

Proteins were isolated after treatment and subjected to SDS-PAGE sample buffer and resolved on an 8% or 10% SDS–PAGE. Immunoblotting was performed with antibodies directed against MMP-1 (1:1000, overnight 4 °C, The binding site), collagen type I 1 (1:200, overnight 4 °C, Santa Cruz), actin (1:50,000, overnight 4 °C, Sigma), tubulin (1:50,000, Dianova) and GAPDH (1:50,000, overnight 4 °C, Abcam). Antibodies were detected by the enhanced chemiluminescence system (Amersham).

2.5. Statistical analysis

Results are given as means ± standard error of the mean. Results were compared using a Mann–Whitney rank-sum test. A significance level of p < 0.05 was considered to be statistically significant.

3. Results

3.1. Effects of Trx on UVA, UVB and IRA induced MMP-1 mRNA

UVA, UVB and IRA all penetrate into the skin. In order to investigate the effects of UVA, UVB and IRA on MMP-1 expression, we irradiated dermal fibroblasts with 30J/cm² of UVA, 33J/cm² of UVB and 240J/cm² of IRA. These doses are physiologically relevant and can penetrate into the skin after sun exposure. All three doses did not induce cell death (data not shown). Twenty-four hours after irradiation, MMP-1 expression was measured by real-time PCR. UVA, UVB and IRA increased MMP-1 expression (Fig. 1A–C). It has previously been demonstrated that upregulation of MMP-1 expression by UV and IRA irradiation is dependent on ROS formation (Schroeder et al., 2007a; Yan et al., 2005). One important intracellular anti-oxidant is the protein Trx. Inhibition of Trx protein leads to dramatic cell death (Haendeler et al., 2004a) and Trx deficient mice are embryonic lethal (Matsui et al., 1996), demonstrating the absolute requirement of Trx for the organism. However, the effects of Trx in dermal fibroblasts have never been investigated. Therefore, we incubated dermal fibroblasts after UVA, UVB and IRA irradiation with 10 ng/ml Trx for 24 h. Surprisingly, Trx inhibited the upregulation of MMP-1 induced by UVA, but not by UVB (Fig. 1A and B). It has to be noted that the dose of IRA investigated here showed only a slight, but significant increase in MMP-1 expression (n = 6). Due to the huge interindividual differences of the donors, we did not observe a statistically significant effect of Trx on MMP-1 regulation after IRA exposure probably due to the minor effects observed (Fig. 1C).

3.2. Effects of Trx on collagen type 1 mRNA

Col1a1 is the most abundant protein in skin connective tissue, which also contains other types of collagen (III, V and VII), elastin, proteoglycans, fibronectin, and other extracellular matrix proteins.
These extracellular matrix proteins are mainly synthesized by the dermal fibroblasts. Therefore, we investigated the expression of CollIα1 after UVA, UVB and IRA irradiation. UVA, UVB and IRA significantly reduced the expression of CollIα1 (Fig. 1D–F). Next, we analysed the effect of Trx on CollIα1. Unexpectedly, we found that Trx did not inhibit UVA induced reduction of CollIα1, but abrogated the effects of UVB on CollIα1 (Fig. 1D and E). Trx also inhibited the reduced CollIα1 expression in IRA irradiated samples (n = 6, p = 0.051, Fig. 1F).

3.3. Effects of Trx on MMP-1 and pro-CollIα1 protein levels after exposure to UVA, UVB and IRA

In order to confirm our data obtained by real-time PCR, we performed immunoblot analyses of MMP-1 and pro-CollIα1. As expected from the mRNA data, UVA, UVB and IRA also increased MMP-1 and decreased pro-CollIα1 protein levels (Fig. 2A–C). Incubation with Trx starting from 1 ng/ml to 10 ng/ml inhibited UVA induced upregulation (UVA vs. UVA + 10 ng/ml Trx, p < 0.05, Fig. 2A). However, reduction of pro-CollIα1 protein levels by UVA was not blocked by all Trx concentrations (Fig. 2A). In contrast, Trx blunted UVB-induced pro-CollIα1 downregulation starting from 0.1 ng/ml, whereas MMP-1 upregulation was not affected (Fig. 2B). Finally, Trx inhibited IRA induced upregulation of MMP-1 and downregulation of CollIα1 (Fig. 2C).

3.4. Blockade of NFκB abrogated the protective effect of Trx on UVA induced MMP-1 upregulation

In order to get insight in one of the mechanisms how Trx regulates MMP-1, we investigated the involvement of NFκB. It has previously been demonstrated that Trx binds to and thereby enhances DNA binding of the p50 subunit of NFκB and that UVA induces nuclear translocation of the p65 subunit of NFκB (Matthews et al., 1992; Wenk et al., 2004). Thus, Trx may alter the composition of the NFκB complex. Therefore, we incubated dermal fibroblasts with Trx in presence of the well established NFκB inhibitor PDTC. Indeed, incubation with PDTC abrogated the effects of Trx on UVA induced upregulation of MMP-1 (Fig. 3). In contrast, blockade of NFκB did not change pro-CollIα1...
protein levels, indicating that regulation of collagen synthesis is not dependent on NFκB.

4. Discussion

The findings of our present study demonstrate that the expression of two major players in the process of photoaging, namely MMP-1 and ColIα1, are regulated by UVA, UVB and IRA. Trx inhibited the UVA induced expression of MMP-1, but not of UVB. In contrast, Trx abrogated the reduction of ColIα1 mRNA by UVB, but not by UVA. Moreover, Trx affected the IRA induced effects on ColIα1 expression (p = 0.051).

Skin aging manifests as wrinkles, diminished structural integrity, and impaired wound healing, due to alterations in the extracellular matrix formed predominantly of ColIα1 (for review see Jenkins, 2002). Decreased structural ColIα1, increased elastin and higher levels of MMP-1, which degrades collagen, have been demonstrated in aged skin relative to young skin (Jenkins, 2002). Several applications have been shown to inhibit wrinkle formation by restoration of collagen formation, e.g. retinoic acid (Griffiths et al., 1993). The dermal fibroblasts investigated in our study are the predominant cells responsible for the alterations in extracellular matrix. Trx inhibited UVA induced MMP-1 expression in our study. This is in accordance with data from Yan et al., who demonstrated that tempol, one of the nitroxides, reduced MMP-1 induction by UVA (Yan et al., 2005). Moreover, we found that the protective effects of Trx on UVA induced upregulation of MMP-1 depend on NFκB. It has previously been shown that Trx enhanced DNA binding of the p50 subunit of NFκB and that blockade of the thioredoxin reductase inhibited Trx NFκB binding and reduced expression of a number of anti-apoptotic genes (Matthews et al., 1992; Lan et al., 2007). In contrast, Wenk et al. demonstrated that nuclear translocation of the p65 subunit occurred when dermal fibroblasts were exposed to UVA which consequently resulted in an increase of IL-6 production and MMP-1 upregulation (Wenk et al., 2004). Therefore, we hypothesized that enhanced binding of Trx to the p50 subunit of NFκB could change subunit composition of the NFκB complex, which may result in activation of a different subset of NFκB target genes. However, Trx did not influence reduction of ColIα1 by UVA, suggesting that ColIα1 expression is not regulated by NFκB. This is in accordance with our data presented here and with findings by Buttner et al., demonstrating that transcriptional regulation of ColIα1 was independent of NFκB in fibroblasts (Buttner et al., 2004). Thus, one may speculate that UVA induced regulation of MMP-1 and NFκB occurred via different signaling pathways and that Trx only influenced the NFκB signaling.

Furthermore, we found that Trx prevented UVB induced reduction of ColIα1 expression, but did not influence upregulation of MMP-1 expression by UVB. Thus, similar as for UVB separate pathways exist, which are regulated by UVB, but differentially influenced by Trx. Brenneisen et al. demonstrated that the UVB induced increase in MMP-1 is completely inhibited by LY294002, a specific inhibitor of PI3K, in dermal fibroblasts (Brenneisen et al., 2002). We have previously been shown that Trx increased PI3K/Akt pathway activity in endothelial cells (Haendeler et al.,...
The upregulation of MMP-1 and downregulation of ColI strongly influence both MMP-1 and ColI expression. Moreover, Trx can influence other pathways that regulate either MMP-1 or ColI expression.

In conclusion, our study demonstrates that MMP-1 and ColI are at least in part regulated by different signaling pathways and that UVB and UVA induce several pathways, which regulate either the expression of MMP-1 or ColI. With respect to IRA, Trx can influence both MMP-1 and ColI expression. Moreover, Trx can influence distinct signaling pathways induced by UVA and UVB. In the case of UVA induced MMP-1 expression, Trx seems to alter NFkappaB signaling. Thus, Trx in combination with other therapeutics, which affect other pathways, may serve as an anti-photoaging “cocktail”.

Acknowledgements

This work was supported by a grant from the Deutsche Forschungsgemeinschaft to J.H. (HA2868/3-2), by the Collaborative Research Center SFB 728 projects B4 (to J.K.) and B5 (to J.H.). C.C. and T.M. are graduate students supported by the Graduiertenkolleg 1033. We thank Diane Schmiegel and Maren Schneider for expert technical assistance. The authors have nothing to disclose.

References


Nuclear Protein Tyrosine Phosphatase Shp-2 Is One Important Negative Regulator of Nuclear Export of Telomerase Reverse Transcriptase


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Nuclear Protein Tyrosine Phosphatase Shp-2 Is One Important Negative Regulator of Nuclear Export of Telomerase Reverse Transcriptase*[^5]

Received for publication, July 7, 2008, and in revised form, September 11, 2008. Published, JBC Papers in Press, October 1, 2008, DOI 10.1074/jbc.M805138200

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Aging is one major risk factor for numerous diseases. The enzyme telomerase reverse transcriptase (TERT) plays an important role for aging and apoptosis. Previously, we demonstrated that inhibition of oxidative stress-induced Src kinase family-dependent nuclear export of TERT results in delayed replicative senescence and reduced apoptosis sensitivity. Therefore, the aim of this study was to investigate mechanisms inhibiting nuclear export of TERT. First, we demonstrated that H2O2-induced nuclear export of TERT was abolished in Src, Fyn, and Yes-deficient embryonic fibroblasts. Next, we wanted to identify one potential negative regulator of this export process. One candidate is the protein tyrosine phosphatase Shp-2 (Shp-2), which can counteract activities of the Src kinase family. Indeed, Shp-2 was evenly distributed between the nucleus and cytosol. Nuclear Shp-2 associates with TERT in endothelial cells and dissociates from TERT prior to its nuclear export. Overexpression of Shp-2 wt inhibited H2O2-induced export of TERT. Overexpression of the catalytically inactive, dominant negative Shp-2 mutant (Shp-2(C459S)) reduced endogenous as well as overexpressed nuclear TERT protein and telomerase activity, whereas it had no influence on TERT(Y707F). Binding of TERT(Y707F) to Shp-2 is reduced compared with TERTwt. Ablation of Shp-2 expression led only to an increased tyrosine phosphorylation of TERTwt, but not of TERT(Y707F). Moreover, reduced Shp-2 expression decreased nuclear telomerase activity, whereas nuclear telomerase activity was increased in Shp-2-overexpressing endothelial cells. In conclusion, Shp-2 retains TERT in the nucleus by regulating tyrosine 707 phosphorylation.

Telomeres are the physical ends of the chromosomes. They maintain chromosome stability, genetic integrity and cell viability in a variety of different species (1, 2). Telomeres can also function as a mitotic clock, because telomeres are progressively shortened during each cell division. The enzyme telomerase, with its catalytic subunit telomerase reverse transcriptase (TERT),[^5] counteracts the shortening of telomeres. Introduction of TERT into human cells extended both their life-span and their telomeres to lengths typical of young cells (3–5). In addition to this well known function of TERT, functions independent of telomere shortening have been described. TERT has been shown to inhibit apoptosis (6, 7). Recently, it has been demonstrated that TERT is also localized in the mitochondria, but its function there is discussed controversially (8–10). TERT is regulated by transcriptional and post-translational mechanisms. Phosphorylation, binding proteins, and cellular localization have been described for post-translational modifications of TERT (11–15). TERT can be phosphorylated and its activity is regulated by kinases like c-Abl, PKC, ERK1/2, and Akt (16–20). We demonstrated that TERT is tyrosine-phosphorylated by the Src kinase family under conditions of oxidative stress (21, 22). Functionally, this results in nuclear export of TERT disrupting the anti-apoptotic and telomere-extending potential of the enzyme, which subsequently leads to enhanced apoptosis sensitivity and accelerated senescence of cells (21, 22).

One important regulator of the Src kinase family activity is the protein tyrosine phosphatase Shp-2 (Shp-2). The importance of Shp-2 for survival has been documented by the Shp-2 knockout mice which are embryonic lethal (23). All the described mechanisms for the regulatory mechanisms of Shp-2 are associated with its ability to dephosphorylate target molecules such as Src kinase family members, which are bound to growth factor receptors at the membrane to inhibit the permanent activation of the receptors by kinase phosphorylation (for review see Ref. 24). Recently, it has been demonstrated that Shp-2 is also localized in the nucleus, where it binds to the transcription factor STAT5a and thereby regulates its function (25). Because the regulation, which leads to nuclear export of TERT has to occur in the nucleus, we hypothesized that Shp-2 might counteract the nuclear export of TERT.

[^5]: The abbreviations used are: TERT, telomerase reverse transcriptase; Shp-2, protein tyrosine phosphatase Shp-2; EC, human umbilical venous endothelial cells; HEK, human embryonic kidney cells; MEF, mouse embryonic fibroblasts; wt, wild type; GFP, green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Nuclear Shp-2 Regulates TERT

Our data demonstrate that nuclear export of TERT is triggered by Src and/or Yes. One negative regulator of this export is Shp-2. Shp-2 is localized in the nucleus, is associated with TERT and dissociates from TERT prior to its export. The catalytic activity of Shp-2 is crucial for retaining TERT in the nucleus.

EXPERIMENTAL PROCEDURES

Cell Culture—Endothelial cells (EC) were cultured in endothelial basal medium supplemented with hydrocortisone (1 μg/ml), bovine brain extract (12 μg/ml), gentamycin (50 μg/ml), amphotericin B (50 ng/ml), epidermal growth factor (10 ng/ml), and 10% fetal calf serum. After detachment with trypsin, cells were grown for at least 18 h (26, 27). Human embryonic kidney cells (HEK) were cultured in DMEM basal medium with 10% heat-inactivated fetal calf serum. Mouse embryonic fibroblasts deficient of Src, Fyn, and Yes and their wild-type counterparts were cultured in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal calf serum and gentamycin.

Plasmids—Human Shp-2 was cloned out of endothelial cell-derived cDNA incorporating EcoRI and BamHI restriction sites. The amplified PCR product was subcloned into pcDNA3.1 (−) vector containing a Myc tag (Invitrogen) or into pGFP vector creating a Shp-2 GFP fusion. The catalytically inactive mutant of Shp-2 (Shp-2(C459S)) was generated by site-directed mutagenesis (Stratagene) out of Shp-2 wt. The hTERT construct was kindly donated by Dr. Weinberg (28). TERT was subcloned into pcDNA3.1 (−)Myc-His vector (TERTwt) containing the Myc tag at the C terminus. TERT(Y707F) was generated by site-directed mutagenesis. ShRNA vectors were obtained from Sigma (Mission shRNA series).

Transfection—HEK were transiently transfected with Lipofectamine/Plus according to the manufacturer’s protocol (Invitrogen) or into pGFP vector creating a Shp-2 GFP fusion. The catalytically inactive mutant of Shp-2 (Shp-2(C459S)) was generated by site-directed mutagenesis (Stratagene) out of Shp-2 wt. The hTERT construct was kindly donated by Dr. Weinberg (28). TERT was subcloned into pcDNA3.1 (−)Myc-His vector (TERTwt) containing the Myc tag at the C terminus. TERT(Y707F) was generated by site-directed mutagenesis. ShRNA vectors were obtained from Sigma (Mission shRNA series).

Telomerase Enzyme Activity Measurement—Telomerase enzyme activity was measured using a commercially available PCR-based assay according to the manufacturer’s protocol (Roche Applied Science) as previously described (29) with a transfection efficiency of 90 ± 4%. EC were transiently transfected with Superfect (Qiagen) as described previously. Mouse embryonic fibroblasts were transiently transfected with Lipofectamine/Plus according to the manufacturer’s protocol (Invitrogen). Transfection of siRNA was performed by using INTERFER™ ThunderTM according to the manufacturer’s protocol (POLYPLUS transfection). Sequence of scrambled siRNA: 5′-AACCUGAGAAGUCGCCUGAA-3′, sequence of Shp-2 siRNA: 5′-GAAGCAAGUCCAGAUUUU-3′.

Separation of Nuclear and Cytosolic Extracts—Nuclear and cytosolic extracts were separated using a commercially available kit according to the manufacturer’s protocol (Pierce). In brief, cells were scraped off the dish and centrifuged at 800 × g for 5 min at 4 °C. The resulting pellet was resuspended in cytosolic extraction reagent I (CERI buffer) and incubated for 10 min at 4 °C. After adding cytosolic extraction reagent II (CERII buffer) and further incubation for 1 min at 4 °C, samples were centrifuged at 16,000 × g for 5 min at 4 °C. The resulting supernatant contained the cytosolic fraction. The resulting pellet was washed with phosphate-buffered saline and resuspended in nuclear extraction reagent (NER buffer) and incubated for 60 min at 4 °C. After centrifugation for 15 min at 16,000 × g at 4 °C, the resulting supernatant was obtained as nuclear fraction. Purity of the nuclear and cytosolic extracts was always assured by immunoblotting with topoisomerase 1 (nuclear) and HSP70 (cytosolic).

Immunoprecipitation and Immunoblotting—Lysates (250 μg) were immunoprecipitated with 2.5 μg of Shp-2 antibody or 2.5 μg of Myc antibody overnight at 4 °C. After incubation with A- and G-Sepharose (Amersham Biosciences) for 2 h at 4 °C, resulting beads were washed, subjected to SDS-PAGE sample buffer, and run on a 10% SDS-PAGE.

Immunoblotting was performed with antibodies directed against TERT (1:200, overnight, 4 °C, Calbiochem or 1:500, overnight, 4 °C, Rockland), actin (1:8000, overnight 4 °C, Sigma) and Shp-2, Myc, Hsp70, Ref-1, topoisomerase 1 (2 h, 1:250, all Santa Cruz). Antibodies were detected by the enhanced chemiluminescence system (Amersham Biosciences). Semi-quantitative analyses were performed on scanned immunoblots using Scion Image 1.6 (Scion Corp.).

Immunostaining—Cells were fixed in 4% paraformaldehyde and permeabilized using 0.3% Triton X-100 and 3% bovine serum albumin in phosphate-buffered saline. For immunostaining, cells were incubated with an antibody against TERT and stained with an anti-rabbit Texas-Red-conjugated Fab fragment. Nuclei were counterstained with Sytox Blue or DAPI (1:1000, 5 min, Molecular Probes). Cells were visualized with confocal microscopy (Zeiss, LSM 510 META, magnification 1:40 oil).

Statistics—Statistical analysis was performed with Student’s t test or Wilcoxon, Mann-Whitney test using XLSTAT 2008.

RESULTS

Nuclear Export of TERT Depends on Src and Yes—Recently, we demonstrated that short term exposure to oxidative stress as well as aging-induced reactive oxygen species formation led to a Src kinase family-dependent nuclear export of TERT. Loss of nuclear TERT resulted in increased apoptosis sensitivity and accelerated senescence (21, 22). To reduce the number of candidates of the Src kinase family, which are responsible for oxidative stress-induced nuclear export of TERT, we used embryonic mouse fibroblasts deficient for Src, Fyn, and Yes and overexpressed TERTwt in these cells. Treatment with 500 μM H2O2 for 6 h resulted in a reduction of nuclear TERT protein and an increase in cytosolic TERT protein in wildtype fibroblasts. In contrast, in fibroblasts deficient for Src, Fyn, and Yes, nuclear export of...
Nuclear Shp-2 Regulates TERT

TERT was completely abolished (Fig. 1a) demonstrating that these three kinases play a role in this process.

The functional relevance of these kinases in EC is substantiated by the fact that Src and Yes could be detected in nuclear extracts of them (Fig. 1b). Having demonstrated that Src and/or Yes are responsible for oxidative stress induced nuclear export of TERT, we next wanted to identify one of the counter players, which inhibits nuclear export of TERT. One known inhibitor of Src kinase family functions is Shp-2. However, to inhibit nuclear export of TERT, we next wanted to identify one of the counter players, which led to a decrease of total Shp-2 protein and activity (Fig. 2, b and c).

Shp-2 Associates with TERT and Retains It in the Nucleus—To get first insights whether Shp-2 could be a direct player in the regulation of TERT, we next determined whether TERT and Shp-2 associate with each other in the nucleus. Therefore, we co-immunoprecipitated endogenous Shp-2 and TERT-myc from nuclear extracts of TERT-myc-transfected cells. Indeed, the majority of TERT is associated with Shp-2 in the nucleus (Fig. 3). This association seems to be specific as we did not find

If Shp-2 is involved in TERT nuclear export by oxidative stress, we hypothesized that Shp-2 itself must be regulated by H2O2 in EC. Indeed, incubation with 200 \mu M H2O2, which led to a nuclear export of TERT, resulted in a minor, but significant decrease of total Shp-2 protein and activity (Fig. 2, a and c).

FIGURE 1. Absence of Src, Fyn, and Yes completely abrogates nuclear export of TERT. a, Src, Fyn, Yes-dependent nuclear export of TERT. Embryonic fibroblasts from mice deficient for Src, Fyn, and Yes (SFY−/−) and from their wild-type counterparts (WT) were transfected with TERT-myc and incubated with 500 \mu M H2O2 for 6 h. Nuclear and cytosolic extracts were prepared as described under “Experimental Procedures” and used for immunoblotting; shown is a representative blot of three independent experiments. Upper panel, detection of TERT-myc with an anti-Myc antibody. The middle and lower panel show the purity of the nuclear and cytosolic extracts with the nuclear marker topoisomerase I and the cytosolic protein Hsp70. b, nuclear localization of Src and Yes. Nuclear and cytosolic extracts were prepared from EC and used for immunoblotting. The following proteins were detected with specific antibodies (from top to bottom): Src, Yes, Fyn, topoisomerase I (nuclear marker), and GAPDH (cytosolic marker).

FIGURE 2. Subcellular localization of Shp-2 and regulation by H2O2. a, endogenous and overexpressed Shp-2 are evenly distributed between nucleus and cytoplasm in EC. Shp-2 wt-myc was overexpressed in EC. Nuclear and cytosolic extracts were prepared as described under “Experimental Procedures” and used for immunoblotting. Upper panel shows an immunoblot with an anti-Shp-2 antibody, middle and lower panels demonstrate the purity of the extracts using antibodies against the nuclear marker topoisomerase I and the cytosolic marker GAPDH. One representative immunoblot is shown (n = 6). EV, empty vector; b, H2O2 reduces Shp-2 protein levels. EC were incubated with 200 \mu M H2O2 for 6 h and immunoblots were performed with an anti-Shp-2 antibody and anti-actin for normalization. Lower panel, semi-quantitative analysis of four independent experiments. Shown are the Shp-2 levels relative to actin, data are means ± S.E. (relative levels in untreated cells set to 1; *, p < 0.05). c, H2O2 reduces Shp-2 activity. EC were incubated with 200 \mu M H2O2 for 6 h, lysed, and Shp-2 activity was measured in the lysates. Data are means ± S.E. (n = 6) and are shown relative to Shp-2 activity in untreated cells (1, p < 0.05).
complexes between TERT-myc, Shp-2, and the transcription factor Ref-1 (supplemental Fig. S2). Having demonstrated that nuclear Shp-2 interacts with TERT, we next examined whether Shp-2 has an inhibitory effect on oxidative stress-induced nuclear export of TERT.

We overexpressed Shp-2 and TERT-myc in EC and incubated them for 6 h with 200 μM H₂O₂ to induce nuclear export of TERT. As shown in Fig. 4a, overexpression of Shp-2 completely abolished nuclear export of TERT. In line with this finding, overexpression of Shp-2 inhibited H₂O₂-induced reduction of endogenous, nuclear telomerase activity (Fig. 4b).

**Nuclear Retention of TERT Requires Catalytically Active Shp-2.** To determine whether the catalytic activity of Shp-2 is required for retaining TERT in the nucleus, we investigated the effects of the catalytically inactive, dominant negative Shp-2 mutant, Shp-2(C459S). First we verified that this mutant like the wild-type protein is evenly distributed between nucleus and cytoplasm (supplemental Fig. S3).

Overexpression of Shp-2(C459S) induced already under basal conditions reduction of nuclear TERT protein (Fig. 5a). In addition, we transfected EC with Shp-2 wt or Shp-2(C459S), incubated them with H₂O₂ and measured telomerase activity in
Nuclear Shp-2 Regulates TERT

Shp-2 wt increased, whereas Shp-2(C459S) reduced endogenous, nuclear telomerase activity under basal conditions. This effect seems to be specific for Shp-2, because overexpression of another phosphatase, namely the protein phosphatase 2a (PP2A) did not alter nuclear TERT protein and telomerase activity (supplemental Fig. S4). Shp-2 wt completely abrogated the effect of H₂O₂ on endogenous, nuclear telomerase activity, which is in accordance with our data presented in Fig. 4e. Interestingly Shp-2(C459S)-overexpressing cells showed a stronger effect on endogenous, nuclear telomerase activity compared with empty vector transfected cells under H₂O₂ treatment (Fig. 5b). These data clearly demonstrate that nuclear retention of TERT depends on the presence of Shp-2 protein and its activity. This is in accordance with the findings shown in Fig. 2 that H₂O₂ treatment reduces total Shp-2 protein and activity, providing one possible explanation for the H₂O₂-induced nuclear export of TERT.

We previously reported that TERT(Y707F) is retained in the nucleus under H₂O₂ treatment, suggesting that this tyrosine is crucial for nuclear export (21). The involvement of Shp-2 in this process suggests that phosphorylation/dephosphorylation regulates subcellular TERT localization. As expected, Shp-2 wt did not alter nuclear telomerase activity in TERT(Y707F)-overexpressing cells even after H₂O₂ treatment because this mutant cannot be phosphorylated at position 707 and thus does not require dephosphorylation for nuclear retention (Fig. 6a and Ref. 21). Moreover, under basal conditions, overexpression of dominant negative Shp-2(C459S) reduced nuclear telomerase activity in TERT-wt transfected cells, whereas it had no influence on TERT(Y707F) (Fig. 6b), suggesting that tyrosine 707 in TERT is the target for Shp-2 phosphate activity. Next, we investigated the influence of tyrosine 707 on the TERT Shp-2 association in the nucleus in the presence and absence of H₂O₂. Association of TERT(Y707F) to Shp-2 is markedly reduced compared with TERT-wt. This is also reflected in the supernatants of the immunoprecipitations where more unbound TERT(Y707F) is found (Fig. 7a). Finally, ablation of Shp-2 increased tyrosine phosphorylation of nuclear TERT-wt, whereas TERT(Y707F) was unaffected (Fig. 7b). Similarly, reduced Shp-2 expression decreased nuclear telomerase activity and TERT protein already under basal conditions (supplemental Fig. S5 and Fig. 7b).

DISCUSSION

The present study demonstrates that nuclear Shp-2 is associated with nuclear TERT and dissociates from TERT prior to its export, that Shp-2 inhibits nuclear export of TERT and that tyrosine 707 in TERT is a target for Shp-2. Shp-2 was identified as a cytosolic SH2 domain containing tyrosine phosphatase, which is ubiquitously expressed. The wide distribution of the enzyme indicates that it might regulate various physiological functions. Homozygous Shp-2 knockout mice are embryonic lethal underscoring the importance of the enzyme. It is clear that Shp-2 plays a critical role in regulating signal transduction; however, a profound biochemical basis for the different functions of this phosphatase remains to be elucidated. Our data now add another important issue to the list of...
Nuclear Shp-2 Regulates TERT

unaddressed questions: What is the function for nuclear Shp-2? Chughtai et al. (25) reported that Shp-2 exists in a complex with the transcription factor Stat5a. The authors speculate that active Shp-2 and a tyrosine-phosphorylated Stat5a translocate to the nucleus and potentially function in the nucleus to regulate transcription as both Shp-2 and Stat5a as a complex bind DNA. Our data now provide evidence that Shp-2 is localized in the nucleus already under basal conditions, where it is associated with TERT. Thus, it is tempting to speculate that some of the physiological functions of Shp-2 have to be addressed to its nuclear localization. The question remains how Shp-2 is imported into the nucleus, because it lacks a nuclear localization sequence. Therefore, a binding protein for Shp-2 could be required to induce its nuclear import. A possible candidate is Gab1, which has a nuclear import sequence (31) and has been shown to bind to Shp-2 (32). Preliminary data from our laboratory indicate that overexpression of a nuclear localization deficient Gab1 mutant prevents nuclear import of Shp-2.6

Under oxidative stress, TERT is tyrosine-phosphorylated and thereby exported from the nucleus in a Src kinase family/CRM1-dependent manner (21). In this study we demonstrate that Src and Yes are also localized in the nucleus of EC and thereby could be responsible for tyrosine phosphorylation of TERT. Moreover, the Src kinase family has been implicated in aging processes. This is mainly due to the fact that reactive oxygen species, which accumulate with age and induce lipid peroxidation, protein modification, DNA strand breaks, and cause oxidative damage, enhance the activity of the Src kinase family, which results in a progressive loss of cell function, a hallmark for aging processes (22, 33–35). We provide functional evidence that Src, Fyn, and Yes could play a role in aging processes, because cells deficient in these kinases can retain TERT in the nucleus under oxidative stress.

It has to be assumed that the export of nuclear TERT is regulated. Here, we demonstrate that Shp-2 is associated with TERT in the nucleus under basal conditions. More importantly, endogenous Shp-2 dissociates from nuclear TERT prior to its export and down-regulation of endogenous Shp-2 expression reduces nuclear telomerase activity. Thus, one may speculate that Shp-2 protects TERT from nuclear export by complex formation with TERT. Our data also suggest that tyrosine 707 in TERT is a target for the phosphatase activity of Shp-2. Therefore, Shp-2 might indeed be the unknown counterplayer for oxidative stress-induced nuclear export of TERT. However, we still do not know whether it acts solely via dephosphorylation. However, our data presented here strongly suggested that tyrosine 707 in TERT is a target of Shp-2 because the phosphorylation status of TERT(Y707F) is unaffected by Shp-2 and more importantly tyrosine phosphorylation of TERT-wt is negatively regulated by Shp-2. As a functional consequence nuclear Shp-2 could protect the cell either against accelerated senescence or increased apoptosis sensitivity or even both.

To our knowledge Shp-2, or more precise nuclear Shp-2, has not been implicated in aging processes until now. It has only been suggested that Shp-1 is responsible for aging-related attenuation of EGFR receptor signaling in dermal fibroblasts (36), which suggests that Shp-1 negatively contributes to aging processes. This is in line with findings that Shp-1 plays a negative role in transducing signals for cellular responses (24). Previous biochemical evidence has shown that the enzymatic activity of Shp-2 is required for its function in signal transduction (37, 38). Replacing cysteine 459 with serine completely abolished its enzymatic activity. Binding of this mutant to other proteins via its SH2 domains remained unaltered. However, introduction of this mutant markedly inhibited the activation of MAP kinases in response to EGF and insulin (37, 39). This is in line with our data presented here that Shp-2(C459S) has also a nuclear localization (data not shown), but reduced nuclear telomerase activity and TERT protein already under basal conditions.

In summary, our study demonstrate a new important function for nuclear Shp-2 in retaining TERT in the nucleus. In addition, tyrosine 707 is a target for Shp-2. It counteracts the Src and Yes effects on TERT protein and telomerase activity in the nucleus under conditions of oxidative stress, most likely by regulating the net phosphorylation status of tyrosine 707 in TERT. This suggests that keeping Shp-2 in the nucleus delays aging processes and inhibits apoptosis.

Acknowledgments—We thank Christine Goy, Barbara Wiegand, Kerstin Kunze, Jennifer Birke, and Diane Schmiegel for expert technical assistance.

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33160 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 283 • NUMBER 48 • NOVEMBER 28, 2008

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supplementary figure 1: Jakob et al.

Shp-2 localization in endothelial cells. Shp-2 wt-GFP was overexpressed in EC and detected by fluorescence microscopy. Left panel: nuclear staining with DAPI, middle panel: Shp-2 wt GFP, right panel: merge. Transfected cells show an even distribution of Shp-2 wt-GFP between nucleus and cytoplasm.
The nuclear transcription factor Ref-1 does not associate with TERT-myc and endogenous Shp-2. HEK cells were transfected with TERT-myc, lysed and the lysates used for immunoprecipitations (IP) with an anti myc-antibody. Immunoblots with the precipitates (left panels) and 25 µg of total protein (IB) (right panels) were performed with an anti-myc antibody (TERT-myc, upper panels), anti-Shp-2 (middle panels) and anti-Ref-1 (lower panels). IgG denotes the antibody used for immunoprecipitation.
Dominant negative Shp-2 reduces nuclear TERT protein. Endothelial cells were transfected with Shp-2(C459S)-GFP and analyzed by fluorescence microscopy. Endogenous TERT was detected by immunostaining using a Texas Red coupled secondary antibody. Top left panel: Shp-2(C459S)-GFP, top right panel: nuclear staining with Sytox Blue, bottom left panel: endogenous TERT, bottom right panel: merge. In Shp-2(C459S)-GFP transfected cells (white arrow) the nuclear staining of TERT is reduced in comparison to non-transfected cells.
PP2A did not alter endogenous nuclear TERT protein. HEK cells were transfected with empty vector (EV) or PP2A. Immunoblots of lysates from nuclear and cytosolic extracts were performed with an anti-TERT antibody (upper panel) and an anti-PP2A antibody (lower upper panel). Anti-topoisomerase I antibody (upper lower panel) was used as nuclear marker and anti-GAPDH antibody as cytosolic marker (lower panel).
Downregulation of Shp-2 reduces nuclear telomerase activity. HUVEC were transfected with Shp-2 siRNA (Shp-2) and scrambled siRNA (scr). Upper panel verifies downregulation of Shp-2. Middle panel shows equal loading using an anti-actin antibody. Nuclear telomerase activity was measured as described under experimental procedures. Data are means +/- SEM (n=3, *p< 0.05 vs scr, n=3) (lower panel).
Mitochondrial Telomerase Reverse Transcriptase Binds to and Protects Mitochondrial DNA and Function From Damage


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Mitochondrial Telomerase Reverse Transcriptase Binds to and Protects Mitochondrial DNA and Function From Damage

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Objective—The enzyme telomerase and its catalytic subunit the telomerase reverse transcriptase (TERT) are important for maintenance of telomere length in the nucleus. Recent studies provided evidence for a mitochondrial localization of TERT. Therefore, we investigated the exact localization of TERT within the mitochondria and its function.

Methods and Results—Here, we demonstrate that TERT is localized in the matrix of the mitochondria. TERT binds to mitochondrial DNA at the coding regions for ND1 and ND2. Binding of TERT to mitochondrial DNA protects against ethidium bromide–induced damage. TERT increases overall respiratory chain activity, which is most pronounced at complex I and dependent on the reverse transcriptase activity of the enzyme. Moreover, mitochondrial reactive oxygen species are increased after genetic ablation of TERT by shRNA. Mitochondrially targeted TERT and not wild-type TERT revealed the most prominent protective effect on H2O2-induced apoptosis. Lung fibroblasts from 6-month-old TERT+/− mice (F2 generation) showed increased sensitivity toward UVB radiation and heart mitochondria exhibited significantly reduced respiratory chain activity already under basal conditions, demonstrating the protective function of TERT in vivo.

Conclusion—Mitochondrial TERT exerts a novel protective function by binding to mitochondrial DNA, increasing respiratory chain activity and protecting against oxidative stress–induced damage. (Arterioscler Thromb Vasc Biol. 2009;29:929-935.)

Key Words: aging • apoptosis • mitochondrial functions • mitochondrial DNA • reactive oxygen species • telomerase reverse transcriptase

To date several theories exist to explain the phenomenon of normal and pathological aging. The free radical theory of aging proposes that reactive oxygen species (ROS) in biological systems attack molecules and thereby cause functional decline of organ systems that eventually leads to death. This damage accumulates over time and may contribute to diseases associated with aging like atherosclerosis, neurodegeneration, or cataracts. Recently, Schriner et al produced transgenic mice that overexpressed human catalase localized to the peroxisome, the nucleus, and the mitochondria. Only mice overexpressing mitochondrially targeted catalase showed a significant increase in life span and a reduction in oxidative damage to DNA and consequently in apoptosis. Thus, these data define the mitochondria as compartment of ROS formation, which contributes to aging processes. Further evidence supporting the importance of mitochondria and formation of ROS in the mitochondria comes from findings that overexpression of mitochondrially localized antioxidant enzymes lengthens lifespan of Drosophila and that deletion of manganese superoxide dismutase results in the age-related decline of mitochondrial function, culminating in increased apoptosis. Recent studies using isolated complex I of the respiratory chain clearly demonstrated that superoxide production into the mitochondrial matrix is predominantly dependent on flavine-mononucleotide within complex I. The enzyme telomerase counteracts the shortening of the physical ends of chromosomes and, thereby, prevents the onset of replicative senescence and genetic instability. The catalytic subunit, which antagonizes telomere-shortening, is the telomerase reverse transcriptase (TERT). Several studies suggested that TERT exerts functions independently of its net telomere lengthening (for review see ). TERT increased cell survival and resistance against oxidative stress after short term incubation of cells with different stimuli, which did not affect telomere shortening. TERT has been shown to have a genuine mitochondrial leader sequence which targets this protein to the mitochondria. However the exact role of mitochondrial TERT remains...
controversial, with 1 group showing that it exacerbates oxidative injury\textsuperscript{19,20} and another group demonstrating a protective effect after oxidative stress.\textsuperscript{21} Taking into account that aging processes are associated with dysfunctional mitochondria, the goal of the present study was to elucidate the localization of TERT within the mitochondria and its functional role in the mitochondria with specific respect to respiratory chain activity, apoptosis, and formation of ROS.

Methods

Please see http://atvb.ahajournals.org for expanded Methods section.

Cell Culture

Human umbilical vein endothelial cells (HUVECs; Lonza, Cologne, Germany) were transfected with Superfect as previously described.\textsuperscript{22} After detachment with trypsin, cells were grown for at least 18 hour prior to all further manipulations.

Isolation of Primary Mouse Lung Fibroblasts

A small piece of lung tissue was minced with 2 scalpels in a culture dish and incubated in HEK 293 medium at 37°C. After 5 days the fibroblasts grown out from tissue fragments were passaged to culture flasks.

Proteinase K Digestion of Mitochondria

To determine whether TERT is localized in the mitochondrial matrix or the intermembrane space, 700 μg of mitochondria was distributed in 3 equal aliquots. Mitochondria were pelleted and incubated at 4°C on a shaker in 40 μL of 3 different buffers for 20 minutes. Buffer 1 (isotonic buffer): 250 mmol/L sucrose, 1 mmol/L EGTA, 10 mmol/L HEPES, pH7; Buffer 2 (hypotonic buffer): 1 mmol/L EGTA, 10 mmol/L HEPES, pH7, 25 μg/mL proteinase K; Buffer 3 (hypotonic buffer with detergent): 1 mmol/L EGTA, 10 mmol/L HEPES, pH7, 1% (v/v) Triton-X100, 25 μg/mL proteinase K. After 20 minutes, digestion was stopped by adding phenylmethylsulfonyl fluoride to a final concentration of 2 mmol/L and incubated for further 5 minutes with shaking. Aliquot 3 was boiled for 5 minutes in Laemmli-buffer. Aliquot 1 and 2 were washed once with Buffer 1 and resuspended in 40 μL RIPA-buffer (50 mmol/L Tris/HCl pH8, 1% [v/v] Nonidet, 150 mmol/L NaCl, 0.1% [wt/vol] SDS, 0.5% [wt/vol] Desoxycholate) and boiled for 5 minutes in Laemmli-buffer. Western blot analyses were performed using antibodies against endogenous TERT (Epitomics, 1:500), TOM40 (Santa Cruz, 1:250), and TIM23 (BD Transduction laboratories 1:1000).

Results

TERT Is Localized in Mitochondria

It has been demonstrated that mitochondria contain measurable telomerase enzymatic activity,\textsuperscript{19} suggesting that a fraction of cellular TERT is localized in these organelles. After having previously shown that telomerase activity is found in mitochondria of HUVECs,\textsuperscript{23} we confirmed that this is also true for HEK cells. As shown in Figure 1A, telomerase enzyme activity was detectable in all 3 compartments under basal conditions. Because commercially available antibodies against endogenous TERT could not be used for immunoprecipitation, we next tested whether overexpressed TERT wild-type protein containing a myc-tag at the C terminus (TERTwt-myc) is detectable in mitochondria. From our previous studies, we knew that TERTwt-myc is exported from the nucleus on stress and thereby behaved identical to endogenous TERT in HEK cells and HUVECs.\textsuperscript{17} Overexpressed TERTwt-myc was detected in the mitochondria by immunoblot (supplemental Figure 1A). The biochemical fractionation was always controlled by proteins, which are known to be located in the nucleus or in the mitochondria.

To determine the exact localization of TERT, which has a genuine mitochondrial leader sequence at the N terminus, within these organelles, we digested the outer mitochondrial membrane with proteinase K. With this approach we could demonstrate that a large portion of endogenous TERT is localized in the mitochondrial matrix as indicated by its presence in the proteinase K resistant fraction (Figure 1B). This requires an import into the mitochondria, as the protein is encoded by the nuclear genome and thus translated in the cytoplasm. The best-characterized pathway to import a protein into the mitochondria of higher eukaryotes is by binding to a complex containing the translocases of the outer membrane 20 and 40 (TOM20 and TOM40) and the translocase of the inner membrane 23 (TIM23). We therefore investigated...
whether TERT can associate with this complex by coimmunoprecipitation studies. As stated above, TERTwt-myc was detected in the mitochondria, and all antibodies directed against endogenous TERT could not be used for immunoprecipitation. Therefore, we performed all further experiments with overexpressed TERTwt-myc. The fact that TERTwt-myc physically interacts with the TOM20/TOM40 complex (supplemental Figure IIIB) and with TIM23 (Figure 1C) strongly suggests a transport mechanism involving these proteins. However, these experiments do not exclude participation of other translocases, like for instance TIM22.

**TERT Binds to mtDNA**

One important component of the mitochondrial matrix is the mtDNA. This prompted us to test whether TERTwt-myc can associate with the genome of the organelle. Therefore, we performed a modified chromatin-immunoprecipitation assay, using primers amplifying segments of mtDNA. After careful examination of the different regions of the circular mitochondrial genome, we identified 2 regions containing the coding sequences for NADH:ubiquinone oxidoreductase (complex I) subunit 1 and 2 (ND1, ND2) as interaction sites for overexpressed TERTwt-myc protein. In contrast, another region of the mtDNA containing the coding sequence for ATP synthase subunit 6 (ATP6) did not show interaction with TERTwt-myc (supplemental Figure II).

**TERT Protects mtDNA From Damage**

It has previously been shown that telomerase can protect mitochondrial function under oxidative stress. As a number of mitochondrial proteins are encoded by the mitochondrial genome it was suggestive to analyze whether TERT bound to mtDNA could protect it against damage. Therefore we first assessed in vitro whether TERT can prevent UV-induced destruction of mtDNA. We treated isolated total cellular DNA (containing nuclear and mtDNA) in vitro in the presence or absence of TERTwt-myc for 3 minutes with short-wave UV light (254 nm) and analyzed intactness of mtDNA by long-range PCR. Indeed, preincubation of the DNA with in vitro translated TERTwt-myc effectively protected mtDNA against UV-induced degradation, whereas immediate irradiation showed only weak protection. Preincubation with β-galactosidase, a protein of comparable size, had no effect (Figure 2A).

To assess whether this protection of mtDNA is of relevance in living cells, we irradiated primary lung fibroblasts from homozygous TERT knockout (TERT−/−) mice and their wild-type littermates (TERT+/+) with different doses of UVB and mitochondrial function was measured. Data are presented as mean±SEM, n=3 with 8 replicates in each experiment, *P<0.05 vs TERT+/+ cells.

**TERT Protects the Respiratory Chain and Specifically Complex I in Cells and in Mice**

To further investigate whether TERT binding to mtDNA has an impact on mitochondrial function, we next measured respiratory chain activity in TERT-transfected cells. We included a TERT mutant (TERTD868A-myc) lacking reverse transcriptase activity, from which we know that it can still bind to mtDNA (data not shown), to elucidate whether catalytic activity of the protein is required for maintaining mitochondrial functions. Measuring the respiratory chain activity in general, we discovered a 30% reduction in succinate-dependent respiration in cells overexpressing TERTD868A-myc (Figure 3A and supplemental Figure III), suggesting that not only the mtDNA binding but also the activity of TERT plays a role for mitochondrial functions. Besides the overall decrease found in respiratory chain activity, we wanted to determine whether the effect was most pronounced for complex I. To emphasize the latter, we normalized all other values to the same rate of succinate-respiration that was set to 1 (Figure 3A) and found a significant reduction in complex I activity. To further underscore these findings, we used a second method to measure complex I activity. Indeed, a significant increase in complex I activity was only found in cells overexpressing TERTwt-myc. (Figure 3B). Of note, the data shown in Figure 4b demonstrate that although all respiratory rates were decreased in TERTD868A, the mitochondria showed no differences in respiratory chain control factors ie, in the ratios of state 4/state 3 or state 4/FCCP-uncoupled rates, which essentially also excludes changes in membrane permeability. To determine whether our results obtained by overexpression of TERT are of relevance in vivo, we next analyzed the respiratory chain activity of intact heart and liver mitochondria isolated from TERT−/− mice and wild-type animals. Indeed, we found that heart mitochondria from TERT−/−
mice showed a significant reduction of the complex I–dependent respiration with the NADH-generating substrates malate and glutamate, which was most obvious in state 3 (ADP-stimulated) respiration (Figure 4a). In contrast, no difference between TERT+/H11002+/H11002 or TERT+/H11001+/H11001 mice and their wild-type littermates was observed in liver mitochondria. This indicated that the protective effect of TERT in vivo might be more important in tissues with a high respiratory rate like the myocardium and with no or weak regenerative capacity.

TERT Reduces Formation of ROS in Mitochondria

Recent studies from Santos et al using a retroviral approach showed that reintroduction of human TERT into human fibroblasts increased oxidative stress, mtDNA lesions, and apoptosis.19,20 In contrast, Ahmed et al showed that TERT protects mitochondria from damage and reduces oxidative stress.21 Therefore, we investigated the effect of TERT under basal and EtBr-induced conditions on ROS formation. Overexpression of TERTwt-myc reduced ROS formation compared to LacZ– and TERTD868A-myc–overexpressing cells (Figure 4B). This is in contrast to Santos19 but in agreement with Ahmed et al.21 Moreover, ablation of endogenous TERT by shRNA (Figure 4C and supplemental Figure IVA) revealed increased mitochondrial ROS formation as measured with Mitosox, a specific compound, which is imported into the mitochondria for ROS detection (Figure 4D and supplemental Figure IVB), suggesting that the presence of TERT in the mitochondria protects from respiratory chain dysfunction.

Mitochondrially Targeted TERT Is More Protective Than TERTwt Against mtDNA Depletion and Oxidative Stress–Induced Apoptosis

Because mitochondrial damage importantly contributes to apoptosis induction, we cloned TERT into a mitochondrial target vector (mitoTERT-myc) to specifically investigate the effect of the mitochondrial localization of TERT on mtDNA protection and apoptosis inhibition.

After having shown that TERT binds to mtDNA and protects cells against UVB-induced decrease of mitochondrial function, we first assessed the effect of mitoTERT-myc on mtDNA protection in living cells. Therefore we reduced mtDNA content in HEK cells transfected with an empty control vector, TERTwt-myc, or mitoTERT-myc by treating...
Figure 5. Mitochondrially targeted TERT rescues cells from ethidium bromide-induced mtDNA depletion and oxidative stress–induced apoptosis. A, HEK cells were transfected with an empty control vector (EV), TERTwt-myc, or mitoTERT-myc and treated with EtBr. mtDNA content was measured by quantitative real-time PCR and normalized to a unique nuclear DNA sequence using the ΔΔCt method. Values are mean±SEM and are shown relative to untreated cells. n=2 to 6 with 2 duplicates in each experiment, *P<0.05 vs EV + EtBr. B, HUVECs were transfected with the constructs indicated and treated with 200 μmol/L H2O2. Apoptosis was measured with annexin V using FACS analysis. Data are mean±SEM, n=4, *P<0.05 vs mitoTERT-myc + H2O2, **P<0.05 vs mitoTERT-myc + H2O2.

cells with low doses of ethidium bromide (EtBr). Expression of TERTwt-myc completely rescued the loss of mtDNA content observed in control transfected cells. This effect was even more pronounced in cells expressing mitoTERT-myc (Figure 5A).

We previously described that H2O2 induces apoptosis in endothelial cells.25 Overexpression of TERTwt-myc in HUVECs showed a slight but significant inhibition of H2O2-induced apoptosis, whereas mitoTERT-myc completely abrogated H2O2-triggered apoptosis (Figure 5B). These data suggest that protection of mtDNA integrity, mitochondrial function, and apoptosis protection by TERT can be mainly ascribed to the mitochondrial function of this protein.

Discussion

Our results presented here demonstrate that a large portion of endogenous mitochondrial TERT is localized in the mitochondrial matrix. TERT binds to mtDNA and thereby protects mtDNA from damage. Concomitantly we observe a reduction in the formation of mitochondrially produced ROS. Overexpression of mitochondrially targeted TERT is more protective than TERT wild-type against EtBr-induced DNA damage and ROS-induced apoptosis. Strikingly, the protective function of TERT was also evident in vivo from the fact that heart mitochondria from TERT<sup>−/−</sup> mice exhibited significantly reduced respiratory chain activity.

Previous studies have shown that TERT contributes significantly to processes of aging by protecting telomeres from critical shortening and by inhibiting apoptosis and decreasing oxidative stress. At the same time, oxidative stress and mitochondrial dysfunction have been demonstrated to be important players of aging processes. Our study now shows that mitochondrial TERT inhibits formation of mitochondrially produced ROS and can contribute to increased energy metabolism. Thus, it is tempting to speculate that not only nuclear TERT has an impact on aging processes, but also mitochondrially localized TERT. A reduction in mitochondrially produced ROS has recently been demonstrated to increase the lifespan of mice. Schriner et al produced mice that overexpressed mitochondrially targeted catalase as well as nuclear targeted catalase and showed that in both mice strains catalase was able to reduce H2O2, but only mice which overexpress mitochondrially targeted catalase exhibited an increased lifespan compared to their wild-type littermates.3 Likewise, overexpression of manganese superoxide dismutase, a mitochondrially localized antioxidative enzyme reducing O2<sup>−</sup>- to H2O2, increased the lifespan of adult Drosophila melanogaster.25 However, recent studies on mice deficient for the mitochondrially localized DNA polymerase γ (Pol-γ) did not find an increase in oxidatively damaged proteins or a reduction in mitochondrial aconitase activity, a classic marker for oxidative damage of proteins. Nevertheless, an accumulation of mitochondrial mutations and the development of many of the phenotypes characteristic of human premature aging were reported.26,27 Thus, it was concluded that the Pol-γ-deficient mice exhibited an aging phenotype in the absence of enhanced ROS production. However, it was also demonstrated that embryonic fibroblasts of the Pol-γ-deficient mice show a 95% reduction in oxygen consumption,27 which would suggest that the extensive mutations lead to a complete breakdown of the respiratory chain. This would exclude the formation of mitochondrially produced ROS in these mice. Therefore, these data do not prove that mitochondrially produced ROS are not involved in aging processes.

Recently, it has been reported that flavine-mononucleotide in complex I of the respiratory chain is the major site where ROS are produced.7,8 Our data now demonstrate that TERT protects the genes for subunits ND1 and ND2 in the mtDNA specifically. Thus, it is tempting to speculate that an increase in ROS production by complex I leads to more damaged mtDNA spurring a vicious cycle finally resulting in dysfunctional respiratory chain and mitochondria. Mitochondrial TERT may counteract ROS production by complex I by binding to the ND1 and ND2 genes, thereby increasing the synthesis of functional complex I subunits which in turn can reduce the formation of damaged complex I that leaks electrons onto oxygen. Unfortunately, too little is still known about the structure and function of complex I.28 Therefore, we do not know whether protection of ND1 and ND2 subunit genes by TERT is a key prerequisite for reducing complex I produced ROS.
Several studies discuss an important role for apoptosis in the process of aging and age-related diseases. Here, we demonstrate that TERT protects mtDNA from damage and that mitochondrial targeted TERT is protective against oxidative stress induced damage. Our data could implicate that mitochondrial TERT also plays an important role in apoptotic processes leading to pathological aging. Key processes during the onset of apoptosis are outer mitochondrial membrane permeabilization, release of cytochrome c into the cytosol, and activation of caspases. Of note, Bayir et al demonstrated that a mitochondrial pool of cytochrome c exists which acts as a cardiolipin oxygenase. This cytochrome c/cardiolipin oxygenase is activated during apoptosis, uses ROS, and causes selective oxidation of cardiolipin. The oxidized cardiolipin is required for the release of proapoptotic factors from mitochondria to the cytosol. Thus, a reduction of ROS by mitochondrial TERT could result in a reduced activation of the cytochrome c/cardiolipin oxygenase, reduced oxidized cardiolipin, and therefore inhibition of the release of proapoptotic factors into the cytosol. Taking this into account, it is not surprising that nuclear as well as mitochondrial TERT can inhibit apoptotic processes, because one may speculate that mitochondrial TERT inhibits the formation of ROS by complex I, blocks cardiolipin oxidation and release of proapoptotic factors from the mitochondria, whereas nuclear TERT protects telomeres from shortening, interacts with the antiapoptotic proteins PARP and Akt and is regulated by p53, a known gatekeeper of apoptotic processes.

Moreover, it has been shown that cells depleted of mtDNA (p0 cells) are resistant to apoptosis induction by several stimuli. Dissipation of mitochondrial potential and release of cytochrome c into the cytosol was absent in these cells. These data clearly demonstrate that mitochondria are necessary for the onset of apoptosis. Similar to these findings, it has been demonstrated that mitochondrial dysfunction is a prerequisite for the onset of aging processes. Therefore, it is tempting to speculate that apoptosis is also a prerequisite for aging processes or that apoptosis and aging processes are tied together. This hypothesis is strengthened by data from Kujoth et al who found that many tissues of Pol–/– deficient mice show an increase in TUNEL-positive cells. Similar to this study in mice, patients who carry a high load of mitochondrial mutations show an increase in TUNEL-positive muscle fibers. We show here that protection of mtDNA by TERT is also associated with a higher protection from apoptosis.

Conclusion/Perspectives

Therefore, one may consider revising the old dogma that only the nuclear function of TERT is important for aging and apoptotic processes. Mitochondrial TERT may also exert a significant contribution to the antiaging and antiapoptosis function of TERT.

Acknowledgments

We thank Barbara Wiegand, Ilka Siebels, Diane Schmiegelt, and Kerstin Kunze for expert technical assistance.

Sources of Funding

This work was supported in part by grants from the Deutsche Forschungsgemeinschaft to J.H. (SFB 728 B5 and HA2868/2-3) and by the Bundesministerium fuer Bildung und Forschung (03NUK003C).

Disclosures

None.

References


Mitochondrial telomerase reverse transcriptase binds to and protects mitochondrial DNA and function from damage

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Running title: Functions of mitochondrial TERT

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Methods

Cell Culture
Primary human umbilical vein endothelial cells (HUVEC) were cultured in endothelial basal medium supplemented with hydrocortisone (1 µg/ml), bovine brain extract (12 µg/ml), gentamicin (50 µg/ml), amphotericin B (50 ng/ml), epidermal growth factor (10 ng/ml), and 10 % fetal calf serum (FCS). HEK 293 cells (HEK cells) were maintained in DMEM containing 10 % FCS and were transfected with Lipofectamine/Plus according to the manufacturer's protocol (GibcoBRL) as previously described \(^1\) with a transfection efficiency of 90 ± 4 %.

cDNA cloning, plasmids and PCRs
A human cDNA TERT construct was kindly donated by Dr. Weinberg \(^2\). Human TERT cDNA was subcloned into pcDNA3.1myc-his vector (TERTwt-myc) or into mitochondrial pShooter vector, which targets TERT into the mitochondria (mitoTERT-myc) (Invitrogen). The mitochondrial pShooter mammalian expression vector incorporates signal sequences into TERT to direct TERT to its localization. TERTD868A-myc was generated by site-directed mutagenesis (Promega). Moreover, we cloned shRNA TERT under the control of an U6 promoter into a GFP-containing vector to control the transfection efficiency. shRNA TERT target sequence: 5'-'TCCTGCGTTTGGATGATT'-3'.

CDNA was prepared from total cellular RNA using reverse transcriptase and used as template for gene specific PCRs with intron spanning primers. TERT specific PCRs were performed with primers derived from exon 6 (5'-'CCGCCTGAGCTGTACTTTGT'-3') and exon 7 (5'-'ATGTACGGCTGGAGGTCTGT'-3'). As housekeeping gene L32 was used (primer sequences: 5'-'GTGAAGCCCAAGATCGTCAA'-3' and 5'-'TTGTTCACATCAGCAGCAC'-3').

Detection of oxidative stress
Living cells were incubated for dye uptake with 20 µM 2',7' dichlorodihydrofluorescein diacetate (H₂DCF-DA) or 5 µM MitoSox for 30 min (Molecular Probes, Germany). Cells were trypsinized for 2 min and reaction was stopped with PBS containing 10 % FCS and cells were pelleted by centrifugation. Cells were resuspended in PBS and measured using FACS analysis.

Cellular fractionation and Immunoprecipitation
Cellular fractionation was performed as described previously \(^3\) and nuclear, cytosolic or mitochondrial lysates were immunoprecipitated with 5 \(\mu\)g TOM20-antibody, 5 \(\mu\)g TIM23-antibody or 5 \(\mu\)g myc-antibody overnight at 4\(^\circ\)C in the corresponding lysis buffers \(^3\). After incubation with A/G Plus agarose (Santa Cruz) for 2 h at 4\(^\circ\)C, resulting beads were washed three times with the same buffers and subjected to SDS-PAGE.

**Measurements of TERT-mitochondrial DNA binding**

Living cells were incubated with 1\% formaldehyde in cell culture medium for 1 h at 4\(^\circ\)C. Cross-link reaction was terminated by adding 0.125 M glycine and cells were scraped off the plate. DNA-protein immunoprecipitation was performed according to the manufacturer’s instructions (Upstate Biotechnology) and after separating of protein and DNA, PCR was performed with primers specific for human ND1 (5\' - ATACCCATGGCCAACCTCTACTCTCATT-3', 5\' - CCCTGATCATGAGTTAAGCGCTAG-3'), ND2 (5\' - CGACTGATTTTTACCTGAGGCCTAG-3', 5\' - AGAATTGCGGTAGGTAGTAGGTTAAG-3'), and ATP6 (5\' - GTACTGATCTCTATTTCCTCTATTTGATCC-3', 5\' - CTTAGAAGTGAAAAACGTAGGGCTGATTAGA-3') coding regions.

**Preparation of mouse heart and liver mitochondria**

Intact heart and liver mitochondria from 6 months old TERT-/- mice and their wildtype littermates (F2 generation) were prepared as described earlier for mitochondria from rat organs \(^4\). Buffer volumes were reduced by a factor of ~ 2 due to the lower mouse organ sizes and one washing step was omitted to reduce preparation dependent losses. Upon completion of the respective preparation, the mitochondrial suspensions were generally stabilized by the addition of 40 mM malate and 50 mM glutamate. The substrate addition ensured that the mitochondria retained high respiratory control factors (ratio between state 3 respiration after ADP addition and state 4 respiration; for heart mitochondria ~ 10; for liver mitochondria > 7) over a longer time period.

**Mitochondrial respiration**

The rate of mitochondrial respiration was monitored at 25 \(^\circ\)C using an Oxygraph-2k system (Oroboros) equipped with two chambers and DatLab software. Mitochondria (depending on the yield of the preparations 130-338 \(\mu\)g protein of TERTwt or 72-296 \(\mu\)g protein of TERTD868A mitochondria) were added to 2 ml of a buffer containing 200 mM...
sucrose, 10 mM potassium phosphate, 10 mM Tris-HCl, 10 mM MgSO$_4$, and 2 mM EDTA, pH 7.0. State 4 respiration was measured after the addition of the NADH-generating substrates malate (6 mM) and glutamate (7 mM). Then, 0.5 mM ADP was added to measure state 3 respiration. After determining coupled respiration, 50 nM carbonyl cyanide $p$-trifluoromethoxyphenylhydrazone (FCCP) was added to the reaction chamber, and respiration was measured in the absence of a proton gradient. To inhibit complex I activity, a total 0.1 μM rotenone was added. Then, 25 mM of succinate were added, and complex II dependent respiration was determined. Finally, 2 mM KCN was added to inhibit complex IV activity. TERTD868A and TERTwt mitochondria were measured in parallel pairs using the same conditions (crossover design). Measurements with intact heart and liver mitochondria followed the same conditions in the identical buffer, but the concentrations of substrates/inhibitors were: 6 mM malate + 7 mM glutamate, 1 mM ADP, 50 nM FCCP, 1 μM rotenone, 5 mM succinate and 2 mM KCN. Heart mitochondria were added in the range of 173–214 μg protein from wt mice and 180–252 μg protein from TERT$^+$ mice, liver mitochondria at 452–728 μg protein from wt mice and 440–686 μg protein from TERT$^{-/-}$ mice. For each preparation, duplicates (heart) or triplicates (liver) were measured.

**Complex I activity**
For the determination of complex I activity, mitochondria (223 – 442 μg protein of TERTwt or 147 – 356 μg protein of TERTD868A) were solubilized using sodium cholate. The final concentration in the assay was 0.01% sodium cholate. NADH:ubiquinone oxidoreductase activity was measured at room temperature in a buffer containing 2 mM Na$^+$/Mops, 50 mM NaCl, and 2 mM KCN, pH 7.2, using 100 μM $n$-decylubiquinone (DBQ) and 100 μM NADH as substrates. Oxidation rates of NADH were recorded with a Shimadzu UV300 dual-wavelength spectrophotometer (OD$_{340-400}$ nm = 6.1 mM$^{-1}$ cm$^{-1}$). After ~ 10 min, 50 μM rotenone were added to inhibit complex I activity.

**Measurements of mitochondrial DNA (mtDNA)**
Total cellular DNA (including nuclear and mtDNA) was isolated from HEK 293 cells by standard methods. To determine the relative mtDNA content 250 ng were used as template for real-time PCR. PCR reactions were carried out on an Abi Prism 7000 (Applied Biosystems, Foster City, CA, USA) using SYBRGreen PCR Master Mix (Applied Biosystems, Darmstadt, Germany). Primers for mtDNA (5'...
GATTGGGTACCACCCAAGTATTG-3', 5'-AACATCTGCTGCTGGCGAGTA-3') amplified a 83 bp segment of the mtDNA. For normalization with the ΔΔCT method a unique genomic segment of 104 bp from the chromosomal region 9p21.3 was amplified (primers: 5’-GCAGAAACGGAGAGACATAAC-3’, 5’-TTTTGGGTGAACATATTG-3’).

Measurements of intact mtDNA after UV-irradiation in vitro were performed with a long range PCR yielding a 6.3 kb amplification product (primers: 5’-ATACCCATGGCCAACCTCCTACTCCTCATT-3’, 5’-CTAGAAGTGTGAAAACGTAGGCTTGGATTAAGGC-3’), which was analyzed by agarose gel electrophoresis.

Measurement of mitochondrial activity with MTT
10000 lung fibroblasts per well were seeded on 96well microtiter plates. 24 h later the cells were serum deprived for 24 h. Before exposure to UVB irradiation cells were washed with PBS. The cells were exposed to UVB irradiation in 100 µl PBS per well. UVB output was measured with a UV-Dosimeter Type II equipped with a UV6 sensor (Waldmann Medizintechnik, Villingen-Schwenningen, Germany) and found to be 0.56 mW/cm² at a tube to target distance of 30 cm. After irradiation PBS was exchanged to serum free medium and cells were incubated at 37°C for 24 h. Cells were incubated for four hours in medium containing 0.25 mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Aldrich). Medium was exchanged to 200 µl DMSO before measurement of absorption at 550 nm.

Apoptosis
Apoptosis was determined by FACS analysis using annexin V-PE binding and 7-Amino-actinomycin (7AAD)-FITC staining (Pharmenting). Apoptotic cells were defined as annexin V-positive, 7AAD-negative cells. In brief, cells were trypsinized of the dish and pelleted. After washing twice with annexin binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4), cell pellets were resuspended in 50 µl of annexin binding buffer and incubated with 2.5 ng/ml annexin V-PE and 2.5 ng/ml 7AAD-FITC for 20 min. The reaction was terminated by adding 250 µl of annexin binding buffer and analyzed by FACS.

Telomerase enzyme activity measurement
Telomerase enzyme activity was measured using a commercially available PCR-based assay according to the manufacturer’s protocol (TRAPeze assay, Roche, Germany).
Telomeric repeat amplification protocol\textsuperscript{6} assays were performed using biotin-labelled TS primers as previously described\textsuperscript{7}.

**Statistics**

All values are expressed as mean ± SEM. Statistical analysis was performed using student t-test.

**References**


**Figure Legends:**

Figure I: TERT is localized in mitochondria. (A) TERTwt-myc overexpressed in HUVEC was detected by immunoblot. Purity of fractions was controlled with topoisomerase I (nuclear fraction) and cytochrome c (mitochondrial fraction). (B) After fractionation of HEK cells transfected with TERTwt-myc into nuclear, cytosolic and mitochondrial fractions, an immunoprecipitation for TOM20 was performed. The immunoprecipitates were immunoblotted with antibodies directed against TERTwt-myc (upper panel),...
TOM40 (middle panel) and TOM20 (lower panel). The absence of precipitated TOM20 in nuclear and cytosolic fractions demonstrates the purity of fractions.

Figure II: TERT binds to the region of mtDNA encoding ND1 and ND2. (A) After cross-linking protein and DNA in HEK cells transfected with TERTwt-myc or an empty control vector (EV), TERTwt-myc was immunoprecipitated. (a) PCRs were performed with the eluted DNA formerly bound to TERTwt-myc using ND1, ND2 and ATP6 specific primers. The left panel (IP: myc) shows the fragments amplified from the co-immunoprecipitated DNA, the right panel (input) the bands obtained after amplification of the material before immunoprecipitation. (B) Success of immunoprecipitation assessed by immunoblot.

Figure III: TERT alters respiratory chain activity in mitochondria. Respiratory chain activity was measured in HEK cells transfected with TERTwt-myc (upper plot) or TERTD868A-myc (lower plot).

Figure IV: TERT inhibits mitochondrial derived ROS. (A and B) HEK cells were transfected with vectors expressing GFP and a TERT-specific shRNA (shRNA TERT) or a scrambled control sequence (scr). The transfection efficiency (=GFP-positive cells) was 54 +/- 9 %. Immunoblot analysis was performed out of whole cell lysates. (B) The transfected living HEK cells from (A) were incubated with Mitosox. GFP/Mitosox double positive cells were measured by FACS analyses as indicated. Panel shows representative FACS plots.
Supplemental Figure I

A

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- EV
- H2O
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- TERTwt-myc
Supplemental Figure III
Physical Exercise Prevents Cellular Senescence in Circulating Leukocytes and in the Vessel Wall


Circulation 120: 2438-47, 2009

Kooperation mit Homburg Saar.
In Düsseldorf wurden folgende Arbeiten für die Publikation durchgeführt:

N. Büchner: Zucht der TERT defizienten Mäuse, Organentnahme und Etablierung der cGMP-Messungen, Experimente zu cGMP-Spiegeln und eNOS-Aktivitäten in Aorten von Mäusen.

Physical Exercise Prevents Cellular Senescence in Circulating Leukocytes and in the Vessel Wall

Christian Werner, MD; Tobias Fürster, MD; Thomas Widmann, MD; Janine Pöss, MD; Cristiana Roggia, MD; Milad Hanhoun, MD; Jürgen Scharhag, MD; Nicole Büchner, DBBSc; Tim Meyer, MD; Wilfried Kindermann, MD; Judith Haendeler, PhD; Michael Böhm, MD; Ulrich Laufs, MD

Background—The underlying molecular mechanisms of the vasculoprotective effects of physical exercise are incompletely understood. Telomere erosion is a central component of aging, and telomere-associated proteins regulate cellular senescence and survival. This study examines the effects of exercising on vascular telomere biology and endothelial apoptosis in mice and the effects of long-term endurance training on telomere biology in humans.

Methods and Results—C57/B16 mice were randomized to voluntary running or no running wheel conditions for 3 weeks. Exercise upregulated telomerase activity in the thoracic aorta and in circulating mononuclear cells compared with sedentary controls, increased vascular expression of telomere repeat-binding factor 2 and Ku70, and reduced the expression of vascular apoptosis regulators such as cell-cycle–checkpoint kinase 2, p16, and p53. Mice preconditioned by voluntary running exhibited a marked reduction in lipopolysaccharide-induced aortic endothelial apoptosis. Transgenic mouse studies showed that endothelial nitric oxide synthase and telomerase reverse transcriptase synergize to confer endothelial stress resistance after physical activity. To test the significance of these data in humans, telomere biology in circulating leukocytes of young and middle-aged track and field athletes was analyzed. Peripheral blood leukocytes isolated from endurance athletes showed increased telomerase activity, expression of telomere-stabilizing proteins, and downregulation of cell-cycle inhibitors compared with untrained individuals. Long-term endurance training was associated with reduced leukocyte telomere erosion compared with untrained controls.

Conclusions—Physical activity regulates telomere-stabilizing proteins in mice and in humans and thereby protects from stress-induced vascular apoptosis. (Circulation. 2009;120:2438-2447.)

Key Words: aging ■ exercise ■ nitric oxide synthase ■ prevention ■ telomeres

Physical training is associated with improvements in exercise capacity, blood pressure regulation, insulin sensitivity, abdominal fat reduction, lipid profile, and psychosocial, hemodynamic, and inflammatory parameters. These effects contribute to an augmentation of endothelial function, delayed atherosclerotic lesion progression, and enhanced vascular collateralization in patients with diabetes mellitus, coronary artery disease, and chronic heart failure. However, despite the wealth of evidence, our understanding of the underlying molecular mechanisms, especially with regard to cellular survival and senescence, is limited.

Clinical Perspective on p 2447

Aging is a predominant and independent risk factor for the development of atherosclerotic diseases. Vascular aging is characterized by impaired endothelial function and arterial stiffening. On the cellular level, telomere biology is a central regulator of the aging process. Telomeres and their regulatory proteins compose t-loop structures at both ends of eukaryotic chromosomes and protect the genome from degradation during repetitive cellular divisions. The enzyme telomerase with its catalytic protein subunit telomerase reverse transcriptase (TERT) is the main component of the telomere complex. Other important proteins in the t loop include the telomere repeat-binding factors (TRFs), which interact with telomere-associated proteins and serve as binding platforms. Recent clinical data suggest that parameters of telomere biology in circulating mononuclear cells (MNCs) are associated with cardiovascular morbidity and can be used as indicators for the effect of therapeutic interventions.

Received March 1, 2009; accepted October 9, 2009.

From the Klinik für Innere Medizin III, Kardiologie, Angiologie und Internistische Intensivmedizin (C.W., T.F., J.P., M.H., M.B., U.L.), Klinik für Innere Medizin I, Onkologie, Hämatologie, Klinische Immunologie und Rheumatologie (T.W.), and Institut für Pathologie (C.R.), Universitätsklinikum des Saarlandes, Homburg/Saar, Germany; Institut für Sport und Präventivmedizin, Universität des Saarlandes, Saarbrücken (J.S., T.M., W.K.); and Institut für Umweltmedizinische Forschung at the Universität Düsseldorf gGmbH, Düsseldorf (N.B., J.H.), Germany.

The online-only Data Supplement is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.109.861005/DC1.

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DOI: 10.1161/CIRCULATIONAHA.109.861005

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other studies revealed a close correlation of blood and vascular leukocyte telomere DNA content.\(^7\) Our previous work demonstrated beneficial effects of physical exercise on myocardial telomere-regulating proteins.\(^8\) However, the effects of physical exercise on vascular telomere biology and aging are unknown.

**Methods**

**Animals and Exercising**

Eight-week-old male C57/B16 (Charles River Laboratories, Wilmington, Mass), endothelial nitric oxide (NO) synthase–deficient (eNOS\(^{−/−}\); B6.129/P2-NoS3, Charles River) mice, TERT\(^{−/−}\) (B6.129S-Tert\(^{−/−}\)I, The Jackson Laboratory, Bar Harbor, Me; mutant generation 2) mice, and strain-matched controls were studied. Exercising mice were kept in individual cages equipped with a running wheel and a mileage counter. The mean voluntary running distance of wild-type mice was 4280±670 m/24 h and did not differ significantly between TERT\(^{−/−}\) mice and eNOS\(^{−/−}\) mice. Indicated mice were treated with paraquat or lipopolysaccharide (Sigma-Aldrich, Munich, Germany).

**Track and Field Athletes and Untrained Control Subjects**

Blood MNCs were studied in professional young middle- and long-distance runners (German National track and field team; n=32; mean age, 20.4±0.6 years; 25 male, 7 female subjects; average running distance, 73±4.8 km/wk). In addition, we studied middle-aged athletes (marathon runners, triathletes) performing regular endurance training and competitions (n=25; mean age, 51.1±1.6 years; 19 male, 6 female subjects; average running distance, 80±7.5 km/wk; 35±2.7 years of training history). Two groups of nonsmoking healthy volunteers (26 young control subjects; mean age, 21.8±0.5 years; 15 male, 11 female subjects; 21 middle-aged control subjects; mean age, 50.9±1.6 years; 14 male, 7 female subjects) who reported <1 hour of exercise per week in the last year served as controls (the Table). Endurance capacity was assessed in all subjects by standardized ECG stress test. Blood samples were taken in the morning in the fasting state before training. Leukocytes were isolated from sodium citrate blood by Ficoll density gradient centrifugation. All subjects gave informed consent, and the study was approved by the ethics committee of the Ärztekammer des Saarlandes (No. 116/07).

**Telomerase Activity and Telomere Length Analysis**

Telomerase activity was assessed with the quantitative telomerase repeat amplification protocol, (B) protein expression of the murine TERT, (C) mRNA expression of TRF1, (D) TRF2 protein, (E) mRNA expression, and (F) mRNA expression of the 70-kDa subunit of the DNA repair protein Ku, each shown with representative Western blots/PCR images and standardized for the housekeeping gene GAPDH. Box plots represent median and 25% to 75% percentiles; whiskers represent range; n=8 per group. ***P<0.001.

**Figure 1.** Voluntary physical exercise increases telomere-regulating proteins in the aorta. Effects of voluntary running exercise in C57/B16 mice for 21 days vs sedentary controls on aortic (A) telomerase activity determined by the telomerase repeat amplification protocol, (B) protein expression of the murine TERT, (C) mRNA expression of TRF1, (D) TRF2 protein, (E) mRNA expression, and (F) mRNA expression of the 70-kDa subunit of the DNA repair protein Ku, each shown with representative Western blots/PCR images and standardized for the housekeeping gene GAPDH. Box plots represent median and 25% to 75% percentiles; whiskers represent range; n=8 per group. ***P<0.001.

**Western Blot Analysis, ELISA, Real-Time PCR, Aortic Ring Preparation and Tension Recording, and Quantification of Apoptosis by Hairpin Oligonucleotide Assay**

These protocols are described in detail in the online-only Data Supplement.

**Results**

**Exercise Increases Aortic Telomerase Activity and Telomere-Stabilizing Proteins in Mice**

Voluntary running for 3 weeks had no effect on lipid levels, body weight, blood pressure (controls, 116/91 mm Hg; exercise, 119/92 mm Hg; n=10 per group), or resting heart rate (controls, 478 bpm; exercise, 474 bpm) in C57/B16 mice but induced a 2.9-fold increase in aortic telomerase activity as determined by the telomere repeat amplification protocol a general linear model for repeated measures and Bonferroni posthoc tests. SPSS software version 17.0 (SPSS Inc, Chicago, Ill) was used. Differences were considered significant at P<0.05. Absolute values are shown for P>0.001.

**Statistical Analysis**

Band intensities were analyzed by densitometry. In mouse experiments, median values between 2 independent groups were compared by use of the Mann–Whitney test. The Kruskal–Wallis test was used to compare median values across ≥3 groups, and Bonferroni posthoc analyses were performed to account for multiple testing. In the figures, boxes represent medians and 25% and 75% percentiles; whiskers represent range. Means of the human data were compared by use of ANOVA and the Bonferroni test for posthoc analyses. Endothelial function data were subjected to regression analysis with
Physical training upregulated the protein expression of the murine TERT by 3.2-fold (Figure 1A). TRF1 mRNA expression was upregulated by 1.5-fold (Figure 1B). TRF2 protein expression increased by 2.6-fold, and TRF2 mRNA increased by 2.0-fold (Figures 1D and 1E). Exercise also upregulated mRNA expression of the 70-kDa subunit (3.0-fold; Figure 1F) but not of the 80-kDa subunit of the DNA repair protein Ku (data not shown).

Exercise Downregulates Vascular Cell-Cycle Inhibitors and Apoptosis Regulators

Mice supplied with a running wheel for 3 weeks were characterized by decreased aortic expression of cell-cycle inhibitors and apoptosis regulators compared with animals without a running wheel (Figure 2A). Protein expression of p16 was reduced to 45%, of cell-cycle–checkpoint kinase 2 (Chk2) to 57%, and of p53 to 42%.

Effects of Exercise Are Absent in TERT<sup>−/−</sup> Mice

TERT<sup>−/−</sup> mice and their strain-matched wild types were subjected to 21 days of voluntary exercise. Similar to the C57/Bl6 mice, B6.129S wild-type mice exhibited a marked upregulation of aortic TRF2 or Ku70 expression (Figure 3). However, running had no effect on TRF2 or Ku70 expression in TERT<sup>−/−</sup> mice. Importantly, the exercise-induced down-regulation of p16, Chk2, and p53 expression in B6.129S wild types was completely absent in TERT<sup>−/−</sup> mice (Figure 3).

Long-Term Exercise for 6 Months Does Not Alter Aortic Telomere Length

Telomere length in aortic sections was examined by FISH (Figure 2B and 2C). There was no significant difference in aortic telomere length between mice after 3 weeks and 6 months of exercise and those in the sedentary condition. As a positive control, we studied a group of 18-month-old mice, which showed shorter telomeres.

Figure 3. Effects of exercise on aortic telomere biology and cell cycle in TERT<sup>−/−</sup> mice. Representative Western blot analysis and quantification of the aortic effects of voluntary exercise for 21 days in B6.129S-Tert<sup>tm1Yjc</sup>/J and their corresponding wild types (WT) on the expression of (A) TRF2 protein, (B) TRF2 mRNA, (C) Ku70 mRNA, (D) p16 protein, (E) Chk2 protein, and (F) p53 protein. Standardization for GAPDH. P values are calculated vs median of wild-type control; n=6 to 8 per group. ***P<0.001.
Effects of Voluntary Exercise Are Mediated by eNOS

Increased availability of endothelial NO is a hallmark of physical exercise. Therefore, the experiments were repeated in eNOS mice. Figure 4A through 4C and Figure I of the online-only Data Supplement show that there was no modification of aortic telomerase activity (telomere repeat amplification protocol) by exercise in eNOS mice. PCR analysis showed no changes in TRF1, TRF2, Ku70, Ku80, or p53 mRNA expression. Similarly, Western blots found no significant differences in aortic TRF2, p16, Chk2, and p53 protein levels between running and sedentary eNOS mice. Therefore, eNOS is a necessary mediator of the exercise-induced regulation of telomere proteins. Aortic cGMP content was not different in TERT compared with wild-type mice (Figure 4D). Both B6.129S and TERT mice showed an increase in the ratio of phospho-eNOS to total eNOS in the thoracic aorta after 3 weeks of exercise (Figure 4E). Endothelium-dependent vasodilation improved markedly in both wild-type and TERT mice after 3 weeks of exercising but was completely absent in eNOS animals (Figure 4F), whereas endothelium-independent vasodilation was comparable in all groups (Figure 4G).

Exercise Reduces Endothelial Apoptosis

To test whether the exercise-induced regulation of telomere regulators and survival proteins would be physiologically relevant, endothelial cell apoptosis (hairpin oligonucleotide assays) was induced by treatment with lipopolysaccharide (120 mg IP for 48 hours; Figure 5A) or paraquat (25 mg/kg IP for 24 hours). Both agents strongly induced apoptosis (lipopolysaccharide by 7-fold, paraquat by 5-fold). Preconditioning with 3 weeks of voluntary exercise potently decreased the number of apoptotic aortic endothelial cells in the lipopolysaccharide experiments (2.1-fold; Figure 5B) and to a similar degree in the paraquat experiments (2.9-fold; P<0.05; data not shown). Importantly, in C57/B16 mice exercise training conferred potent protection from oxidative stress-induced endothelial apoptosis. When these experiments were repeated in TERT mice, exercising partially abolished the exercise-induced antiapoptotic effect (Figure 5B). In eNOS mice, a complete loss of the protective effect of exercise was observed (Figure 5B).
Exercise Decreases Senescence Proteins in MNCs via Upregulation of Telomere Proteins

Exercise potently activated telomerase in MNCs isolated from the spleen by 3.3-fold (Figure 6A). This activation was also observed in circulating blood MNCs and MNCs isolated from bone marrow. Running led to an upregulation of TRF2 protein and mRNA expression (Figure 6B and 6C). In parallel to aorta, TRF2 protein levels were increased in the spleen-derived MNCs of exercised B6.129S wild-type animals (1.7-fold) but not in trained TERT knockout animals (Figure 6D). The exercise-induced downregulation of p53 was abolished in MNCs of TERT-deficient mice (Figure 6E). These data suggest that physical training positively influences telomere biology to similar degrees in MNCs and the vascular wall.

Comparison of Telomere Biology in Athletes With Untrained Individuals

To evaluate the effects of physical activity on telomere biology in humans, we compared young professional athletes from track and field disciplines and middle-aged athletes with a history of continuous intense endurance exercise since their youth with untrained control subjects of similar ages. All study participants were healthy nonsmokers. The fitness level of the athletes was superior; they were characterized by a lower resting heart rate, lower blood pressure, lower body mass index, and more favorable lipid profile.

Western blot analysis of circulating MNCs revealed an upregulation of TRF2 protein in young (1.8-fold) and middle-aged (2.2-fold) athletes compared with the untrained control groups (Figure 7A). TRF2 mRNA was upregulated in the young athletes (1.7-fold) and the middle-aged athletes (2.2-fold) (Figure 7B). Chk2 mRNA was downregulated in both the young and middle-aged athletes to less than half that of the controls and did not significantly differ between the age groups (Figure 7C). p53 Protein was not influenced by sports in the MNCs of young subjects, but its expression was upregulated in the untrained middle-aged individuals by 2.1-fold (Figure 7D). Interestingly, this upregulation was not observed in MNCs from middle-aged athletes. In young individuals, training did not change the expression of p16 or Ku proteins. However, in older individuals, training was associated with a marked downregulation of p16 and an increase in Ku 70 and 80 mRNA (Figure 7E through 7G).

Long-Term Exercise Training Activates Telomerase and Attenuates Telomere Attrition in Human Leukocytes

Exercise was associated with a marked increase in telomerase activity in the MNCs of both the young (2.5-fold) and middle-aged (1.8-fold; Figure 8A) athletes. Telomere length was measured in blood leukocytes cells through the use of 2 independent protocols. The Flow-FISH method allows differentiation between granulocytes and lymphocytes in the scatterplot (Figure 8C and Figure II of the online-only Data Supplement). In lymphocytes and granulocytes, telomere length was not different between professional athletes and untrained young subjects. However, older untrained individuals exhibited shorter MNC telomeres ($P<0.001$ versus all other groups; Figure 8B). This age-dependent telomere loss was attenuated in lymphocytes ($P<0.001$; Figure 8B) and granulocytes ($P<0.001$; Figure 8D) from individuals who had performed endurance exercise for several decades.
Vascular Antiaging Effects of Physical Exercise

To confirm these results, a real-time PCR method for measuring telomere length was used (online-only Data Supplement) and showed a reduction in leukocyte telomere length in middle-aged control subjects ($P=0.006$ versus young control subjects). This telomere erosion was reduced in middle-aged athletes (Figure 8E).

Discussion

Our animal data show that physical exercise upregulates telomere-stabilizing proteins in the vascular wall and in MNCs. The underlying mechanism is the increase in endothelial NO, which synergizes with activation of telomerase to protect against cellular senescent and apoptotic signaling events. In mice, these effects are observed after only 3 weeks of voluntary running. Circulating leukocytes of track and field athletes show similar changes in telomerase activity, telomere-stabilizing proteins, and senescence markers compared with untrained individuals.

Improvement in endothelial NO availability is central to the vascular protection observed in the mouse model of voluntary running by mediating antioxidant effects and increasing circulating endothelial progenitor cells. These data are now extended by the observed effects on the regulation of telomere proteins. One of the key findings of the present study is the marked upregulation of aortic and MNC telomerase activity. In accordance with this observation, protein expression of the catalytic subunit, the aortic TERT, is increased. Both telomerase and TERT have been shown to regulate endothelial cell growth and survival, and they act as antiapoptotic factors. Defects in mice lacking the RNA component of telomerase involve apoptosis, and telomerase directly protects cells against programmed cell death. As a consequence, these mice show a hypertensive phenotype. In addition to telomerase, exercise upregulated the expression of TRF1 and TRF2. Interestingly, TRF2 has been suggested to serve as a binding platform for additional telomere-associated proteins, mediating signal transduction to DNA damage checkpoint controls. TRF2 mediates proapoptotic signaling in cardiomyocytes and was shown to signal independently of telomere length in endothelial progenitor cells. In progenitor cells, TRF2 was identified as a regulator of clonogenic potential and migratory capacity. In agreement with the literature, it seems likely that TRF2 serves as a regulator of cellular aging and function beyond and potentially independently of protecting telomere length.

The regulation of aortic telomere regulating proteins by exercise was paralleled by an inhibition of the expression of the DNA damage checkpoint kinase, Chk2, and the regulators of cell-cycle progression and survival, p16 and p53. The data agree with reports suggesting a role of these transformation-related proteins downstream of the telomere complex. Our data identify voluntary running as a novel and potent inhibitor of Chk2, p16, and p53 in the vessel wall. To test whether the exercise-induced regulation of the telomere complex and the regulation of p53 represent a coincidence or may be causally related, the experiments were repeated in TERT-deficient mice. Running induced a very similar regulation of the transformation-related proteins in TERT-deficient mice. Stress-induced endothelial cell apoptosis has been suggested to cause endothelial dysfunction and is linked to the
upregulation of p16- and p53-dependent signaling pathways. In cultured endothelial cells, TERT gene transfer reduces replicative senescence and apoptosis. We therefore tested whether the observed upregulation of telomere-protecting proteins would be meaningful by protecting cellular survival in the presence of vascular injury. Two separate sets of experiments applying the bacterial endotoxin lipopolysaccharide and the herbicide paraquat, which induce endothelial cell apoptosis in vivo, revealed that running mice were potently protected from endothelial cell death. This protection may be mediated in part by NO-dependent telomerase activation during lipopolysaccharide stress in the mitochondria. Of note, the only intervention to achieve this marked effect was supplying mice with a running wheel in their cages for only 3 weeks before their intoxication.

One of the hallmarks of signaling induced by exercising is the increased bioavailability of endothelial NO. Reduced NO bioavailability has been proposed as a major component of the endothelial aging process. Recent evidence shows that active eNOS regulates TERT expression in the endothelium. Our experiments show that the observed protective effects were completely absent in eNOS \(^{−/−}\) animals. Functional assays showed a normal endothelium-dependent vasodilation and upregulation of phospho-eNOS in TERT \(^{−/−}\) mice; however, they exhibit reduced protection against endothelial apoptosis by exercising. These data prove the importance of telomere proteins for exercise-mediated endothelial survival benefits. Because the protection by exercise in TERT \(^{−/−}\) was incomplete but fully absent in eNOS \(^{−/−}\) mice, it seems likely that eNOS exerts additional beneficial effects in this situation independently of TERT regulation, eg, benefits related to antioxidant effects. The data are consistent with the concept that exercise-dependent improved vascular stress resistance reduces the need for reparative turnover and thus telomere attrition. Taken together, the data demonstrate the crucial role of eNOS for the exercise-mediated protection against endothelial apoptosis upstream of telomere-regulating proteins.
control group of 18-month-old C57/Bl6 mice exhibited significantly shorter telomeres. Rodents have large telomeres, a fact that impedes the measurement of this parameter. Nevertheless, the regulation of telomerase activity, telomere-regulating factors, and downstream survival signaling may be independent of telomere length.

The functional capacity of bone marrow–derived MNCs and endothelial progenitor cells depends on age-related changes. Thus, the functional improvement seen in murine endothelial progenitor cells after running wheel exercise may be linked to changes in telomere biology. Our experiments show a robust regulation of telomerase activity, telomere-associated proteins, and senescence markers in leukocytes of running mice. The results were comparable in MNCs isolated from blood, bone marrow, and spleen, suggesting a systemic effect on these cells. Parameters of telomere biology in circulating MNCs are associated with the incidence and severity of cardiovascular disease in humans. Recently, Ornish et al showed a positive effect of lifestyle changes on MNC telomerase activity. Importantly, Wilson et al reported that leukocyte telomere length correlates with aortic telomere length. Circulating MNCs from our young athletes and from middle-aged athletes with a long history of endurance exercise were characterized by a profound upregulation of telomerase activity and telomere proteins and downregulation of proapoptotic proteins compared with untrained individuals. In agreement with the animal data, this regulation occurred in the absence of a detectable change in telomere length in the young athletes. Recently, Cherkas and colleagues reported a positive correlation between leukocyte telomere length and physical activity in 2401 twin volunteers. Our study population may have been too small and too young to detect subtle differences in telomere length, but the data show that beneficial antisenescent effects of physical activity are observed more rapidly than effects on telomere length itself. Indeed, long-term vigorous physical exercise in the older athletes is associated with a conservation of telomere length, as shown by the use of 2 independent methods of telomere length measurement, namely Flow-FISH assays and quantitative real-time PCRs. The published data on the effects of exercise on vascular telomere length in older subjects are limited. One study in a cohort of Chinese individuals >65 years of age reported no differences in leukocyte telomere length between physically active and inactive participants. Another small study in 16 obese middle-aged women showed no differences in leukocyte telomere length after 6 months of aerobic exercise training. However, these studies may have been limited by the use of questionnaires to detect physical activity or by the late onset of exercise. Presumably, the continuity, cumulative

Figure 8. Long-term exercise training activates MNC telomerase and prevents telomere attrition. Telomere biology in young (n = 26) and older (n = 21) untrained volunteers vs young (n = 32) and older (n = 25) athletes. A, Human telomerase activity measured by telomere repeat amplification protocol. B, Lymphocyte telomere length measured by Flow-FISH assays. C, Representative fluorescence-activated cell sorter scan for an individual from the older control group (top row) and older athlete group (bottom row). Left column, Scatterplot with lymphocyte gate; middle column, cells gated by FSC vs intensity of telomere staining (FITC channel); right column, histogram analysis of telomere FITC intensity vs median intensity. D, Granulocyte telomere length measured by Flow-FISH assays. E, MNC telomere length determined by real-time PCR. FSC indicates forward scatter; SSC, sideways scatter. **P < 0.001.
duration, and intensity of endurance training were significantly higher in our study population.

Our study has limitations. We cannot rule out that the extent of telomere shortening in the older control group is due in part to an unknown selection bias. However, the key novel finding is the protection against telomere shortening by intensive exercise that is independent of the absolute telomere length of the control group. As expected, the athletes were characterized by a lower resting heart rate, blood pressure, and body mass index and a more favorable lipid profile. In the mouse studies, the regulation of telomere proteins was independent of blood pressure, heart rate, or lipid profile; however, in our clinical study, we cannot determine whether or to what extent the exercise-induced beneficial effects on metabolism, heart rate, and blood pressure affect the telomere proteins compared with exercising itself. It is conceivable that long-term exercise training has protected their cardiovascular system by increasing stress resistance and their maintenance systems in a way that vascular damage and subsequent reparative endothelial cell turnover over the years have been reduced and thus telomere attrition has been minimized. On the other hand, because mice lacking the RNA component of telomerase develop hypertension,13 one could speculate that exercise-induced long-term upregulation of telomere-stabilizing proteins may exert direct beneficial vascular effects in humans.

Conclusions

The data identify voluntary physical exercise as a powerful intervention to upregulate telomere-stabilizing proteins in circulating cells and the vasculature, thereby protecting against endothelial apoptosis in mice. In agreement with the animal data, long-term continuous exercising leads to an attenuation of telomere erosion in the leukocytes of middle-aged athletes. Our data improve the molecular understanding of the vasculo protective effects of exercise and underline the potency of physical training in reducing the impact of age-related diseases.

Acknowledgments

We thank Ellen Becker, Simone Jüger, and Irina Hartmann for excellent technical assistance and Dr Stefan Gräber from the Institute of Medical Biometry, Epidemiology and Medical Informatics of the Universitätsklinikum des Saarlandes for help with the statistical analyses.

Sources of Funding

This study was supported by the Deutsche Forschungsgemeinschaft (KFO 196), the Universität des Saarlandes (HOMFOR), the Ministerium für Wirtschaft und Wissenschaft des Saarlandes, and the European Stroke Network.

Disclosures

None.

References

Telomere erosion is a central component of aging, and telomere-associated proteins regulate cellular senescence and survival. To elucidate the cellular mechanisms of the vasculoprotective effects of physical exercise, mice were randomized to voluntary running or no running wheel conditions. Exercise upregulated telomerase activity and telomere regulating proteins in the thoracic aorta and in circulating mononuclear cells compared with sedentary controls and reduced the expression of vascular apoptosis regulators such as cell-cycle–checkpoint kinase 2, p16, and p53. Mice preconditioned by voluntary running exhibited a marked reduction in lipopolysaccharide-induced endothelial apoptosis. Transgenic mouse studies showed that endothelial nitric oxide synthase and telomerase reverse transcriptase synergize to confer endothelial stress resistance after physical activity. To test the significance of these data in humans, telomere biology in circulating leukocytes of young and middle-aged track and field athletes was analyzed. Peripheral blood leukocytes isolated from endurance athletes showed increased telomerase activity, expression of telomere-stabilizing proteins, and downregulation of cell-cycle inhibitors compared with untrained individuals. Older athletes were characterized by reduced leukocyte telomere erosion compared with untrained controls. We therefore conclude that physical activity represents an “antiaging” intervention mediating cellular antisenescent and antiapoptotic vascular effects.
A – Expanded Materials and Methods

Animals

Animal experiments were approved by the animal ethics committee of the Universität des Saarlandes and conformed with NIH Pub. No. 85-23, revised 1996.

Telomerase activity

Telomerase activity was quantified using the Telomerase Repeat Amplification Protocol.\(^1\)\(^-\)\(^4\) Protein extracts (1µg) of mouse thoracic aorta or 10,000 mononuclear cells in 1 x CHAPS lysis buffer (Trapeze, MP Biochemicals, Germany), 0.1µg of Primer TS (template), 0.05µg Primer ACX in 20µl Lightcycler Fast Start SYBR Green PCR Master Mix (Roche, Mannheim, Germany) containing 1.5mM MgCl\(_2\) were incubated at 30°C for 30 minutes to allow template elongation by telomerase activity. After immediate transfer to the Lightcycler instrument (Roche, Mannheim, Germany), telomerase activity was terminated and hotstart DNA polymerase activated by incubation at 95°C for 10 minutes. 40 cycles of amplification were carried out with 20s at 95°C, 30s at 60°C and 50s at 72°C. Protein extracts from human embryonic kidney (HEK 293, Gibco, Karlsruhe, Germany) cells were measured as positive controls in each assay (intra-assay variability 5.2%). A standard titration curve of HEK 293 cells was established from 0 to 10,000 cells to ensure linearity of the assay (\(R^2=0.99\), Suppl. Fig. 3A). Furthermore, serial dilutions of aortic protein extracts, Suppl. Fig. 3B) and human MNCs (5,000 to 20,000 cells, Suppl. Fig. 3C) were tested to ensure linearity of the PCR reaction. A positive result of the telomerase assay was considered if the quantity of telomerase activity was 3 times above the standard deviation of the mean negative control’s background level (1 x CHAPS lysis buffer). To reduce the risk of systematic errors in the human study samples from each of the four experimental groups were present in each run.

Telomere length analysis by Flow-FISH assays

Telomere length was determined by the Flow-FISH method.\(^1\)\(^\,\)\(^2\)\(^\,\)\(^5\) In brief, 600,000 peripheral blood leukocytes from athletes and controls were washed once (5% dextrose, 0.1% BSA, 10mM HEPES) and resuspended in hybridisation buffer (75% deionised formamide, 20mM Tris (pH 7.1), 20mM NaCl, 1% BSA) for 10 minutes. After denaturing cells, 90 minutes incubation with
either no probe (unstained control) or 0.18µg FITC labelled, telomere-specific \((C_3TAR)_3\) DNA probe (Cambridge Research Biochemicals, Cleveland, UK) was performed. After 3 rounds of washing (75% deionised formamide, 0.1% BSA, 10 mM Tris, 0.1% Tween 20), cells were resuspended in PBS, 0.1% BSA, RNase A at 10µg/ml and 0.1µg/ml LDS 751 (Exciton, Ohio, USA) for DNA counterstaining. Proper gating and DNA counterstaining with LDS751 ensured that only single, mononuclear and euploidic cells were analyzed (Online Suppl. figure 2). Flow cytometric analysis was carried out on a FACSCalibur (Becton Dickinson, Heidelberg, Germany). Telomere length was expressed as mean fluorescent signal intensity. To control for interday variation, FITC-labelled beads (Quantum-FITC low level, Polysciences, Eppelheim, Germany) with defined amounts of fluorescence (MESF = molecules of equivalent soluble fluorochrome) were run in parallel each day. To further reduce the risk of systematic errors all study samples were measured on only two experimental days. In addition, a reference MNC sample isolated from a donor not included in the study was run in parallel for each experiment and yielded identical median fluorescence intensities in all runs. A previously established standard curve \(^2\) was used for the conversion of the median telomere fluorescence intensities into kilobase pairs (kbp).

**Histological telomere length analysis by quantitative fluorescence in-situ hybridization**

Telomere length is directly related to its integrated fluorescence intensity when marked with a specific telomere peptide nucleic acid (PNA) probe using quantitative fluorescence in-situ hybridization as described.\(^6\) 5µM cryosections of the thoracic aorta were fixed in 4% paraformaldehyde for 30min at room temperature followed by treatment with 0.1% Pepsine solution (Sigma-Aldrich, Germany) for 10min at 37°C. After washing and dehydration, 25µl of the hybridisation mix (70% formamide, 0.13µg Cy3-conjugated telomere-specific PNA probe \((C2TA3)_3\) (Applied Biosystems, Germany), blocking reagent (Roche, Germany), MgCl2 buffer and 1mM Tris pH 7.2) was added to the sections. Denaturation was carried out at 80°C for 3min and preceded incubation for 2 hours in a humid chamber and washing with wash buffer (70% formamide, 10mM Tris pH 7.2, 0.1% bovine serum albumin), tris-buffered saline (+ 1% Tween) and phosphate-buffered saline for a total of 1 hour. Nuclei were then counterstained with DAPI. All sections were analyzed using a Nikon E600 epifluorescence microscope (Nikon, Germany) by an investigator blinded to the study. At least 3 representative images of the Cy3 and DAPI channel from each section were recorded at 1000 X magnification with Lucia G software with
identical exposure times. Telomere fluorescence intensity was calculated with TFL-Telo freeware version 2.2.07.0418 – 2002 (URL: http://www.bccrc.ca/tfl/research_lansdorp/Applications.htm).7

Measurement of telomere length by real-time PCR
Genomic DNA was extracted from the mononuclear cells of athletes and controls with the QIamp DNA blood mini kit (Qiagen, Germany) according to the manufacturer’s protocol. Mononuclear cell telomere length was measured with a real-time PCR-based assay that compares telomere repeat sequence copy number to the single-copy gene 36b4.8 During optimisation of the assays, a six-point standard curve (two-fold dilution from 40 ng - 1.25 ng) was run from a pool of 10 control DNAs for both the telomere and 36b4 PCRs to ensure linearity of the reaction (R^2 > 0.99). PCR efficiencies for target and reference gene were approximately equal (data not shown). In each run 20ng of sample DNA was measured in duplicate. The pooled control DNA (20ng) was tested in all assays to allow comparability of the results. The mastermix for each 25µl PCR reaction was prepared with 12,5 µl Platinum SYBR Green qPCR Supermix-UDG (Invitrogen, Germany), 0.5 µM/l ROX reference dye, 150 nmol/L of telomere-specific primers (Forward: CCGTTTGTTTGGTTTGGTTTGGTTTGGTT; Reverse: GGCTTGCTTACCCTACCTACCCTTACCCTACCTTACCCT) or 100 nmol/L 36b4 primers (Forward: CAGCAAGTGGAAGGTTAATCC; Reverse: CCCATTCTATCATCAACGGGTACAA).9 All Real-Time PCR reactions were carried out on a 7900 HT Sequence Detector (Applied Biosystems, Germany). The thermal cycling profile for both reactions consisted of a 95°C activation step, followed by 40 cycles of 95°C for 15 seconds and 58°C for 60 seconds. The PCR data were analyzed as age- and gender-matched pairs with the comparative Ct method (2^{ΔΔCt}) to calculate the relative differences in the amount of telomere repeat DNA between the athletes and their corresponding controls.

Western blot analysis
Aortic tissue from mice or mononuclear cells from mice and humans were homogenized with 200µl lysis buffer (100mM Tris pH 6.8, 4% SDS, 20 Glycerol) containing the protease inhibitors PMSF 0.1mM, leupeptin 0.5µl and Aprotonin 0.5µl. 50µg protein were separated on 10% SDS–PAGE. Proteins were transferred to nitrocellulose membranes (Biorad 162-0112), blocked with 5% dry milk for 30 minutes and exposed to rabbit polyclonal IgG TRF2 (H-300 Santa Cruz, sc-9143, dilution 1:200), mouse monoclonal IgG P16 (F-12 Santa Cruz, sc-1661, dilution 1:250),
rabbit polyclonal IgG anti-P53 (FL-393 Santa Cruz, sc-6243, dilution 1:500 in dry milk 1%), mouse monoclonal IgG Chk2 (A-11 Santa Cruz, sc-17747, dilution 1:1000 in dry milk 1%) and mouse monoclonal IgG GAPDH (6C5 Santa Cruz, sc-32233, dilution 1:1000), rabbit polyclonal IgG eNOS (Acris Antibodies, Germany) and mouse monoclonal IgG p-eNOS S1177 (BD1250 Acris Antibodies, Germany). For analysis of TERT, immunoprecipitation was performed on 150µg total protein in HNTG buffer (20mM HEPES, 150mM NaCl, 0.1% Triton-X-100, 10% Glycerol) using polyclonal IgG anti-TERT (H-231 Santa Cruz, sc-7212) and Agarose-A Protein goat anti-rabbit IgG (Sigma, Germany). Immunodetection was accomplished using goat anti-rabbit IgG (1:4000 dilution) (Sigma, Germany) and goat anti-mouse IgG (1:5000 dilution) (Biorad, Germany) secondary antibodies, and an enhanced chemiluminescence kit (ECL, Amersham).

**cGMP Measurement by ELISA**

For cGMP measurements 2 mm² pieces from murine aortas were frozen in liquid nitrogen and homogenized. The obtained powder was dissolved in 5% trichloroacetic acid. After centrifugation, trichloroacetic acid was removed from the supernatant by ether. Equal amounts of the supernatant were used in a commercially available cGMP ELISA according to the manufacturer’s instructions (IBL Immuno-Biological Laboratories, Hamburg), and cGMP levels were obtained using a standard included in the assay.

**Aortic Ring Preparations and Tension Recording**

After excision, the descending thoracic aorta was immersed in Tyrode's solution (118 mM NaCl, 2.5 mM CaCl₂, 4.73 mM KCl, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 2.5 mM NaHCO₃, 0.026 mM Na EDTA, and 5.5 mM D-(+)
Glucose, pH 7.4). Adventitial tissue was carefully removed. Per animal, four 3mm rings were mounted in organ bath chambers filled with Tyrode's solution (37°C, continuously aerated with 95% O₂ and 5% CO₂) and were attached to a force transducer recording isometric tension. Aortic rings were gradually stretched to a resting tension of 10 mN, which was maintained throughout the experiment, and were allowed to equilibrate for further 30 min.. Pharmacologically induced contraction of aortic rings was performed with a β-agonist, Phenylephrine-HCl (5 µM). Drugs were added in increasing concentrations to obtain cumulative concentration-response curves for carbachol (carbamylcholine-chloride, 1 nM–100 µM) as an endothelium-dependent relaxing agent, and glyceryl trinitrate (1 nM–10 µM) as an NO donor.
The drugs were washed out before adding the next substance. The relaxing effect of carbachol was abolished by adding N-nitro-L-arginine methyl ester (1 µM).

**RT-PCR**

Reverse transcription-polymerase chain reaction standardized to GAPDH was performed using the following primers (designed to match mouse cDNA unless otherwise specified):

- **TRF1-for** 5'-CAT GGA CTA CAC AGA CTT AC-3'  55°C, 27 cycles
- **TRF1-rev** 5'-ATC TGG CCT ATC CTT AGA CG-3'
- **TRF2-for** 5'-TGT CTG TCG CGC ATT GAA GA-3'  55°C, 26 cycles
- **TRF2-rev** 5'-GCT GGA AGA CCT CAT AGG AA-3'
- **TRF2-human-for** 5'-TGT CTG TCG CGG ATT GAA GA-3'  53°C, 35 cycles
- **TRF2-human-rev** 5'-GCT GGA AGG TCT CAT ATG AA-3'
- **Ku70-for** 5'-GAG CAT CCA GTG TAT CCA GA-3'  55°C, 26 cycles
- **Ku70-rev** 5'-CAG CAT GAT CCT CTT GTG AC-3'
- **Ku80-for** 5'-TCA CAG TGT GCA GAC ACC TG-3'  56°C, 26 cycles
- **Ku80-rev** 5'-AAC TGC AGA GAG ATG CCA AA-3'
- **Ku70-human-for** 5'-GAG CAT CCA GTG TAT CCA AA-3'  52°C, 35 cycles
- **Ku70-human-rev** 5'-CAG CAT GAT CCT CTT ATG AC-3'
- **Ku80-human-for** 5'-TCA CAG TGC ACA GAC ATC TG-3'  56°C, 40 cycles
- **Ku80-human-rev** 5'-AAT TGC AGA GAG ATG TCA CA-3'
- **Chk2-human-for** 5'-GCA GCA GTG CCTGT CAC A-3'  55°C, 35 cycles
- **Chk2-human-rev** 5'-TGG ATA TGC CCT GGG ACT GT-3'
- **GAPDH-for** 5'-ACC ACA GTC CAT GCC ATC AC-3'  60°C, 30 cycles
- **GADPH-rev** 5'-TCC ACC ACC CTG TTG CTG TA-3'

**Quantification of apoptosis by hairpin oligonucleotide assay**

To detect apoptosis, 3 µm thick paraffin sections of formalin-fixed murine thoracic aortae were stained using the ApopTag Peroxidase In Situ Oligo Ligation Kit (Millipore). This in-situ oligoligation assay specifically detects apoptosis by staining only cells that contain double-stranded breaks that are blunt-ended or have a one base 3' overhang (apoptosis). Cells containing nicked, gapped, 3'-recessed, 3'-overhanging ends longer than one base and single-stranded ends are not detected. Thus, unlike conventional terminal transferase-based labelling (TUNEL), the hairpin assay specifically stains apoptotic but not necrotic or transiently damaged cells.
References for Materials and Methods


Supplementary Figure 1 –

No effects of exercise on aortic telomere biology and apoptosis regulators in eNOS\(^{-/-}\) mice

**Suppl. Figure 1**
Quantification of the aortic effects of voluntary exercise for 3 weeks in B6.129/P2-Nos3 mice on (A) TRF1 mRNA (p=0.377), (B) Ku70 mRNA (p=0.774), (C) Chk2 (p=0.711) and (D) p16 protein (p=0.748) expression. Standardization for GAPDH. N=8 per group.
Supplementary figure 2 – Standard gating procedure for Flow-FISH assays

A - FACS gates for the fluorescent reference beads

B - FACS gates for the negative control (no telomere probe)

C - All FACS gates for a representative aged control (lymphocytes)

D - All FACS gates for a representative aged athlete (lymphocytes)

Suppl. figure 2

(A) Beads: Microbeads of defined size and fluorescence are depicted in scattergraphs. Median intensity values are measured for each bead (M1-M5) and settings are adjusted to allow comparable measurements between runs. Bead fluorescence intensity correlates with molecules of soluble fluorochrome (MESF, $R^2>0.99$); (B) Neg. controls: MNCs with LDS751 but no PNA probe are present in each run; lymphocytes / granulocytes are gated, marked by a weak FITC autofluorescence and DNA staining. The histogram shows a minor FL1-intensity below 10 arbitrary units. (C and D) Gating for an aged control and an aged athlete: R1 – lymphocytes / granulocytes; R2 – cells with DNA stain are gated; R3 – cells with high LDS fluorescence are excluded (cell clusters); R4 – LDS751-stained cells with sufficient PNA hybridization are selected. Analysis histograms show mean fluorescence intensity values of cells in R1-R4 (ranging between 40 and 120 arbitrary units).
Supplementary figure 3 – Standard curves for Telomere-Repeat Amplification Protocols

Suppl. figure 3

Telomerase activity was measured by TRAP assay in serial dilutions of (A) 0-10,000 HEK 293 cells, (B) 0.31-2.5µg aortic CHAPS protein lysates and (C) 1,000-20,000 human MNCs. Correlation coefficients ($R^2$) were > 0.99 for all experiments.
**Nuclear Redox Signaling**


**Antioxid Redox Signal 12: 713-42, 2010**

Autoren:

M. Lukosz: Erstautorin, schrieb den Teil über nukleäre Redoxregulatoren und redoxregulierte Transkriptionsfaktoren.


**N. Büchner:** Erstellte die Abbildungen und Legenden

T.C. Zschauer: Führte Immunfärbungen durch und erstellte die dazugehörigen Abbildungen und schrieb den Teil zu APEX-1 und Glutaredoxin.

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Nuclear Redox Signaling

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Abstract

Reactive oxygen species have been described to modulate proteins within the cell, a process called redox regulation. However, the importance of compartment-specific redox regulation has been neglected for a long time. In the early 1980s and 1990s, many in vitro studies introduced the possibility that nuclear redox signaling exists. However, the functional relevance for that has been greatly disregarded. Recently, it has become evident that nuclear redox signaling is indeed one important signaling mechanism regulating a variety of cellular functions. Transcription factors, and even kinases and phosphatases, have been described to be redox regulated in the nucleus. This review describes several of these proteins in closer detail and explains their functions resulting from nuclear localization and redox regulation. Moreover, the redox state of the nucleus and several important nuclear redox regulators [Thioredoxin-1 (Trx-1), Glutaredoxins (Grxs), Peroxiredoxins (Prxs), and APEX nuclease (multifunctional DNA-repair enzyme) 1 (APEX1)] are introduced more precisely, and their necessity for regulation of transcription factors is emphasized. Antioxid. Redox Signal. 12, 713–742.

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Reviewing Editors: Aron Fisher, Pascal Goldschmidt-Clermont, Arne Holmgren, Toren Finkel, Junichi Sadoshima, Albert van der Vliet, and Junji Yodoi

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interplay of ONOO$^-$ irreversibly to form 3-nitrotyrosine (95). The ONOO$^-$ acts with target molecules even in neighboring cells.

Because it is readily converted to two other radical species, ONOO$^-$ is reduced by superoxide dismutases (SODs) to H$_2$O$_2$. H$_2$O$_2$ is then metabolized to H$_2$O by several enzymes, mainly by catalase, but also by glutathione peroxidase (GPx), which in turn results in oxidation of glutathione. The glutathione disulfide (GSSG) itself is then reduced to glutathione by the glutathione oxidoreductase (GR) to feed back into this cycle (Fig. 2). Another important antioxidative system, the thioredoxin/thioredoxin-reductase (Trx/TR) also has been described to metabolize H$_2$O$_2$ directly. However, this seems to be a rather rare event in cells, and the Trx/TR system mainly reduces oxidized proteins. In the course of this process, reduced Trx itself is oxidized. Reduced Trx is then regenerated by TR by using the cofactor NADPH to be further available for the reduction of oxidized proteins (Fig. 3).

Therefore, a controlled redox balance exists in cells. Perturbation of this balance either by increased production of ROS or by reduced antioxidative capacity will result in so-called oxidative stress (Fig. 1B). Increased ROS lead to modifications of biological molecules, including proteins, DNA, and lipids. In line with this, under conditions of oxidative stress, O$_2^-$ can directly react with nitric oxide (NO) to form peroxynitrite (ONOO$^-$), which readily crosses cell membranes. Although possessing a short half-life, ONOO$^-$ interacts with target molecules even in neighboring cells. Peroxynitrite is involved in protein nitration by nitrating tyrosine residues irreversibly to form 3-nitrotyrosine (95). The interplay of ONOO$^-$ with ROS to damage cells is then termed nitrosative stress. ONOO$^-$ itself is extremely toxic to cells because it is readily converted to two other radical species, OH$^-$ and NO$_2^-$, by hemolytic decomposition (92). Therefore, these species are often referred to as ROS/RNS (reactive nitrogen species). Oxidative and nitrosative stress–induced modifications of biological molecules have been implicated in a variety of diseases, such as cardiovascular diseases, neurologic disorders, and cancer. Over a long period, it was believed that the production of ROS is, in principle, bad for cells, and therefore, the term “redox signaling” was underestimated. However, several lines of evidence have established that redox signaling exists and is required for organisms to survive. Moreover, ROS have a wide range of action because of their ability to interact with almost all biological molecules. The mode of action of ROS is also dependent on the localization of their target molecules. Thus, the modifications induced by ROS can be separated depending on the cellular compartments they affect. Several studies investigated whether mitochondrial and nuclear redox signal new exist, in addition to the well-described cytosolic (previously termed cellular) redox-dependent events. Under physiologic conditions, ROS can induce changes in gene expression (203), whereas under conditions of oxidative and nitrosative stress, ROS and RNS can directly damage DNA in the nucleus, which can result in apoptosis or malignant transformation (140, 233).

This review focuses on nuclear redox signaling and oxidative stress–induced nuclear translocation of proteins and their resultant nuclear modes of actions. The antioxidative enzymes, Trx-1 and the APEX nuclease (multifunctional DNA repair enzyme) 1 (APEX1, also known as Ape/Ref-1), and their important role in nuclear redox signaling are highlighted. A number of proteins, which belong to the classes of transcription factors, chromatin-modifying enzymes, kinases, and phosphatases, are discussed in detail. All of the mentioned proteins have in common that they have a nuclear localization and function and are redox regulated or regulated by oxidative stress in higher eukaryotes. Although this review tries to be as comprehensive as possible, we are aware that we might not have discussed all of them.

II. Nuclear Redox Regulators

A. Redox state of the nucleus

The term redox state describes the balance between the oxidized and reduced forms of biologically relevant redox pairs, including NADH/NAD$^+$, NADPH/NADP$^+$, and GSH/GSSG. It is reflected in the balance of several sets of
metabolites (e.g., lactate and pyruvate) whose interconversion is dependent on these ratios. An abnormal redox state can develop in a variety of deleterious situations, such as hypoxia, shock, and sepsis. Determination of the relative concentrations of the components of these redox pairs showed that the GSH/GSSG and NADPH/NADP⁺ ratios are >1 (234, 236), whereas the NADH/NAD⁺ ratio is <1 (221, 261). Generally, the nucleus provides a reductive environment.

NAD⁺/NADH is required as a coenzyme for metabolic processes. The high NAD⁺/NADH ratio allows this coenzyme to act as both an oxidizing and a reducing agent. In contrast, the main function of NADP⁺ is as a reducing agent in anabolism. Since NADPH is needed to drive redox reactions as a strong reducing agent, the NADPH/NADP⁺ ratio is kept high. Because no barrier exists to diffusion of these dinucleotide coenzymes across the nuclear membrane (160, 261), it is assumed that the NAD⁺/NADH and NADP⁺/NADPH ratios are the same in the cytoplasm and the nucleus. The majority of these coenzymes are protein bound, and the concentration of free NADH in the nucleus has been calculated to be 130 nM (261). It is assumed that the NADPH/NADH ratio is *4 (210); therefore, the concentration of free NADPH in the nucleus must be *500 nM.
Glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is a ubiquitous thiol tripeptide and is the most abundant thiol present inside the cell. GSH has multiple direct and indirect functions in many critical cellular processes like synthesis of proteins and DNA, amino acid transport, enzyme activity, and metabolism (149). GSH also serves as a reductant to destroy free radicals, hydrogen peroxide, and other peroxides and as a storage form of cysteine. The redox state of the GSH/GSSG couple is often used as an indicator of the overall redox environment of the cell (200). GSH is found in a fairly high concentration of ~10 mM within cells. As it can freely diffuse (160, 172), the cytoplasmic and nuclear concentrations are similar (215, 236). Interestingly, the nuclear levels change during the cell cycle, with the highest levels found in the S and G2/M phases (137). In line with these findings, depletion of GSH leads to reduced proliferation and apoptosis (138, 151). Taken together, one would hypothesize that during the G2/M phase, a more reductive nuclear environment is required for cell proliferation to proceed. This was corroborated by a combined *in vitro*/*in vivo*informative investigation, which showed that 69 proteins containing redox-sensitive motifs have functions in central cell cycle processes like transcription, nucleotide metabolism, (de)phosphorylation, and (de)ubiquitylation. The majority of these oxidant-sensitive proteins function during the G2/M phase, indicating that oxidant-sensitive proteins may be temporally regulated by oscillation of the intracellular redox environment (39).

**B. Antioxidative enzymes in the nucleus**

As mentioned earlier, this review focuses on nuclear redox signaling. Therefore, it is important to introduce the antioxidative systems that have been described to be localized in the nucleus. Already in the late 1980s, Cu/Zn SOD and catalase were detected in the nuclear fractions of the developing rat cerebral cortex (43). In contrast, in adult Langerhans cells in the nuclear envelope (220). Several lines of evidence demonstrated that, under certain conditions, Trx-1 accumulates in the nucleus and to be present in their active forms (88). These findings point out that the presence of catalase in the nucleus seems to be dependent on cell type and perhaps also on age.

Several studies investigated the nuclear localization and activity of enzymes of the glutathione system, including GR, GPxs, and glutathione S-transferases (GSTs). In rat liver cells, all proteins were found to be localized in the nucleus. Moreover, enzymatic activity could be measured in nuclear extracts (191). Recently, the exact localization of GSTa-2 was determined to be at the nuclear membrane. Thus, the authors speculated that GSTa-2 probably has a role as a defense barrier at the nuclear envelope (220). Several lines of evidence demonstrated that, at the end of a β-strand and in the beginning of an α-helix (88). This structure defines a large superfamily of proteins (124). It is adopted by bacterial glutaredoxins (Grx) and appears in the other members of the family as a substructure or domain (9, 50, 139). Besides Trxs and Grxs, protein disulfide isomeras, GSTs, GPxs, peroxiredoxins, and chloride intracellular channels (CLICs) are members of the Trx superfamily (12, 139, 202). Mammalian cells contain two Trxs, Trx-1 and Trx-2 (exclusively localized in mitochondria) and three Trxs: cytosolic TR1, mitochondrial TR2, and the testis-specific thioredoxin glutathione reductase (TGR). In 1985, Holmgren (87) introduced Trx-1 as a small, ubiquitous protein with two redox-active cysteine residues in an exposed active center, having the same amino acid sequence as *E. coli* Trx-Cys-Gly-Pro-Cys- (Cys 32 and Cys 35 within Trx-1), which is essential for its redox-regulatory function. The thioredoxin-1 system consists of Trx-1 and TR1. The regeneration of reduced Trx-1 from its oxidized form is catalyzed by TR1 by using NADPH (162). One important function of Trx-1 is the reduction of oxidized proteins, which depends on cysteine 32 and cysteine 35. The functions of Trx-1 are dependent on its cellular localization. Cytosolic Trx-1 interacts with its active-site cysteines with several proteins by forming disulfide bridges and thereby inhibits apoptosis induction. Trx-1 associates with ASK1 and the vitamin D3–upregulated protein 1 (Txnip, also named VDU-P1) (194, 258). Thereby, reduced Trx-1 protects cells from apoptosis through an inhibitory binding to ASK1, whereas this binding is lost when Trx-1 is oxidized (194). Similarly, binding of Trx-1 to Txnip completely abrogates the antiproliferative function of Txnip (204). It has been demonstrated that, under certain conditions, Trx-1 accumulates in the nucleus and directly or indirectly interacts with different transcription factors (203). In the case of direct association, Trx-1 reduces oxidized transcription factors and thereby allows them to interact with DNA. The capacity of Trx-1 to interact with several transcription factors and thereby alter their functions is discussed in more detail later in this review. In case of an indirect action on transcription factors,
D. Glutaredoxins (Grxs) and peroxiredoxins (Prxs)

Glutaredoxins (Grxs) belong to the Trx superfamily of proteins. Grxs were first described as glutathione-dependent reductases three decades ago (86). They play an important role in cellular redox-dependent processes, mainly through deglutathionylation of proteins. In addition to the early-discovered dithiol Grxs, another group of Grxs has been identified. These monothiol Grxs lack the C-terminal active-site cysteine but contain all structural and functional elements to bind and use GSH. Therefore, two distinct mechanisms are known, the dithiol reaction in which the two cysteines in the active center take part in reducing both low-molecular-weight and protein disulfides and the monothiol mechanism in which only one cysteine in the active center is present (for review, see ref. 123).

In humans, four Grx isoforms have been identified: the dithiol Grx1 and Grx2 and the monothiol isoforms Grx3 (PICOT/TXNL-2) and Grx5 (94, 131, 249, 250). Grx1 is considered mainly to be a cytosolic protein, although some studies show a nuclear localization (133, 219) and a presence in the intermembrane space of mitochondria (171). Several studies have shown that Grx1 is able to deglutathionylate a variety of proteins, like actin, protein tyrosine phosphatase-1B, glyceraldehyde-3-phosphate dehydrogenase, Ras, and caspase 3 (3, 19, 126, 173, 240) and thereby influences several important cellular functions, including actin polymerization, hypertrophy, and apoptosis (3, 173, 240, 242). Furthermore, Grx1 regulates transcription factor activity directly as well as indirectly, which is discussed later in this review in the respective transcription factor sections. Grx2 is known to exist in at least three different splicing variants, Grx2a, Grx2b, and the newly discovered Grx2c. Grx2a is considered a mitochondrial protein, and Grx2b was detected in nuclear fractions (62, 131). A recent screening of diverse tissues showed a ubiquitous expression of Grx2a, whereas Grx2b and Grx2c were found exclusively in testis and some cancer cell lines (129). Grx3, also termed PICOT (protein kinase C–interacting cousin of thioredoxin), was first identified in 2000 as an interaction partner of protein kinase Cθ (PKCθ) and described to exist in the cytosol (250). Grx5 is a monothiol enzyme with a mitochondrial localization signal and therefore resides within the mitochondria (190). Thus, only Grx1 and Grx2b are localized in the nucleus, and regulation of transcription factor activity in the nucleus has been demonstrated only for Grx1.

Peroxiredoxins (Prxs) are members of a superfamily of Se-independent peroxidases. Six members have been identified and characterized in mammals. Prxs execute enzymatic degradation of H$_2$O$_2$ and organic hydroperoxides by using electrons donated by Trx-1 (Prx1 to Prx5), cyclophilin A (Prx1 to Prx4), or glutathione (Prx6). Prxs are divided into three classes: typical 2-Cys Prxs; atypical 2-Cys Prxs; and 1-Cys Prxs (for review, see refs. 102 and 187). These enzymes share the same basic catalytic mechanism, in which the single, redox-active cysteine is oxidized to a sulfenic acid by the peroxide substrate (38). The recycling of the sulfenic acid back to a thiol distinguishes the three enzyme classes: 2-Cys Prxs are reduced by thiols, particularly thioredoxin (188), whereas the 1-Cys enzymes are reduced by glutathione (136) and ascorbic acid (152). By using crystal structures, a detailed catalytic cycle has been derived for typical 2-Cys Prxs, including a model for the redox-regulated oligomeric state proposed to control enzyme activity (251). Prxs have a wide tissue distribution and specific subcellular localization. Nuclear localization has been demonstrated for Prx1, Prx2, Prx4, Prx5, and Prx6, at least in tumor cells (109). However, only for Prx5 has a nuclear function been demonstrated. Nuclear Prx5 significantly reduced nuclear DNA damage induced by H$_2$O$_2$ (15).

![FIG. 4. APEX1. General model of APEX nuclease (multifunctional DNA-repair enzyme) 1 (APEX1)](image-url)
The second abbreviation, Ref-1, stands for redox effector factor-1 and reflects its function as a redox regulator of transcription factors (Fig. 4). Through its redox function, APEX1 maintains transcription factors in an active, reduced state required for DNA binding and transcriptional activation. The two functions of APEX1, repair and redox regulation, are independent and located in separate domains of the protein, which was demonstrated by deletion analysis. The N-terminal portion of APEX1 that is not present in functionally related proteins from other organisms is required for the redox activity, whereas the DNA-repair activity requires conserved C-terminal sequences. Chemical alkylation or oxidation of cysteines inhibits the redox activity of APEX1 without affecting its DNA-repair activity (255). In addition, mutation of cysteine residue 65 (Cys 65), which is unique to mammalian APEX1, abrogates the redox function of the human protein. The wild-type zebrafish APEX has a threonine residue in the corresponding position (Thr 58) and is redox inactive, but can be converted to a redox enzyme by conversion of Thr 58 to cysteine (59). Conversely, when Cys 65 in human APEX1 is converted to alanine, the resulting protein is redox deficient (132).

Two reports uncovered a third function of APEX1, the stimulation of transcription factor reduction independent of its intrinsic redox activity. In vitro analysis of mouse APEX1 mutated at the cysteine previously identified as the redox catalytic site revealed a surprising result. Unlike APEX1-null mice, which die very early in embryonic development, homozygous APEX1(64A) mice were viable with no overt phenotype. Although APEX1 is the major redox regulator of activator protein 1 (AP-1) in murine cells, AP-1 DNA binding activity and reduction of Fos and Jun were unaltered compared with wild-type mice, demonstrating that the redox activity of APEX1 is not required for redox regulation of AP-1 (169). A more-robust proof was obtained by the analysis of human APEX1, in which all seven cysteine residues were substituted to serine (APEX C/S; Fig. 5). It was demonstrated that APEX1 can directly reduce in vitro oxidized recombinant p50, a subunit of nuclear factor-kappa B (NF-kB), at relatively high concentrations (i.e., at concentrations >50-fold higher than that of p50). However, when Trx-1 or GSH was included in the reaction, a much lower concentration of APEX1 was sufficient to stimulate p50 DNA binding activity, indicating that APEX1 might facilitate reduction of transcription factors by other reducing molecules such as Trx-1. Surprisingly, unlike the direct reduction of p50, this effect was not dependent on the redox activity of APEX1 because APEX1 C/S increased p50 DNA binding activity as efficiently as APEX1 wt in the presence of GSH or Trx-1. This activity of APEX1 was not restricted to the NF-kB transcription factor complex, as also the DNA binding activity of AP-1 was stimulated by APEX1 C/S. Interestingly, a physical interaction of APEX1 and the C/S mutant occurs only with one subunit of these heterodimeric transcription factors (with p50, p52, c-Rel, and c-Jun, but not with p65 and c-Fos) (8).

Thus, APEX1 binding to certain subunits of heteromeric transcription factors leads to reduction of the transcription factor subunits by other reductive systems. Therefore, APEX1 can function as a redox chaperone. This activity may be important for the modulation of the activity of a subset of transcription factors.

F. Trx-1/APEX1 interactions

A direct interaction between overexpressed APEX1 and Trx-1 has been demonstrated in the nucleus. Detailed analysis...
of the cysteines in Trx-1 in a yeast two-hybrid system revealed that cysteines 32 and 35 are required for direct interaction with APEX1 (79). Conversely, cysteines in APEX1 were not mapped for Trx-1 interaction. We found that colocalization of the two endogenous proteins, APEX1 and Trx-1, is detectable mainly in the nucleus in human endothelial cells (Fig. 6). Mapping of the interaction domain within APEX1 is under further investigation. It must be noted that, under certain conditions, Trx-1 and APEX1 act in concert to regulate transcriptional activation (8, 244), whereas in other cases, APEX1 or Trx-1 alone is sufficient to control DNA binding of transcription factors. This is discussed in closer detail for the redox-regulated transcription factors in the following sections of this review.

Table 1. Redox-regulated Transcription Factors

<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>Critical amino acid</th>
<th>Affected function</th>
<th>Compartment of modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-1</td>
<td>Cys 269 (c-Fos)</td>
<td>DNA binding (1)</td>
<td>Nucleus (79)</td>
</tr>
<tr>
<td></td>
<td>Cys 154 (c-Jun)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPV E2</td>
<td>Cys 340</td>
<td>DNA binding (146)</td>
<td>n.d.</td>
</tr>
<tr>
<td>CBP/PEBP2</td>
<td>Cys 115, Cys 124</td>
<td>DNA binding (5)</td>
<td>n.d.</td>
</tr>
<tr>
<td>c-Myc</td>
<td>Cys 130</td>
<td>DNA binding (67, 155)</td>
<td>n.d.</td>
</tr>
<tr>
<td>CREB</td>
<td>Cys 300, Cys 310</td>
<td>DNA binding (64)</td>
<td>Nucleus (91)</td>
</tr>
<tr>
<td>Egr-1</td>
<td>n.d.</td>
<td>DNA binding (91)</td>
<td></td>
</tr>
<tr>
<td>Estrogen receptor</td>
<td>n.d. (DBD)</td>
<td>DNA binding (122)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Glucocorticoid</td>
<td>n.d. (DBD)</td>
<td>DNA binding (93, 229)</td>
<td>Nucleus (135)</td>
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<tr>
<td>receptor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIF-1z</td>
<td>Cys 481</td>
<td>DNA binding (146)</td>
<td>Nucleus import (167)</td>
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<td>HLF</td>
<td>Cys 800</td>
<td>DNA binding (5)</td>
<td>n.d.</td>
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<td>HoxB5</td>
<td>Cys 844</td>
<td>DNA binding (67, 155)</td>
<td>Cytoplasm (167)</td>
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<td>Cys 232</td>
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<td>n.d.</td>
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<td>Cys 427</td>
<td>DNA binding (128)</td>
<td>n.d.</td>
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<td>Cys 85, Cys 89</td>
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<td>n.d.</td>
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<tr>
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<td>DNA binding (156)</td>
<td>Nucleus (76)</td>
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<td>p53</td>
<td>n.d.</td>
<td>DNA binding (26)</td>
<td>n.d.</td>
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<td>Nucleus (76)</td>
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<td>DNA binding (103, 226)</td>
<td>Nucleus (226, 227)</td>
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<td>Sp1</td>
<td>n.d.</td>
<td>DNA binding (6, 7)</td>
<td>Nucleus (36)</td>
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<td>TTF-1</td>
<td>Cys 87</td>
<td>DNA binding (11, 103, 225)</td>
<td>n.d.</td>
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</table>
III. Redox-Regulated Transcription Factors and Chromatin Modifiers

Transcription factors regulate cellular functions through altering the gene expression profile. A number of transcription factors have been shown to be redox regulated through modulation of their DNA binding capacity. Additional layers of regulation are on the level of transcriptional activation by changing cofactor interactions, oligomerization, or subcellular localization. Thereby cells can modulate their transcriptome to adjust to physiologic and pathophysiologic changes in ROS levels and exogenous noxae. Table 1 provides an overview over the mammalian transcriptional regulatory proteins for which a redox regulation has been shown. In the following sections, we review the molecules for which a more detailed knowledge of their redox regulation is available.

A. Activator protein-1 (AP-1)
 Activator protein-1 (AP-1) is one of the first mammalian transcription factors that were identified (119). AP-1 is not a single protein, but represents various homo- or heterodimers formed between the proteins of the basic region-leucine zipper (bZIP) family. They belong to the Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1, and Fra-2), Maf (c-Maf, MaBF, MaF, MaFG/K, and Nrl), and ATF (ATF2, ATF3, LRF1, B-ATF, JDP1, JDP2) subfamilies of the bZIP proteins. Their complexes bind to a canonical AP-1 site, originally described as 12-O-tetradecanoylphorbol-13-acetate (TPA) response element, or variants thereof. Recruitment of other transcription factors, coactivators, and chromatin-remodeling proteins generates a plethora of regulatory complexes with cell- and stimulus-specific transcriptional activities (for review, see ref. 35). Therefore, AP-1 is involved in a wide range of physiologic functions, including proliferation and survival, differentiation, growth, apoptosis, cell migration, transformation, and carcinogenesis. AP-1 itself is target of a variety of upstream kinases like c-Jun NH2-terminal kinases (JNKs), extracellular regulated kinases (ERKs), and p38 mitogen-activated protein kinases (MAPKs) through phosphorylation of Jun and Fos proteins.

DNA binding of AP-1 is regulated by the redox state of a cysteine residue within the DNA binding domains of both proteins. Oxidation of Cys 154 in human Fos and Cys 269 in human c-Jun inhibits DNA binding of AP-1 in vitro (1). This was ascribed to an intermolecular disulfide bridge formed between the two cysteines. Oxidation of these residues could be achieved enzymatically, by using GPx, and DNA binding protected them from oxidation in vitro (16). In the viral homologue of c-jun, the transforming oncogene v-jun, the corresponding cysteine residue is replaced by a serine, which results in an insensitivity to oxidation, leading to constitutive DNA binding and aberrant transcriptional regulation. Similarily, Fos can escape regulation and is converted to a transforming protein when the critical cysteine is mutated (168). Shortly after uncovering the redox sensitivity of AP-1 DNA binding, a nuclear protein was identified that copurified with AP-1 and stimulated DNA binding of oxidized Fos-Jun heterodimers, Jun-Jun homodimers, and AP-1 complexes purified from HeLa cells (253, 254). This protein turned out to be APEX1. Besides its direct redox function, APEX1 can act as redox chaperone for AP-1, as discussed earlier.

B. Cyclic AMP response element–binding protein (CREB)

The transcription factor CREB binds the cAMP response element (CRE) and functions in glucose homeostasis, growth factor–dependent cell survival, and has been implicated in learning and memory. CREB is activated in response to, among other signals, cAMP. The accumulation of cAMP, triggered by extracellular signals induces most cellular responses through protein kinase A (PKA). An increase in cellular cAMP levels liberates the catalytic from the regulatory subunits of PKA, which then translocate into the nucleus and induce cellular gene expression by phosphorylating CREB at serine residue 133. Together with the related activating transcription factor 1 (ATF1) and the CRE modulator (CREM), CREB comprises another family of bZIP transcription factors (for review of the CREB/ATF family, see refs. 145, 175, and 197). Originally, the Fos/Jun and ATF/CREB protein families were regarded as distinct sets of transcription factors that recognize closely related, but different DNA binding sites and form intrafamily dimers. However, it was shown that members of these two families can form selective cross-family heterodimers. These display DNA binding specificities distinguishable from each other and from their parental homodimers (21, 72). Analogously to AP-1, DNA binding of CREB is regulated by its oxidation status. The reduction of two cysteine residues (Cys 300 and Cys 310) located in the DNA binding domain, enhances the binding efficiency of CREB to DNA and regulates CRE-mediated gene expression. Substitution of these residues to serine renders CREB insensitive to reduction. These substitutions, which do not alter the secondary structure of the protein (189), enhance the binding of CREB to its cognate DNA sites under oxidative conditions and CREB dependent gene expression during normoxia (64). Interestingly, this redox regulation is bypassed by the Tax protein of the human T-cell leukemia virus type 1 (HTLV1), which recruits CREB independent of phosphorylation and redox status to the HTLV1 promoter (61, 64) to promote viral transcription.

C. Specificity protein 1 (Sp1)

Specificity protein 1 (Sp1) is one of the best-characterized zinc-finger transcription factors. It is a member of an extended family of DNA binding proteins, harboring three Cys2-His2 zinc-finger motifs, which bind to GC-rich DNA recognition elements. By regulating the expression of a large number of genes that have GC-rich promoters, Sp1-like transcription factors are involved in the regulation of many cellular processes, including proliferation, apoptosis, differentiation, and neoplastic transformation. Individual members of the Sp1 family can function as activators or repressors, depending on which promoter they bind and the co-regulators with which they interact (for review of the Sp1 family, see refs. 100 and 193). A first hint that DNA binding by Sp1 is regulated by the cellular redox status came from the analysis of nuclear extracts from 30-month-old rat tissues, in which the DNA binding efficiency of Sp1 was greatly decreased, although the protein was present in levels comparable to those in younger tissues (6). This was attributed to increased levels of reactive oxygen intermediates in the aged animals because high concentrations of DTT, added to the aged tissue extracts, fully restored Sp1 DNA binding. Conversely, H2O2 treatment of extracts from young tissues strongly decreased the Sp1 DNA binding.
binding activity, which again could be restored with DTT. The same results were obtained with purified Sp1, clearly demonstrating that oxidation directly affected Sp1 and not a co-factor (7). A similar phenomenon was observed during the transition of thymocytes from the resting to the proliferating state, in which production of ROS upon priming with phorbol 12-myristate 13-acetate (PMA) is nearly abolished. In the proliferating state, Sp1 DNA binding activity increased and could be compromised by the addition of H2O2 to extracts from these cells, whereas the binding activity in nuclear extracts from resting cells could be fully restored with DTT (199). This susceptibility to oxidation is conferred by thiol groups, depends on zinc coordination, and is prevented by DNA binding, suggesting that the DNA binding domain is the target for oxidation and is protected when in contact with DNA. This was corroborated in vitro by findings that arsenic treatment of promyelocytic leukemia cells prevented DNA binding of Sp1 to specific promoters. Of note, the nuclear levels of Sp1 did not change with arsenic treatment, suggesting that the oxidation occurs in the nucleus (36). In addition, Sp1 DNA binding was inversely correlated with the GSSG/GSH ratio added to nuclear extracts in vitro (110). Moreover, Trx-1 alone or in conjunction with the full thioredoxin system (Trx-1/TR and NADPH) was able to increase the DNA binding activity of recombinant Sp1 produced in Escherichia coli and of the protein from a mammalian cell line (27). Interestingly, not only DNA binding of Sp1 might be affected by oxidation, but also its transactivation properties; however, this phenomenon has not been investigated in detail (153).

D. Nuclear receptors

Nuclear receptors constitute to a large superfamily of ligand-activated transcription factors (for review, see ref. 147). These intracellular receptors are activated by lipophilic ligands and play crucial roles in development, differentiation, metabolic homeostasis, and reproduction. The prototypic glucocorticoid and estrogen receptor (ER) consist of an N-terminal transactivation domain (TAD), a central DNA binding domain (DBD) containing zinc-finger motifs, and a C-terminal ligand-binding (LBD) domain. In the cytosol, they are complexed with various chaperones of the heat-shock protein family. After steroid binding, glucocorticoid and estrogen receptors are released from these cytosolic complexes and translocate to the nucleus, where they interact as homodimers with their cognate DNA binding sites, the glucocorticoid response element (GRE), and estrogen response element (ERE), respectively, from where they activate transcription.

Early studies with biochemically purified glucocorticoid receptor showed that it changes its conformation under oxidizing conditions, when it seemingly can form intra- and intermolecular disulfide bonds. This conformational change was completely reversible with DTT, and only the reduced form of the receptor was capable of binding DNA (93, 212, 229), which was later confirmed in intact cells (52). A role for Trx-1 in this process was first suggested by experiments that showed that suppression of Trx-1 expression decreases glucocorticoid-inducible gene expression (134). Mammalian two-hybrid and pull-down assays finally demonstrated a direct interaction between the two proteins involving the DNA binding domain of the glucocorticoid receptor. Analysis of the subcellular localization demonstrated that this interaction most likely takes place in the nucleus under oxidative conditions (135). Besides DNA binding, the nuclear import of the glucocorticoid receptor also is under redox control, which affects a cysteine residue in its nuclear localization signal (167). Similar observations were made for the estrogen receptor, whose DNA binding activity is also sensitive to oxidation, which alters the conformation of the DNA binding domain (122). As for the glucocorticoid receptor, the transcription of endogenous and transfected synthetic ER target genes was shown to depend on Trx-1 when cells were placed under oxidative stress (78).

E. Nuclear factor-kappa B (NF-κB)

NF-κB is a collective name for inducible dimeric transcription factors composed of members of the Rel family of DNA binding proteins that recognize a common sequence motif, the κB site. NF-κB is found in essentially all cell types and is involved in activation of an exceptionally large number of genes in response to infections, inflammation, and other stressful situations requiring rapid reprogramming of gene expression. NF-κB was originally identified as a nuclear protein binding to the immunoglobulin kappa light-chain enhancer (208). Shortly thereafter, it was demonstrated by the same investigators that its DNA binding activity in pre-B cells can be induced by bacterial lipopolysaccharide (LPS) with a superinduction after cycloheximide treatment (207), which then provided the first evidence that the activity of transcription factors can be regulated posttranslationally. Later it was shown that NF-κB represents a protein complex composed of hetero- or homodimeric combinations of five different members of the NF-κB/Rel family: NF-κB1 encoding p50 and p105, NF-κB2 encoding the p52 precursor p100, RelA or p65, RelB, and c-Rel. All subunits contain a conserved Rel-homology domain important for nuclear localization, dimerization, and DNA binding (for review see ref. 151). However, only the three Rel proteins contain transactivation domains required for the transcriptional activation of target genes. Therefore, and because each subunit has distinct biologic activities, different dimer combinations regulate specific sets of genes (for review, see ref. 157). This combinatorial mode of action results in diverse effects on cell fate and function.

In unstimulated cells, NF-κB is sequestered in an inactive form in the cytosol. It can be released from these cytosolic pools by two main pathways (for review, see ref. 201), resulting in nuclear translocation of NF-κB complexes. The canonic pathway, which is triggered by several proinflammatory cytokines, pathogen-associated molecules, and antigen receptors, depends on phosphorylation of the inhibitor of NF-κB (IκB) by an IκB kinase (IKK) complex consisting of the catalytic subunits IKKα, IKKβ, and a regulatory IκKγ subunit. Phosphorylated IκB is then subject to proteasomal degradation. This pathway leads primarily to the activation of p50/RelA and p50/c-Rel dimers. The noncanonic pathway engaged by various members of the tumor necrosis factor (TNF)-receptor family selectively requires IKKζ activated by the upstream kinase NF-κB-inducing kinase (NIK). IKKζ in turn phosphorylates p100, leading to proteasomal degradation of its C-terminus to generate p52, which then forms heterodimers predominantly with RelB. Besides these two major pathways, other mechanisms can release cytosolically...
sequestered NF-κB components [e.g., the phosphorylation of p105 by TPL-2, accelerating proteasome-mediated removal of the carboxy terminus yielding p50 (20)].

The observation that increased levels of the oxidoreductase Trx-1 are found in lymphocytes under conditions in which the transcription factor NF-κB is active [e.g., after lymphocyte activation or in EBV- or HTLV-I–infected cells (238)], led to the suspicion that NF-κB might be under redox control. It could be shown that in vitro DNA binding activity of NF-κB is inhibited by agents modifying free sulfhydryls (141, 142, 230). Mutagenesis of conserved cysteine residues in the p50 subunit revealed that Cys 62 is critical for high-affinity binding to the κB motif. DNA binding activity of wild-type p50, but not a mutant, in which Cys 62 was exchanged to serine (C62S), was stimulated by Trx-1. Detection of disulfide cross-linked dimers in p50 wild type but not C62S suggested that Trx-1 stimulates DNA binding by reduction of a disulfide bond involving Cys 62 of the NF-κB subunit p50. Cotransfection of a plasmid expressing human Trx-1 and an NF-κB–dependent reporter construct demonstrated that Trx-1 also can regulate DNA binding and transcriptional activation by NF-κB in living cells (142). Interestingly, depending on its subcellular localization, Trx-1 can have opposing effects on NF-κB. In the cytosol, it interferes with signals to IκBs and thereby blocks the degradation of IκB, resulting in cytosolic retention of NF-κB, which prevents the activation of target genes. In contrast, nuclear Trx-1 has a positive effect on NF-κB transcriptional activity by enhancing its ability to bind to DNA (81). Molecular-modeling studies finally provided a clue to this dual role of Trx-1. Oxidized, disulfide-bridged NF-κB is more compact than the reduced form, which might facilitate its nuclear translocation. However, the inter-subunit disulfide blocks DNA from entering the active site of the oxidized dimer, explaining why reduction in the nucleus is essential for DNA binding and transcriptional activation to occur (32). Similar to Trx-1, Prx1 has the same dual role in the regulation of NF-κB activity (75). Besides disulfide cross-linked dimer formation, another oxidation product of NF-κB subunits has been detected. Both p50 and p65 can be glutathionylated, which leads to reduced NF-κB DNA binding and transactivation (181, 182). In the case of p65, glutathionylation was detected under hypoxic conditions, when the intracellular GSH levels were increased. The modification of p65 required Grxl, and the authors speculated that p65-SSG formation takes place in the cytosol and that modified p65 is still transported to the nucleus (182). However, as Grxl can be detected in the nucleus, it also is possible that glutathionylation of this transcription factor subunit takes place there.

In addition to cysteine oxidation, the modification of specific tyrosine residues in p65 has been reported. Peroxynitrite inhibited NF-κB activity through nitration of p65 at Tyr 66 and Tyr 152, leading to p65 destabilization and nuclear export (176).

An interesting observation was made concerning the role of NF-κB redox regulation in the action of thalidomide, which causes severe malformations, especially of the extremities, in children when taken by the mothers during pregnancy. Thalidomide increases the production of free radicals and elicits oxidative stress. Oxidative stress, as marked by GSH depletion, occurs preferentially in limbs of thalidomide-sensitive rabbits. Activation of an NF-κB–dependent reporter gene is attenuated in limb bud cells of treated rabbits and can be restored by addition of N-acetylcysteine and a free radical spin-trapping agent (74). NF-κB is a key factor in limb development, because it regulates expression of twist and fibroblast growth factor 10 (fgf10), two genes necessary for proper limb outgrowth. Therefore, its oxidative inactivation triggered by thalidomide might explain the malformations occurring in children.

F. p53

The protein p53 is one of the best-characterized tumor suppressors and is constitutively expressed in nearly all cells and tissues. It functions as a tetrameric transcription factor found at very low levels in normal cells. Several genomewide surveys have been undertaken to map genomic binding sites of p53 and thus to identify its target genes (83, 104, 213), yielding a plethora of genes potentially regulated by p53. Various kinds of cellular stress that alter normal cell cycle progression or induce mutations in the genome lead to stabilization and thus accumulation of the protein. Depending on the cell type and tissue and the extent of damage, p53 now either leads to cell cycle arrest to repair the lesions or forces the cell into apoptosis. Therefore, p53 has been termed “guardian of the genome” (117). Somatic mutations in the p53 gene are critical events in a wide variety of malignancies, and p53 is the gene most frequently mutated in human cancers. The majority of the mutations are missense mutations, and a hotspot is the region of the gene coding for the DNA binding domain (for review of p53 and its mutations, see refs. 28, 84, 217, and 239).

Because of its prominent role in human cancers, the regulation of p53 has been at the focus of intensive studies. With respect to redox regulation, it has to be noted that all 10 cysteine residues in p53 are within its DNA binding domain. This prompted very early studies on redox regulation. It was shown that oxidation of in vitro translated and recombinant, baculovirus-produced p53 disrupted its DNA binding, which was attributed to a change in conformation that could be reversed by reduction (44, 73). Interestingly, the redox state of p53 seemingly regulates only sequence-specific DNA binding and activation of p53-dependent genes. In contrast, no difference is found in the binding of oxidized p53 and reduced p53 to double-stranded nonspecific DNA (177).

That APEX1 can modulate the redox-dependent properties of p53 was demonstrated by the stimulation of sequence-specific DNA binding of oxidized p53 in vitro (97). The expression of reporter genes driven by p53-responsive promoters and endogenous p53 target genes, like p21 and cyclin G, was equally stimulated on overexpression of APEX1 (57, 97). Importantly, downregulation of APEX1 caused a marked reduction in p53-dependent induction of p21 and diminished the transcriptional activation of p21 and Bax by p53. In addition, the same authors demonstrated a physical interaction between APEX1 and p53, although only a small portion of both proteins was present in the same complex (57). Interestingly, APEX1 modulates p53 DNA binding, not only as a redox regulator, but also to facilitate formation of p53 tetramers, the most active form in terms of DNA binding (245), independent of its redox activity (77). This is substantiated by the fact that the cysteine residues, which are exclusively found in the DNA binding domain of p53 and are the targets for oxidation/reduction, have no influence on tetramerization
(185). Collectively, these data suggest that APEX1 stimulates p53 by both redox-dependent and -independent means and imply a key role for it in p53 regulation.

G. Nuclear factor I/CAAT transcription factor (NFI/CTF)

NFI/CTF was originally described as being required for the replication of adenovirus DNA (for review, see ref. 42). Later it was shown that NFI can regulate the transcription of a large number of cellular and viral genes. NFI represents a family of four genes in vertebrates (NFI-A, NFI-B, NFI-C, and NFI-X), which are expressed in overlapping patterns. The transcripts of all four genes can be spliced differentially, yielding distinct proteins. NFI proteins have been associated with changes in the growth state of cells and a number of malignancies (for review of NFI proteins, see ref. 65).

As for other transcription factors, it has been shown for NFI that its DNA binding activity is redox sensitive (164). A single cysteine residue (Cys 3) in the DNA binding domain of the NFI-family proteins, which is conserved from Caenorhabditis elegans to humans is the target for this regulation, as was shown by site-directed mutagenesis (13). Oxidized, inactive NFI can be reduced to a DNA binding form by Grx1 in vitro. This requires the GSH/GR system to regenerate reduced Grx1. The in vitro relevance has been shown by treatment of HeLa cells with buthionine sulfoximine, an agent that inhibits GSH synthesis. This GSH depletion potentiated the inactivation of NFI by the oxidizing agent diamide. Similarly, a stronger restoration of NFI activity after oxidation with diamide was observed, when the cells were treated with a stronger restoration of NFI activity after oxidation with NFI-activating NFI by the oxidizing agent diamide. Similarly, its GSH synthesis. This GSH depletion potentiated the inactivation of NFI by the oxidizing agent diamide. Similarly, a stronger restoration of NFI activity after oxidation with diamide was observed, when the cells were treated with N-acetylcysteine, an agent that can replenish intracellular GSH (14).

In the case of NFI, not only DNA binding is subject to redox control. By using fusions with a heterologous DNA binding domain from the Gal4 protein, it was demonstrated that transcriptional activation by NFI also is regulated through oxidation of a cysteine residue (Cys 427) in the transcription-activation domain (TAD) (153) (Fig. 7). These findings were corroborated in living cells subjected to various stress conditions that induce cellular ROS formation, including inflammatory cytokine treatment, GSH depletion, heat and osmotic shocks, and chemical stress. In all cases, suppression of a reporter gene was specific for the NFI TAD, as no effects were observed with the transactivation domains of activator protein 2 (AP-2) and octamer transcription factor 2 (Oct-2). A common target for all these stressors was again Cys 427, leading to the suggestion that the NFI TAD might be a negative sensor of cellular stress (154). Based on the concentrations of exogenously applied H$_2$O$_2$ required to block transcriptional activation and DNA binding by NFI, it was calculated that a 100-fold difference in sensitivity to oxidation exists between the DNA binding domain of NFI and its TAD (153). During this investigation, TADs from other transcription factors (Sp1 and Oct-2) were analyzed to show that redox regulation of TADs is not a general effect. Whereas the Oct-2 TAD was not sensitive to oxidative stress, the analogous domain of Sp1 showed some responsiveness, indicating that other transcription factors could be subject to similar control mechanisms, which might have been overlooked during the characterization of the redox dependency of their DNA binding capacity.

It is not clear whether the thiol moiety of Cys 427 of NFI undergoes an oxidation with a gain of oxygen atoms or if it forms an intra- or intermolecular disulfide bridge, although mutations of a cysteine in the neighborhood (Cys 405) indicate that the formation of an intramolecular disulfide bridge within the TAD is not the mechanism most likely to trigger the effect. The oxidation of Cys 427 could affect the conformation of the TAD, which is the interface for interactions with the TATA-box-binding protein TBP, the coactivator CBP/p300, and histones H1 and H3.

H. Hypoxia-inducible factor 1 (HIF-1)

HIF-1 is the most prominent regulator of genes induced by hypoxia. It is a transcription factor that binds to the hypoxia-responsive element (HRE) in the promoters and enhancers of various hypoxia-inducible genes. HIF-1 is a heterodimer composed of HIF-1α and HIF-1β, which is identical to the aryl hydrocarbon-receptor nuclear translocator (ARNT). Both proteins contain a basic helix–loop–helix (bHLH) and a PAS domain at their N-terminus, the latter being an acronym for Per, ARNT, and Sim, the first three members of the protein family characterized. Whereas the basic domain is essential for DNA binding, the HLH domain and the N-terminal half of the PAS domain are required for heterodimerization and DNA binding. HIF-1β contains a single C-terminal transactivation domain, whereas in HIF-1α, two such domains are found, termed NAD and CAD, according to their location closer to the N- or C-terminus. The NAD is embedded in a region controlling protein stability, the oxygen-dependent degradation domain (ODD). Both subunits of HIF-1 are expressed constitutively, but the α-subunit has an extremely short half-life (<5 min) under normoxic conditions, because of continuous proteolysis by the ubiquitin–proteasome pathway targeting the ODD. As both subunits are required for the activation of HIF-1 target
genes, these are not or only weakly expressed at normal oxygen tension. During hypoxia, HIF-1α is instantaneously stabilized, resulting in a functional heterodimer and expression of hypoxia-inducible genes. HIF-1α is hydroxylated at proline 564 (Pro 564) in the ODD in an oxygen-dependent fashion. This hydroxylated HIF-1α is bound by the von Hippel-Lindau (VHL) protein, which acts as an E3-ubiquitin ligase, tagging HIF-1α for proteasomal degradation. The critical molecules for the rapid turnover of HIF-1α under normoxia are members of a family of prolyl-4-hydroxylases (PHDs). These enzymes require oxygen and 2-oxoglutarate as cosubstrates and contain iron liganded by two histidine and one aspartic acid residues. Oxygen binding requires the vitamin C–dependent maintenance of iron in its ferrous state. When oxygen is low, the rapid turnover of HIF-1α under normoxia is no longer bound by VHL. Similarly, iron in HIF-1α remains unmodified, resulting in stabilization of the protein, which is no longer bound by VHL. Similarly, iron chelation or replacement by transition metal ions (Co²⁺, Ni²⁺, Mn²⁺) can mimic hypoxia-inducible stabilization, explaining the apparent “upregulation” of HIF-1α by these metals (for review of HIF-1, see refs. 205, 206, 246, and 247).

In addition to HIF-1α, two other family members are known, HIF-2α/HLF (HIF-1α–like factor) and HIF-3α. They show a more-restricted expression pattern, but contain domains similar to HIF-1α and have comparable biochemical properties.

In 1996, it was shown that pretreatment of cells with hydrogen peroxide has an inhibitory effect on transcriptional activation by HIF-1α, which suggested an additional redox control mechanism. Sulfhydryl alkylation or oxidation, the latter being reversible, indicated an involvement of cysteine residues in this process. These findings were supported by the fact that purified, oxidized Trx-1 in combination with DTT could stimulate HIF-1α DNA binding in hypoxic extracts. In addition, overexpression of Trx-1 or APEX1 enhanced the hypoxic induction of a HIF-1α–dependent reporter gene (98). Interestingly, DNA binding of HLF, but not HIF-1α–containing complexes was redox dependent. This is explained by the fact that the critical cysteine residue in the basic domain of HLF (Cys 28), which seems to be a target for APEX1, is replaced by a serine residue at the corresponding position (Ser 25) in HIF-1α. However, APEX1 also is important for transcriptional activation by HIF-1α (116). The last finding is in agreement with a previous report that the CADs of HIF-1α and HLF interact with the transcriptional coactivator CBP/p300 and that APEX1 and Trx-1 further enhanced the activity of a luciferase reporter activated by a fusion protein between the Gal4 DNA binding domain and CAD under hypoxic conditions. This potentiation required the catalytic activity of Trx-1, suggesting that a redox reaction is involved. Interestingly, a single cysteine residue is conserved in the 49-amino-acid CAD between HLF (Cys 844) and HIF-1α (Cys 800). Mutations of this cysteine abolished the hypoxia-inducible transcriptional activation by Gal4-CAD and interaction with CBP. The importance of this cysteine residue was confirmed by mutation in the context of full-length HIF-1α and HLF, which markedly reduced the transcription-enhancing activity (51).

I. Nuclear factor erythroid 2–related factor 2/
NF-E2 related factor 2 (Nrf-2)

The NF-E2–related factor 2 (Nrf-2), not to be confused with the GA-binding protein nuclear respiratory factor 2, which is also called Nrf-2, is a transcription factor implicated in the cellular responses to oxidative stress and to chemical compounds that are metabolically transformed to reactive or electrophilic intermediates. Nrf-2–deficient mice show a lower expression of xenobiotic enzymes and are predisposed to tumors induced by carcinogens (186). Nrf-2 is a member of the so-called cap ‘n’ collar basic region leucine zipper (CNC-bZIP) family of transcription factors, the bZIP region of which is distinct from other bZIP families, such as the Jun/Fos family. Nrf-2 heterodimerizes with other bZIP transcription factors, including the small Maf (sMaf) proteins. These heterodimers bind to antioxidant-response elements (AREs) and thereby upregulate numerous genes coding for detoxification enzymes, antioxidants, and the enzymes required for de novo GSH synthesis (148). However, Nrf-2 controls not only inducible, but also low-level gene expression under nonstressed conditions, suggesting that its activity is tightly controlled. It has been shown that Nrf-2 is tethered by the Kelch-like ECH–associated protein 1 (Keap1), and in this complex, is not available as a transcriptional activator. Keap1 serves not only simply to sequester Nrf-2, but also functions as an adaptor for a Cul3–dependent E3 ubiquitin ligase modifying Nrf-2 for proteasomal degradation, which explains the short half-life of Nrf-2 (~15 min). It is still a matter of debate whether Keap1 transiently enters the nucleus and targets Nrf-2 for ubiquitination there or is capable of engaging in a nucleocytoplasmic shuttling of Nrf-2 dependent on CRM-1 (235). Nevertheless, the generally accepted scenario involves a cytosolic retention and degradation of Nrf-2 by Keap1.

Keap1 contains an N-terminal BTB/POZ domain (for broad-complex, Tramtrack and Bric-a-brac; also known as a Poxvirus and zinc finger domain) potentially serving as an interface for homomeric or heteromeric interactions. The C-terminus comprises six Kelch repeats, each of which forms a homomeric or heteromeric complex with an sMaf to activate gene expression. Several kinases have been shown to phosphorylate Nrf-2, but the disulfide bridges most likely are formed crosswise between Cys 273 and Cys 288 (237). After release from Keap1, the disulfide bridges most likely are formed crosswise between Cys 273 and Cys 288 (237). After release from Keap1, Nrf-2 escapes degradation and can bind to AREs in a heteromeric complex with an sMaf to activate gene expression. Several kinases have been shown to phosphorylate Nrf-2, but the molecular consequences of these phosphorylation events have not been elaborated (for review of Nrf-2 and its regulation by Keap1, see refs. 107, 158, and 159).

Besides the cytosolic retention of Nrf-2 by Keap1 and its release upon formation of an intermolecular Keap1 dimer, a second layer of Nrf-2 activity regulation exists. Like other transcription factors, Nrf-2 must be in a reduced state for efficient DNA binding. The critical residue is Cys 506, whose oxidation reduced its affinity for the ARE, leading to decreased expression and antioxidant induction of NAD(P)H/quinone oxidoreductase 1 (NQO1). However, mutation of this residue to serine did not affect the retention of Nrf-2 by Keap1.
in the cytosol or its release in response to antioxidants (26).

It has been shown that this two-layered, compartmentalized regulation of Nrf-2–dependent gene expression involves two cellular redox systems. Whereas cytosolic retention is controlled by GSH/GSSG ratios, the Nrf-2–DNA interactions depend on nuclear Trx-1 (76) (Fig. 8).

**J. Homeobox B5 (HoxB5)**

To our knowledge, only one case exists in which oxidation of a cysteine residue in a transcription factor can enhance DNA binding, homeobox B5 (HoxB5). It is a homeodomain (HD) protein of the antennapedia family and functions as a sequence-specific transcription factor that is involved in lung and gut development. In the cardiovascular system, HoxB5 is an upstream transcriptional switch for differentiation of the vascular endothelium from precursor cells (252). It was shown in vitro that the cooperative stabilization of HoxB5 DNA binding, but not sequence-specific DNA binding, is under redox regulation. Cooperative binding and redox regulation were found to require the presence of a cysteine residue (Cys 232) in the turn between homeodomain helices 2 and 3 and that oxidation of this cysteine is necessary for cooperative binding of the protein to tandem binding sites. This was shown by DTT treatment of purified, recombinant HoxB5, which resulted in loss of cooperativity (58). Multiple clustered HD protein-binding sites are found in the promoters of Dro sophila and vertebrate genes whose expression is regulated by antennapedia-type HD proteins. Thus, cooperative interactions could have a large influence on the DNA binding of HD proteins to these sites.

**K. Other redox-regulated transcription factors**

Besides the transcription factors discussed in detail, DNA binding of several others is inhibited by oxidation: CBP/PEBP2 (5, 101), c-Myb (67, 155), Egr-1 (91), MyoD (218), NF-Y (156), Pax-5 (226, 227), Pax-8 (103, 224), and bovine papilloma virus E2 (BPE E2) (146). In some of these cases, oxidation sensitivity has been mapped to the DNA binding domain; in others, it has been shown that Trx-1 or APEX1 can restore the DNA binding capacity after oxidation.

**L. Histone deacetylase 2 (HDAC2)**

Interestingly, not only transcription factors as bona fide DNA binding proteins are affected by modification of cysteine residues. Recently, it was shown that the chromatin modifier histone deacetylase 2 (HDAC 2) becomes nitrosylated after treatment of rat cortical neurons with neurotrophins. This modification occurs on two cysteine residues (Cys 262 and Cys 274) and is dependent on neuronal nitric oxide synthase (nNOS), as shown in neurons from nNOS-deficient mice and nonneuronal cells expressing nNOS. Intriguingly, nitrosylation of the two cysteines did not change the enzymatic activity of HDAC 2 but rather induced its release from chromatin. This dissociation of HDAC 2 leads to acetylation of histones H3 and H4, activation of BDNF target genes, and dendritic

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**FIG. 8. Activation of Nrf-2: general model of gene induction by the Keap1/Nrf-2 pathway.**

Nuclear factor erythroid 2–related factor 2/NF-E2–related factor 2 (Nrf-2) is sequestered in the cytosol by Kelch-like ECH associated protein 1 (Keap1). In addition to binding Nrf-2, Keap1 functions as an adaptor for an E3 ubiquitin ligase, which ubiquitinates Nrf-2, thereby tagging it for proteasomal degradation. After induction, Keap1 is oxidized to an intermolecular, disulfide-linked dimer involving reciprocal cysteine residues of both monomers. This oxidation requires GSSG. Released Nrf-2 translocates to the nucleus, where it is reduced by thioredoxin-1 (Trx-1). Nrf-2 must be in a reduced state for efficient DNA binding as a heterodimer with a small Maf protein (sMaf). These heterodimers induce transcription of genes, whose promoters contain antioxidant responsive elements (AREs).
growth (163). This mechanistic study could explain why HDAC 2 can negatively regulate memory formation and synaptic plasticity (66).

IV. Redox-Regulated Nuclear Kinases and Phosphatases

A. Nuclear-localized kinases

Protein kinases are involved in many different cellular signaling pathways. Therefore, a strict regulation of these kinases is necessary for the survival of the cell. The most important regulatory mechanism is their activation by phosphorylation (for review, see refs. 98 and 99). Kinases are phosphorylated under physiologic and pathophysiologic conditions. Recently a direct oxidation of Src and the fibroblast growth factor type 1 was demonstrated, leading to their inactivation. This mechanism seemingly only works for kinases containing a cysteine in the Gly loop capable of forming disulfide homodimers and therefore applies only to a small number of human protein tyrosine kinases (106).

In the context of this review, we restrict the detailed discussion to kinases, for which a nuclear localization has been demonstrated, even if the redox regulation takes place outside the nucleus. Nuclear localization has been shown for protein kinase C δ (PKCδ), PKA, JNKs, Akt, ERKs, and some members of the Src kinase family. The following paragraphs discuss the kinases mentioned earlier in more detail; a brief overview is given in Table 2.

1. Protein kinase Cδ (PKCδ). The protein kinase C (PKC) family of serine/threonine kinases is involved in many cellular signaling pathways such as growth, proliferation, and cell death (for review, see ref. 259). This family consists of three groups: (a) the conventional PKCs (cPKCs: α, β, γ, δ, ε, θ, µ, η); (b) the novel PKCs (nPKCs: δ, ε, θ, µ); and (c) the atypical PKCs (aPKCs: ζ, η). PKCδ belongs to the novel PKCs and is redox regulated in several cell types. One well-described role for PKCδ is in mitochondria-dependent apoptosis induction. Overexpression of PKCδ in keratinocytes leads to translocation of PKCδ to mitochondria, alterations in mitochondrial functions, and induction of cell death (121). However, other studies also show translocation of PKCδ into the nucleus in various cell types. In response to cellular stresses, like oxidative stress, PKCδ is activated by tyrosine phosphorylation, and nuclear translocation occurs. PKCδ possesses its own nuclear localization sequence (NLS) (46) and has several tyrosine phosphorylation sites, which regulate its kinase activity. One of these sites, tyrosine 512, is phosphorylated by the apoptosis-related protein DNA-dependent protein kinase (DNA-PK) (22, 60). DNA-PK is essential for repair of DNA double-strand breaks (214). Phosphorylation by PKCδ also its catalytically active fragment induces the dissociation of DNA-PK from DNA, resulting in an inhibition of DNA repair and enhanced DNA fragmentation (22) DeVries et al. (46) showed that caspase 3–dependent cleavage of PKCδ occurs in response to oxidative stress. Active IκKζ translocates into the nucleus.

Table 2. Nuclear Kinases and Phosphatases

<table>
<thead>
<tr>
<th>Nuclear kinase</th>
<th>Critical amino acid</th>
<th>Molecular consequence</th>
<th>Cellular consequence</th>
<th>Compartment of modification</th>
<th>Ref.</th>
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<td>PKCδ</td>
<td>Tyr 512</td>
<td>Kinase activation</td>
<td>Apoptosis</td>
<td>Cytosol</td>
<td>46, 222</td>
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<td>Kinase activation</td>
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<td>Apoptosis inhibition, delayed</td>
<td>Nucleus</td>
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<td>Kinase activation</td>
<td>De novo GSH synthesis</td>
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<td>Kinase activation</td>
<td>Senescence, apoptosis</td>
<td>Cytosol</td>
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<td>ROS induction, apoptosis</td>
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<td>Cys 331, Cys 367</td>
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<td>Cys 377</td>
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(b) the novel PKCs (nPKCs: δ, ε, θ, µ); and (c) the atypical PKCs (aPKCs: ζ, η). PKCδ belongs to the novel PKCs and is redox regulated in several cell types. One well-described role for PKCδ is in mitochondria-dependent apoptosis induction. Overexpression of PKCδ in keratinocytes leads to translocation of PKCδ to mitochondria, alterations in mitochondrial functions, and induction of cell death (121). However, other studies also show translocation of PKCδ into the nucleus in various cell types. In response to cellular stresses, like oxidative stress, PKCδ is activated by tyrosine phosphorylation, and nuclear translocation occurs. PKCδ possesses its own nuclear localization sequence (NLS) (46) and has several tyrosine phosphorylation sites, which regulate its kinase activity. One of these sites, tyrosine 512, is phosphorylated by the proapoptotic tyrosine kinase c-Abl in response to oxidative stress. Phosphorylation results in activation of PKCδ followed by its nuclear translocation (222, 260). After nuclear translocation, activated PKCδ initiates a sequence of events that activates caspase 3, which in turn cleaves PKCδ (25, 46). This cleavage results in a 40 kDa catalytically active fragment and a 38 kDa regulatory fragment of PKCδ. The catalytically active fragment induces apoptosis by phosphorylation of the apoptosis-related protein DNA-dependent protein kinase (DNA-PK) (22, 60). DNA-PK is essential for repair of DNA double-strand breaks (214). Phosphorylation by PKCδ and also its catalytically active fragment induces the dissociation of DNA-PK from DNA, resulting in an inhibition of DNA repair and enhanced DNA fragmentation (22) DeVries et al. (46) showed that caspase 3–dependent cleavage of PKCδ increases the rate of nuclear translocation of the 40 kDa PKCδ cleavage fragment, which results in an amplification of the apoptotic signal (46) (Fig. 9).

PKCδ also interacts with and activates IκKζ in response to oxidative stress. Active IκKζ translocates into the nucleus.
and regulates the transcriptional activity of the tumor-suppressor p53 by phosphorylation at serine 20 (256). This is a recently described mechanism for ROS-induced p53 activation.

2. Protein kinase A (PKA). Cyclic AMP formed by adenylyl cyclases binds the regulatory subunits (R) of the tetrameric PKA holoenzyme and promotes dissociation of the catalytic subunits (C-PKA). A fraction of C-PKA translocates to the nucleus and stimulates cAMP-dependent gene expression in different cell types (174). The best-characterized target of nuclear C-PKA is CREB, which is phosphorylated at serine 133 by C-PKA (71). H$_2$O$_2$ has been shown to initiate an increase in CREB phosphorylation at serine 133 in a nontransformed murine alveolar type II epithelial cell line (18), leading to transcriptional activation (145). The increased phosphorylation of CREB was due to an H$_2$O$_2$-induced increase in nuclear accumulation of C-PKA (17). This was confirmed by pharmacologic inhibition of PKA with H89, which reduced H$_2$O$_2$-mediated phosphorylation of CREB. Because the downregulation of CREB by siRNA increased the sensitivity of cells to H$_2$O$_2$-induced apoptosis and reduced transcription of the antiapoptotic gene B-cell lymphoma protein 2 (Bcl-2), CREB seems to play a role in cell survival in response to oxidative stress (17).

3. c-Jun NH$_2$-terminal kinases (JNKs). JNKs are involved in the regulation of cell proliferation and apoptosis. The activation of these pathways is dependent on the actual stress stimulus and cell type (125, 128). Sustained activation of JNKs leads to apoptosis, whereas the acute and transient activation induces survival pathways and cell proliferation (196). The sustained stress-induced activation of JNKs by phosphorylation through upstream MAP-kinases, ASK1, and MAP kinase kinase (MKK) 4/7 leads to an induction of apoptosis (for review, see ref. 127). JNKs translocate into the nucleus and phosphorylate c-Jun and activating transcription factor 2 (ATF2) (for review, see ref. 41), leading to the formation of an AP-1 complex and to the transcription of genes coding for proapoptotic proteins [e.g., tumor necrosis factor α (TNF-α), Fas-L, and Bak] (53). However, JNKs also regulate physiologic and homeostatic processes. One attractive explanation for these differences is the existence of three isoforms of JNK and the subcellular pools of the JNKs. Only combined siRNA knockdown of all JNKs 1, 2, and 3 provides substantial protection from cell death. In contrast, knockdown or knockout
of individual JNKs or two JNKs together does not protect. Moreover, to determine whether cytosolic or nuclear JNKs are responsible for JNK-dependent cell death, compartment-specific inhibitors for JNKs were generated. Therefore, a nuclear-exclusion sequence (NES) from MKK1 or three NLSs from SV40 large-T antigen were fused in tandem upstream of the JNK inhibitor protein JIP. These targeted proteins localized to cytosolic and nuclear compartments, respectively (23). Overexpression of these compartment-specific JNK inhibitors revealed that cell death requires nuclear but not cytosolic JNK activity, as the nuclear dominant-negative inhibitor of JNK protected against apoptosis, whereas the cytosolic form only blocked physiologic JNK function (24). Thus, any one of the three JNKs is capable of mediating apoptosis, and this is dependent on their nuclear localization (Fig. 10).

4. Protein kinase B (Akt). The activity of the serine/threonine kinase Akt is redox regulated by Grx. By keeping Akt in its reduced form, Grx enables the activation and stimulation of the endothelial NO-synthase/NO signaling pathway (241). However, it is unclear whether this has to be attributed only to cytosolic Akt or also to the nuclear Akt.

We previously reported that nuclear Akt is required for the maintenance of telomerase activity and inhibition of apoptosis in human umbilical vein endothelial cells (70). Mechanistically, after the first assembly of the telomerase holoenzyme, which requires Hsp90 and p23 (89), Akt is recruited into this complex in the nucleus. Upon its recruitment, Akt phosphorylates TERT at serine 823, which increases nuclear telomerase activity (29, 70). Furthermore, the complex formation of TERT with HSP90 and Akt protects Akt from being dephosphorylated by the protein phosphatase 2A and thereby keeps Akt and TERT in their active forms (70, 105). Independent of its telomere-elongation function, nuclear TERT also has antiapoptotic effects. Inhibition of telomerase activity or overexpression of nuclear TERT induces or inhibits cell death.
apoptosis, respectively (68, 166, 192, 262). Thus, maintaining TERT in its active state in the nucleus by Akt protects cells against apoptosis.

It has been demonstrated that, upon its activation, myocardial Akt accumulates in the nucleus (30). Therefore, important studies investigated the specific function of nuclear Akt in cardiomyocytes by using an adenovirus expressing Akt with nuclear localization signals. Nuclear-targeted Akt inhibited cardiomyocyte hypertrophy and apoptosis (211, 232). Moreover, transgenic mice with cardiac-specific expression of nuclear-targeted Akt were protected from ischemia/reperfusion injury (211). Given the fact that constitutive active Akt causes hypertrophy in cardiomyocytes, specifically, nuclear-targeted Akt provides a new opportunity for therapeutic applications.

5. Extracellular regulated kinases 1/2 (ERK1/2). ERK1/2s are a subfamily of the MAP kinases and are involved in many important cellular processes like cell proliferation, survival, apoptosis, and metabolism (165). Their stimulation is triggered by growth factors and different environmental and oxidative stresses. The cytokine epidermal growth factor (EGF) is one of the activators of ERK1/2 through phosphorylation of the EGF receptor (EGFR) (31), whereas H$_2$O$_2$ activates ERK1/2 by EGFR-dependent and -independent pathways (63, 243). The phosphorylation state of ERK2 is more important for its nuclear retention than is the activity of ERK2. This was confirmed with catalytically inactive ERK2, in which lysine 52 was mutated to arginine [ERK2(K52R)]. Like the wild-type protein, microinjected ERK2(K52R) transiently translocated to the nucleus after stimulation. To reduce the susceptibility to dephosphorylation, the two activating phosphorylation sites were thiophosphorylated in vitro. The stably phosphorylated wt and K52R mutant directly moved into and remained in the nucleus, demonstrating that ERK2 activity is not necessary for nuclear accumulation (108). Besides phosphorylation of ERK2, homodimerization is necessary for its nuclear accumulation (108). The dimerization

![FIG. 11. Nuclear Src and Yes induce TERT export under conditions of oxidative stress. Cytosolic and mitochondrial ROS induce increased activity of nuclear Src and Yes. This leads to tyrosine phosphorylation of telomerase reverse transcriptase (TERT) and its nuclear export. The functional consequences are increased apoptosis sensitivity and accelerated senescence.](image-url)
of ERK2 induces a change of conformation and exposes a binding site for a NLS-containing protein. One of the potential candidates for such a NLS protein is growth factor–receptor bound protein 2-associated protein 1 (Gab1), which associates with ERK2. It was suggested that binding of ERK2 and Gab1 regulates the nuclear import of phosphorylated ERK2 (170). Another important role for ERK1 and ERK2 is their involvement in the nuclear localization of Nrf-2, which, among others, activates genes coding for enzymes required for de novo GSH synthesis (263). Thus, ERK1/2 or mainly ERK2 plays a role in ensuring an appropriate GSH supply for the cell.

6. Src kinase family. The Src kinase family consist of at least nine members (178). The most prominent members are the kinases Src, Fyn, and Yes. In the nucleus, only the kinases Src and Yes can be found, but not Fyn (96). In response to oxidative stress or growth factors, Src is phosphorylated at tyrosine 416, and Yes at tyrosine 426, which increases their enzymatic activity. Under conditions of oxidative stress or during the process of aging, these nuclear kinases are involved in tyrosine phosphorylation of TERT, which then leads to nuclear export of TERT. This has been demonstrated by mutation of tyrosine 707 within TERT, resulting in nuclear retention of the enzyme and by pharmacologic inhibition of Src kinase family activation, which inhibits tyrosine phosphorylation of TERT and its subsequent export from the nucleus (70, 96) (Fig. 11). The underlying export mechanism is mediated by a complex formation between TERT, the well-described export receptor CRM-1, and the nuclear GTPase Ran (70). Functional consequences of this Src kinase family–dependent nuclear tyrosine phosphorylation of TERT are increased apoptosis sensitivity and accelerated senescence (68, 69). This is in agreement with other studies, demonstrating that nuclear TERT acts as an inhibitor of apoptosis in several cell types (56, 144, 166). Therefore, an imbalance in the redox status seems to enhance active nuclear Src and Yes, which importantly contribute to apoptosis and accelerated senescence.

Recently a new mechanism for inactivation of the Src kinase was discovered. Src is active only in its reduced form, and oxidation results in complete inactivation. This inactivation is caused by a specific, reversible oxidation of Cys 277, located in the catalytic domain of Src, which results in homodimerization of Src linked by a disulfide bridge. This cysteine residue is conserved only in three of the Src family members, Src, Yes, and Egr, and could therefore be a specific mechanism for regulating a specific subset of Src kinases (106). Interestingly, only Src and Yes could be found in the nucleus, leading to the speculation that the formation of disulfide homodimers might be a prerequisite for nuclear import.

B. Redox-regulated phosphatases

Protein phosphatases can be divided into serine/threonine phosphatases and tyrosine phosphatases (PTPs). Both are important regulators in the activation and inactivation of cell-signaling pathways. Serine/threonine phosphatases are generally regulated by association with regulatory subunits and their own phosphorylation (49, 209). In the last few years, the role of oxidative stress in the regulation of PTPs has received more attention. PTPs have been shown to be redox sensitive and to be inhibited reversibly or irreversibly, depending on the degree and mechanism of oxidation (120, 150, 180, 231). Oxidation of the essential cysteine in the active site by H₂O₂ inactivates phosphatase activity (45). The reversible oxidation of this cysteine residue to sulfenic acid (Cys-SO⁻³) has been identified as a key mechanism for the regulation of many pathways. Higher oxidation to sulfinic (Cys-SO₂⁻³) or sulfonic (Cys-SO₃⁻) acid leads to an irreversible inactivation of the phosphatase (Fig. 12A). Many PTPs prevent a higher oxidation by formation of intracellular disulfide bonds between the active-site cysteine and nearby so-called backdoor cysteines [for example, Cdc25 phosphatases (216) or the phosphatase and tensin homologue (PTEN) (113)]. These intramolecular disulfides can then rapidly and effectively be reduced by FIG. 12. Model for the regulation of PTP activity. (A) Under physiologic conditions, the catalytic cysteine of active protein tyrosine phosphatases (PTPs) is in the thiolate anion form. Oxidation leads to reversible sulfenic acid formation. Further oxidation is irreversible and leads to sulfinic and sulfonic acid formation. To prevent this further oxidation, GSH can form a mixed disulfide with the sulfonic acid, which is then reduced by glutaredoxin (Grx). (B) To prevent PTPs from being irreversibly oxidized, the reversible inactive state is stabilized by formation of an intramolecular disulfide bond between the cysteine in the catalytic center and a backdoor cysteine. This intramolecular disulfide bond can be rapidly and effectively reduced by several reductants.
reductants like dithiothreitol (DTT), GSH, Trx-1, or a combination of these (113, 216) (Fig. 12B). The oxidation of PTPs is important for the regulation of many cellular signaling pathways in response to oxidative stress.

C. Nuclear-localized phosphatases

Recently a new concept emerged, which emphasizes an important role for nuclear tyrosine phosphatases and their signaling pathways in response to oxidative stress. We concentrate on the detailed discussion of PTPs for which a nuclear localization has been shown, even if the redox regulation takes place outside the nucleus: Shp-2, T-cell protein tyrosine phosphatase (TC-PTP), and Cdc25C (an overview is given in Table 2).

1. Protein tyrosine phosphatase Shp-2. The Src homology 2 (SH2) domain containing protein tyrosine phosphatase Shp-2 is involved in many signal-transduction processes induced by cytokines and growth factors (2, 4, 54, 55). Further findings indicated a regulation of Shp-2 by ROS (150). Stimulation of Rat-1 cells with platelet-derived growth factor (PDGF) induced production of intracellular ROS, which leads to oxidation and inactivation of Shp-2. This oxidation requires complex formation between Shp-2 and the activated PDGF receptor (PDGFR). In its basal state, the active site of Shp-2 is covered by the N-terminal SH2 domain (N-SH2). The binding of a specific phosphotyrosyl ligand opens the active site of Shp-2 (82). Meng et al. (150) speculated that binding of PDGFR to Shp-2 promotes an open, active conformation of Shp-2, and that not only substrates but also ROS can interact with this site.

Like other PTPs, Shp-2 is oxidized by ROS. Recently, two backdoor cysteines were found to be involved in the redox regulation of Shp-2. Instead of forming a disulfide bond between the active-site cysteine and a backdoor cysteine to prevent the phosphatase from further oxidation, the reduced catalytic cysteine in Shp-2 (and also in Shp-1) is shielded by a disulfide bond between two backdoor cysteines (33).

In 2002, Chughtai et al. (37) reported a nuclear localization of Shp-2 in connection with an association of the signal transducer and activator of transcription 5 (STAT5) and Shp-2. This nuclear translocation of Shp-2 in a complex with STAT5 is induced by the stimulation with prolactin in mammary cells. Formation of this complex requires the carboxy-terminal SH2 domain and the catalytic activity of Shp-2 and correlates with the tyrosine phosphorylation of STAT5 by Janus kinase 2 (JAK2) on the tyrosine residue 694 (Fig. 13). The authors speculated that the nuclear Shp-2/STAT5 complex binds to DNA and regulates transcription of milk-protein genes (37). In endothelial cells, Shp-2 is localized in nuclear and cytosolic fractions under basal conditions (96). Our findings identified nuclear Shp-2 as a counterplayer for the nuclear export of TERT. Under conditions of oxidative stress, the nuclear export of TERT is blocked by Shp-2 overexpression. This is dependent on the activity of Shp-2 because the dominant-negative Shp-2(2C459S) reduces nuclear TERT protein and telomerase activity. Ablation of endogenous Shp-2 leads to an increased tyrosine phosphorylation of TERT. Tyrosine 707 within TERT (which has previously been shown, once phosphorylated, to be essential for nuclear export of TERT) seems to be the target tyrosine for Shp-2. Thus, Shp-2 inhibits the nuclear export of TERT by regulating the tyrosine 707 phosphorylation (Fig. 14). This implies a new role for nuclear Shp-2 in protecting nuclear TERT, and therefore, nuclear Shp-2 may delay cellular senescence.

2. T-cell protein tyrosine phosphatase (TC-PTP). T-cell protein tyrosine phosphatase (TC-PTP) is a ubiquitously expressed PTP. As a result of alternative splicing, two isoforms of TC-PTP are generated, a 45 kDa isoform located in the nucleus and a 48 kDa isoform targeted to the endoplasmic reticulum (130). Specific cellular stresses cause a reversible cytosolic accumulation of the 45 kDa isoform of TC-PTP (114). For example, oxidative stress or hyperosmolarity induces the nuclear export of TC-PTP, whereas other stresses like heat shock have no effect on the localization of TC-PTP. This change in

FIG. 13. Nuclear translocation of Shp-2 induces STAT5-dependent gene transcription. Prolactin induces tyrosine phosphorylation of STAT5 at tyrosine 694 by the Janus kinase 2 (JAK2), which leads to complex formation of Shp-2 and STAT5. This complex translocates into the nucleus, binds to DNA via STAT5, and induces milk-protein gene transcription.
localization could be observed in different cell types. Non-nuclear TC-PTP is involved in the regulation of cell growth and cell differentiation in endothelial cells (143). There, TC-PTP binds to vascular endothelial growth factor receptor 2 (VEGFR2) and dephosphorylates specific tyrosine residues. Dephosphorylation of tyrosines 1045 and 1059 delays VEGF-induced VEGFR2 internalization and thus reduces sprouting angiogenesis.

Another target tyrosine of TC-PTP is tyrosine 1214, which has been implicated in VEGF-induced actin remodeling and thereby plays a role in endothelial cell migration (115). Together, these data suggest an important role for cytosolic TC-PTP in endothelial cell growth and differentiation.

Several other groups have investigated the functions of nuclear TC-PTP. The 45 kDa form of TC-PTP was first identified as a nuclear STAT1 tyrosine phosphatase. Upon stimulation, STAT1 becomes tyrosine phosphorylated by the family of JAKs and translocates into the nucleus. There it binds to DNA and activates transcription (40). To terminate this transcription, STAT1 must be dephosphorylated to dissociate from DNA. Ten Hoeve et al. (228) were the first to identify the 45 kDa form of TC-PTP to be the tyrosine phosphatase of STAT1. They also investigated the dephosphorylation of other STATs (STAT3, STAT5, and STAT6) and found that, in TC-PTP–deficient mouse embryonic fibroblasts, only the dephosphorylation of STAT1 and STAT3 is affected on interferon stimulation, but not that of STAT5 and STAT6 (228). This is in agreement with findings of Yamamoto et al. (257), who demonstrated dephosphorylated STAT3 after interleukin 6 treatment and showed a direct interaction between STAT3 and nuclear TC-PTP. Similarly, Aoki and Matsuda (10) found that in epithelial cells, stably expressing mouse TC-PTP STAT5a and STAT5b are dephosphorylated after prolactin stimulation (10). TC-PTP has different regulatory roles in diverse pathways, dependent on its localization. Because nuclear export of TC-PTP occurs under specific stresses, one
can imagine stress-specific functions of TC-PTP. This opens up an interesting field for further studies to understand why specific stress inducers like heat shock do not induce the nuclear export of TC-PTP.

3. Cdc25C. In mammalian cells, cell cycle progression is tightly regulated by the cyclin-dependent protein kinases (CDKs). CDK1 (also named Cdc2) is the key component of the checkpoint pathway, which delays mitotic entry after DNA damage or stalled replication. CDK1 forms complexes with cyclin B1 that, in their phosphorylated, inactive form, are retained in the cytosol. During prophase, CDK1/cyclin B1 complexes accumulate in the nucleus and are activated through the phosphatase Cdc25C by dephosphorylation of threonine 14 and tyrosine 15 of CDK1 (48, 112). As mentioned earlier, phosphatases can protect themselves from irreversible oxidation by forming a disulfide bond between their catalytic cysteine and a backdoor cysteine. This protection has also been demonstrated for Cdc25C. Its catalytic cysteine is cysteine 330, and the backdoor is cysteine 377. Mutation of cysteine 377 in Cdc25C leads to irreversible oxidation of cysteine 330, which results in degradation of Cdc25C (198). During interphase of the cell cycle, a formation of the disulfide bond between cysteine 330 and 377 in Cdc25C is induced, and Cdc25C is phosphorylated at serine 216. This phosphorylation leads to binding of Cdc25C to 14-3-3 proteins and results in the nuclear export of Cdc25C (179). Mutation of serine 216 in Cdc25C perturbs mitotic timing and allows cells to escape the G2-checkpoint arrest (179, 195).

For the fate of the cytosolic Cdc25C, different hypotheses exist. One possible mechanism is the degradation of Cdc25C, according to the destruction hypothesis of Savitsky and Finkel (198). It has been demonstrated that vitamin C induces formation of ROS, which leads to decreased Cdc25C levels. Its catalytic cysteine 377 in Cdc25C leads to irreversible oxidation of cysteine 330, which results in degradation of Cdc25C (198). During interphase of the cell cycle, a formation of the disulfide bond between cysteine 330 and 377 in Cdc25C is induced, and Cdc25C is phosphorylated at serine 216. This phosphorylation leads to binding of Cdc25C to 14-3-3 proteins and results in the nuclear export of Cdc25C (179). Mutation of serine 216 in Cdc25C perturbs mitotic timing and allows cells to escape the G2-checkpoint arrest (179, 195).

V. Conclusions and Outlook

Redox regulation plays an important role in intracellular signal transduction. Numerous proteins have been described to be redox regulated. However, it must be noted that, for the nuclear import and export receptors, only initial studies in yeast have shown that these receptors change their localization from the nucleus to the cytosol and can be oxidized on oxidative stress, which could importantly contribute to gene regulation (111, 183, 184). Thus, further studies in higher eukaryotes are required to investigate the redox regulation of the nuclear import and export machinery. Furthermore, many studies do not distinguish between the intracellular compartments in which the redox modification of the protein of interest takes place. This is of special importance, because many antioxidative systems exist in different cellular compartments like the cytosol, the mitochondria, and the nucleus. However, not all antioxidative systems are equally distributed throughout the cell. The distribution strongly depends on the cell type and on the stimulus used. Therefore, it is noteworthy that cellular functions regulated by redox modifications of proteins are intimately associated with their cellular localization. Unfortunately, many previous investigations did not accurately discriminate between the compartments where redox regulation occurs and the corresponding cellular function. Without this differentiation, pharmacologic interventions may exhibit undesirable and unanticipated side effects because the compartment-specific effects of the proteins have been disregarded in the past. Therefore, compartment-specific investigations will help us to determine protein functions in more detail and to uncover misunderstood protein functions.

Finally, compartment-specific delivery of inhibitors and activators will open a new field of drug design to regulate protein actions more precisely and to reduce unwanted side effects.

Acknowledgments

The first two authors contributed equally to this work. We apologize for the failure to cite many of the important and relevant articles in this field due to space limitations. This study was in part supported by the Deutsche Forschungsgemeinschaft (HA-2868/3-2, SFB 612 TP B15, and SFB 728 TP B5 to J.H.). M.L. received a scholarship from the Research Training Group 1089.

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NUCLEAR REDOX SIGNALING


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Date of first submission to ARS Central, March 31, 2009; date of final revised submission September 2, 2009; date of acceptance, September 5, 2009.
### Abbreviations Used

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<tr>
<td>AP-1</td>
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<td>APEX1</td>
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<td>ATF</td>
<td>activating transcription factor</td>
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<td>bZIP</td>
<td>basic region-leucine zipper</td>
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<td>cyclic adenosine monophosphate</td>
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Well-known Signaling Proteins Exert New Functions in the Nucleus and Mitochondria

N. Büchner, J. Altschmied, S. Jakob, G. Saretzki, J. Haendeler

Antioxid Redox Signal, 2010, in press

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Well-known Signaling Proteins Exert New Functions in the Nucleus and Mitochondria

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Abstract

One distinguishing feature of eukaryotic cells is their compartmentalization into organelles, which all have a unique structural and functional identity. Some proteins are exclusively localized in a single organelle, whereas others are found in more than one. A few proteins, whose function was thought to be completely understood, were only recently found to be present in the mitochondria. Although these proteins come from diverse functional classes, their common new denominator is the regulation of respiratory chain activity. Therefore, this review focuses on new functions of the Signal Transducer and Activator of Transcription 3, originally described as a transcription factor, the most prominent Src kinase family members, Src, Fyn, and Yes, which were so far known as plasma membrane-associated molecular effectors of a variety of extracellular stimuli, the tyrosine phosphatase Shp-2 previously characterized as a modulator of cytosolic signal transduction involved in cell growth, development, inflammation, and chemotaxis, and Telomerase Reverse Transcriptase, the key enzyme preventing telomere erosion in the nucleus. Their unexpected localization in other organelles and regulation of mitochondrial and/or nuclear functions by them adds a new layer of regulatory complexity. This extends the flexibility to cope with changing environmental demands using a limited number of genes and proteins. Antioxid. Redox Signal. 13, 000–000.

Introduction

One distinguishing feature of eukaryotic cells in comparison to prokaryotes is their compartmentalization into organelles, which are obvious already at the microscopic level. Each compartment or organelle contains a characteristic set of proteins providing it with a unique structural and functional identity. Therefore, proteins, which—with the exception of a few respiratory chain components in the mitochondria—are translated in the cytoplasm, have to be targeted to their place of final destination. Eukaryotic cells have evolved highly specialized mechanisms to perform this task. Most commonly, specific topogenic sequences within proteins are used to target them to a distinct subcellular localization, such as the nucleus, mitochondria, peroxisomes, and the endoplasmic reticulum, from where they are transported through the Golgi apparatus to become secreted or membrane proteins. All these targeting sequences are characterized by conserved amino acids and are recognized by highly specialized transport complexes that are required to carry their cargo to the respective organelle. Specific sequences of amino acids can easily be recognized by appropriate analysis software and therefore a large number of programs are available to predict the subcellular localization of a protein based on its primary structure (Table 1). However, not all proteins contain such conserved targeting sequences despite a highly specific subcellular distribution (7, 8, 11, 20, 21).

Besides proteins that are exclusively localized in a single organelle, others exist, which are present in more than one compartment. One cellular strategy to achieve distribution to several or different locations is to produce different polypeptides possessing or lacking one or the other targeting sequence, either from separate genes or from a single gene by means of alternative transcription or translation initiation, differential splicing, or post-translational modification. However, several proteins possess two targeting signals leading to distribution between several organelles. In these cases, the desired and/or required localization can be achieved by different relative affinities to the transport machineries, accessibility of the targeting signals, incomplete translocation or redistribution via retrograde transport, leakage out of the organelle, or active export (for review, see Refs. 9 and 23).
As the distribution of single translation products to more than one destination within the cell is less well understood than targeting to a single compartment, this review will be far from comprehensive, but rather a compilation of a few interesting proteins, for which a role in the nucleus and in the mitochondria has been shown just recently. The aim is not to describe the regulation of targeting of these proteins, but rather their function in different organelles. The major emphasis will be on proteins newly discovered in the mitochondria, which play a role in regulating the electron transfer chain. Mitochondria contain the most reducing compartment, have the highest rate of electron transfer, and are highly sensitive to oxidation. They are the most redox-active compartment of mammalian cells, accounting for more than 90% of electron transfer to O\textsubscript{2} as the terminal electron acceptor. Therefore, proteins that have well-described functions in other cellular compartments and were recently shown to be involved in the regulation of respiratory chain regulation are in the focus of this review. Specifically, we will discuss functions of four proteins in compartments where they had not been suspected before: a) the Signal Transducer and Activator of Transcription 3 (STAT3), originally described as a transcription factor; b) the most prominent Src kinase family members, Src, Fyn, and Yes, which were so far known as plasma membrane-associated molecular effectors of a variety of extracellular stimuli; c) the tyrosine phosphatase Shp-2 previously characterized as a modulator of cytosolic signal transduction involved in cell growth, development, inflammation, and chemotaxis; and d) Telomerase Reverse Transcriptase (TERT), the key enzyme preventing telomere erosion in the nucleus.

### 1. Signal Transducer and Activator of Transcription 3

Signal transducers and activators of transcription (STATs) were originally described as key components of a direct signal transduction pathway from the cell surface to the nucleus in response to cytokines and growth factors. For a long time, the tyrosine phosphorylation of STATs by ligand activated receptors was thought to be an obligatory requirement for dimerization in an active conformation, nuclear import, and transcriptional activation (10, 25). More recently it has been shown that nonphosphorylated STATs shuttle between the cytoplasm and the nucleus at all times in a constitutive manner and that also these nonphosphorylated STATs can be transcriptionally active, either as homodimers or in a complex with other transcription factors. However, these nonphosphorylated STATs regulate a different set of target genes than their phosphorylated counterparts (36, 42) (Fig. 1).

Lately, new functions for STAT3 outside the nucleus became evident. STAT3 was shown to be present in the mi-
to mitochondria of cultured cells and primary tissue, although it does not contain a mitochondrial targeting sequence. Immunoprecipitations demonstrated an association with complex I and possibly with complex II of the electron transport chain. On the functional level, an influence of STAT3 on the respiratory chain was demonstrated in STAT3-deficient pro-B cells, where the activities of complexes I and II were reduced by 40% and 85%, respectively, although the mitochondrial content in the STAT3-/- cells was unaltered. These findings were confirmed in hearts of mice with cardiomyocyte specific ablation of STAT3. Reconstitution of STAT3-deficient cells with different STAT3 mutants specifically targeted to the mitochondria revealed that mitochondrial STAT3 is sufficient to modulate respiratory chain activity and that phosphorylation on serine 727 and a monomeric conformation play a crucial role in this process. In addition, the effects of STAT3 on the respiratory chain were unrelated to its actions as a transcription factor (39). A second report described a function of mitochondrial STAT3 in cellular transformation by the nontyrosine kinase oncogene Ras (14). Ras mediated transformation in vitro and tumor growth in mice were impaired in STAT3-deficient cells. Mutational analysis demonstrated that the N-terminal DNA binding domain, the Src homology 2 (SH2) domain, phosphorylation on tyrosine 705, and nuclear localization of STAT3 are dispensable for supporting malignant transformation by Ras. In contrast, tyrosine phosphorylation and presence in the nucleus are required for transformation by the tyrosine kinase oncogene v-Src. This newly discovered function of STAT3 in the nucleus was ascribed to its mitochondrial localization accompanied by augmentation of respiratory chain activity, particularly that of complex II and V, and a dependence on phosphorylation of serine 727. In summary, these results lead to the conclusion that mitochondrial STAT3 can modulate the activity of the electron transport chain and that the structural requirements are completely different than the ones for transcriptional activation in the nucleus (Fig. 2).

2. Src, Fyn, and Yes Kinases

The Src family of nonreceptor protein tyrosine kinases consists of at least 9 members, some of which, like Src, Yes, and Fyn, are ubiquitously expressed, whereas others show more limited expression patterns (28). In this review we will focus on the most prominent kinases, Src, Fyn, and Yes, because they can compensate for each other. These three kinases are important for the regulation of cell proliferation by modulating cell metabolism, division, survival, and migration. Their function as plasma membrane-associated molecular effectors of a variety of extracellular stimuli is well known. However, recent studies demonstrated that at least Src fulfills also important functions in the nucleus and mitochondria. Changes in the chromatin structure indicative of active or inactive transcription are observed during cell cycle, tumorogenesis, and senescence. Increased euchromatic hypocondensation and heterochromatic hypercondensation are detected upon growth factor stimulation. These processes depend on nuclear tyrosine phosphorylation by Src, Fyn, and/or Yes, since they are not observed in Src, Fyn, Yes-triple deficient mouse embryonic fibroblasts (MEFs) (38). Recently, our group revealed a different cellular function for nuclear Src and Yes in endothelial cells by demonstrating that they contribute to the hydrogen peroxide-induced nuclear export of telomerase reverse transcriptase (TERT) (22), which will be discussed in more detail later in this review.

A mitochondrial localization of Src has been demonstrated by several groups (2, 26, 30). In the experiments described in these publications, several complexes of the respiratory chain have been identified as substrates for Src. First, the cytochrome c oxidase, the terminal complex of the electron transport chain was shown to be activated by Src (26). Recently, it has been discovered that Src has also effects on other complexes of the respiratory chain. Arachiche et al. (2) reported an increase of Src activity in response to ATP in rat brain mitochondria. ATP addition induced an autophosphorylation of Src at its catalytic site, which leads to its activation. This activated Src increased the activity of the complexes I, III, and IV, and decreased that of complex V (2). Taken together, these data indicate that respiratory chain activity is partially dependent on tyrosine phosphorylation by Src.

3. Protein Tyrosine Phosphatase Shp-2

The ubiquitously expressed protein tyrosine phosphatase Shp-2 contains two N-terminal SH2 domains and a C-terminal protein tyrosine phosphatase domain. Shp-2 plays an important role in cytosolic signal transduction. It modulates different pathways involved in cell growth, cell development, tissue inflammation, and cellular chemotaxis. These cytosolic functions of Shp-2 are well known and reviewed elsewhere (5). However, over the last years also nuclear and mitochondrial functions of Shp-2 have been identified. In 2002 Chughtai et al. (6) reported a nuclear localization of Shp-2 associated with the signal transducer and activator of transcription 5 (STAT5). After stimulation of mammary cells...
with prolactin, Shp-2 forms a complex with STAT5, which translocates into the nucleus. Formation of this complex is dependent on tyrosine phosphorylation of STAT5 in response to prolactin. Shp-2 binds to tyrosine phosphorylated STAT5 for which it requires the distal SH2 domain and an intact catalytic center. The nuclear Shp-2/STAT5 complex binds to DNA and regulates transcription of milk protein genes (6), demonstrating a transcriptional regulation by nuclear Shp-2. Given the fact that Shp-2 does not dephosphorylate STAT5 and that binding of Shp-2 to STAT5 is required for keeping STAT5 in its tyrosine phosphorylated and thereby active state, nuclear Shp-2 acts as an enhancer of transcription by binding to STAT5 and not as a phosphatase. Thus, these findings revealed a new phosphatase independent function of Shp-2. In contrast, an inhibition of the transcriptional activity of STAT1 by dephosphorylation of tyrosine and serine residues of STAT1 in the nucleus by Shp-2 has been shown (41), demonstrating that nuclear Shp-2 can also function as a phosphatase. In line with these findings are data from our group. We demonstrated that hydrogen peroxide-induced nuclear export of TERT is dependent on the tyrosine kinases Src and Yes. We identified nuclear Shp-2 as the counterplayer for this export, demonstrating again a phosphatase activity of Shp-2 in the nucleus (22). The maintenance of TERT in the nucleus by Shp-2 under conditions of oxidative stress will be discussed in more detail later in this review.

Recently, a tyrosine phosphatase activity was detected in the mitochondria of rat brains. The responsible phosphatase was identified as Shp-2. It was mainly located inside the mitochondria associated with cristae and the intercristal space (31). Arachiche et al. also showed a mitochondrial localization of Shp-2 (2). As mentioned above, the same group also detected the tyrosine kinase Src in the mitochondria and suggested that Src is partially involved in the regulation of respiratory chain activity. For cytosolic Src and Shp-2, it has long been demonstrated that the two proteins regulate each other in their activity. Therefore, it is tempting to speculate that mitochondrial Shp-2 acts as a phosphatase and inhibits Src activity and is thus also involved in the regulation of respiratory chain activity.

4. Telomerase Reverse Transcriptase

Telomeres, the physical ends of chromosomes, are necessary for their stability and integrity. They are shortened during each cell division. This shortening is counteracted by the enzyme telomerase. Telomerase is a large ribonucleoprotein complex and consists of the reverse transcriptase subunit telomerase reverse transcriptase (TERT), which contains the catalytic activity of the enzyme, and the associated RNA component TERC, which serves as the template for synthesis of the telomeric sequence (15, 27). Several telomerase-associated proteins are involved in the assembly and activity of the holoenzyme (Fig. 3).

TERT is differentially regulated on transcriptional and post-translational levels but also via its localization. One major post-translational event is the phosphorylation of TERT by kinases such as Src, Akt, PKC, and ERK1/2. The kinase Akt has a dominant role in the activation of TERT (3). Upon phosphorylation of serine 823 in TERT by Akt, telomerase activity is increased. Also the binding of HSP90 to TERT is essential for telomerase activity via stabilization of a TERT/
apoptosis (13, 17, 19). The anti-apoptotic capacity of TERT occurred within few hours after transfection, which indicates a function independent of direct telomere elongation. Further studies supported telomere-independent functions of TERT. In cell culture models, the suppression of TERT or TERC in cancer and stem cells has been shown to reduce proliferation and render the cells more vulnerable to apoptosis in a largely telomere length independent fashion (12, 34, 43). Similar results were obtained by ectopic expression of TERT. Stewart et al. demonstrated that TERT enhances tumorigenesis independent of its telomeric function although the mechanism for this effect is not entirely clear (37). Importantly, Sarin et al. showed that conditional transgenic induction of TERT can activate epidermal stem cells independent of its catalytic function (35). This demonstrated for the first time that TERT has an important telomere-independent function in stem and progenitor cells. In line with the emerging nontelomeric functions, Santos et al. showed that telomerase activity and TERT protein can be detected in mitochondria (32, 33). Although this came as a surprise to the scientific community, it is supported by the finding that TERT has a N-terminal mitochondrial targeting sequence. In addition, we showed that TERT is imported into mitochondria by the translocases of outer and inner membrane (16) (Fig. 6). However, the functions that TERT fulfills in mitochondria are still controversial. Santos et al. associated the mitochondrial localization of TERT with an increased apoptosis induction and interpreted this as a potential selective mechanism for the elimination of damaged stem cells (33). Recently our laboratories have contradicted these findings by demonstrating a beneficial role of TERT within mitochondria (1, 16). Independent of each other we found an improved mitochondrial function, decreased apoptosis, and reduced mitochondrial reactive oxygen species measured as a decrease in Mitosox fluorescence in cells expressing TERT (Fig. 6). Furthermore, we demonstrated that TERT directly or indirectly binds to mitochondrial DNA. Moreover, we showed that mouse lung fibroblasts from TERT knockout animals are more sensitive to ultraviolet B (UVB)-induced decrease in proliferation and respiration than their

FIG. 4. In the nucleus TERT forms a complex with Akt and Hsp90, which keeps TERT phosphorylated on serine 823 and therefore in its active state. Active nuclear TERT prevents telomere erosion and can inhibit apoptosis. Under conditions of oxidative stress, Src kinases induce phosphorylation of nuclear TERT on tyrosine 707 resulting in nuclear TERT export via the nuclear pore in a CRM1/RanGTP dependent manner. Protein tyrosine phosphatase Shp-2 inhibits phosphorylation and TERT export.

FIG. 5. Regulation of nuclear TERT tyrosine phosphorylation by Src and Shp-2. Active Src, phosphorylated on tyrosine 416, phosphorylates TERT on tyrosine 707, leading to nuclear export of TERT. This is counteracted by catalytically active Shp-2 (C459), which either dephosphorylates TERT directly or indirectly through inactivation of Src by dephosphorylation.

FIG. 6. TERT is imported into the mitochondria via the translocases of outer and inner membrane (TOM, TIM). Here, TERT is located in the matrix and can bind mitochondrial DNA (mtDNA). Mitochondrial TERT improves mitochondrial function, decreases apoptosis, and lowers ROS levels in the cell.
wild-type counterparts. UVB radiation causes cell death and DNA damage. It induces the formation of cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts (44). Together with our finding that TERT associates with mitochondrial DNA, one could speculate that TERT protects mitochondrial DNA against the deleterious effects of UVB. However, there is accumulating evidence that other mechanisms, such as free radical formation, play important roles in the cellular responses caused by UVB radiation (24). This would offer an additional explanation for the protective function of mitochondrial TERT, which reduces reactive oxygen species in this organelle. We also demonstrated that TERT overexpression enhances respiratory chain activity and found that the respiration rate is decreased in heart, but not in liver from TERT knockout animals (16).

In accordance with TERT expressing cells having lower reactive oxidative species levels, it has recently been demonstrated that cells and tissues from mice deficient for the RNA component of telomerase (TERC) have an imbalance in their redox systems, resulting in higher levels of oxidative stress. Perez–Rivero and colleagues (29) found increased MnSOD level in MEFs and tissues from first generation TERC knock-out mice, which do not display telomere shortening, but a level in MEFs and tissues from first generation TERC knock-out mice, which do not display telomere shortening, but a higher oxidative stress is supported by data from our laboratories. We showed a reduced oxygen uptake in heart tissue from TERT knockout mice and a decreased UVB resistance in lung fibroblasts derived from these animals (16). These data are complemented by our in vitro findings demonstrating a decrease of reactive oxygen species and improvement of mitochondrial function in TERT overexpressing fibroblasts (1). In accordance with this, higher catalase protein levels were found in TERT overexpressing fibroblasts while there was no change in the levels of MnSOD (Saretzki, unpublished data). Moreover, the influence of TERT on heart function has been further investigated in a voluntary running mouse model (40). We showed that physical exercise can stimulate telomerase in the heart and has beneficial anti-aging effects measured by a decrease in senescence-associated markers such as p16, p53, and Chk2. In TERT-deficient mice, however, the effect of exercise was absent, pointing to an important role of telomerase in this process. This leads to the conclusion that the running-induced upregulation of telomerase reduces oxidative stress and thereby may slow down senescence. Given the facts that serum levels of insulin-like growth factor 1 (IGF-1) are increased with voluntary running and that IGF-1 has been shown to activate Akt in cardiomyocytes, we wanted to determine whether increased IGF-1 levels serve as a mediator of increased telomerase activity. Therefore, mice were treated with IGF-1 and as expected, the IGF-1 treatment resulted in an activation of Akt in the heart and a substantial increase of telomerase (40). In addition, an increased proliferation rate in cardiomyocytes was observed after voluntary running. One possible explanation might be a change in pro-proliferative transcriptional programs due to increased TERT levels, because changes in the cellular transcriptome have been observed upon overexpression of TERT (4). However, it is undeniable that mitochondrial function is required for cardiomyocyte proliferation, suggesting that increased TERT levels, which result in enhanced respiratory chain activity, are one of the reasons for cardiomyocyte proliferation.

In conclusion, it is tempting to speculate that nuclear and mitochondrial TERT act in concert to improve cardiomyocyte and thereby heart function.

Conclusion

In this review we have summarized recent evidence for several proteins extending their functions to cellular compartments beyond the ones, which have been textbook knowledge for a long time. These proteins can have similar roles in different organelles or can perform completely different, so far unexpected tasks depending on their subcellular localization. These new functions are not restricted to a specific class of proteins, as they have been described for transcription factors, protein kinases and phosphatases, and the only eukaryotic reverse transcriptase, TERT. Interestingly, all these proteins are involved in regulatory processes, which help cells to adapt to changing environmental situations. Thus, one may speculate that such additional functions in other cellular compartments, especially in the mitochondria, are not restricted to the few examples described here, but could be a more general phenomenon, which might have been overlooked in the past. Changing the subcellular distribution of a particular protein and thereby sometimes making use of other functional properties, adds a new layer of complexity in addition to the well described regulatory processes on the transcriptional, translational, or post-translational levels. Thereby cells, organs and whole organisms would extend their flexibility to cope with changing environmental demands using a limited number of genes and proteins.

Acknowledgments

We apologize for the failure to cite many of the important and relevant papers in this field due to space limitations. This study was in part supported by the Deutsche Forschungsgemeinschaft to J.H. (SFB 728 B5 and HA-2868/2-3).

References


Downregulation of mitochondrial telomerase reverse transcriptase induced by H₂O₂ is Src kinase dependent

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Exp Gerontol, 2010, in press

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Downregulation of mitochondrial telomerase reverse transcriptase induced by H$_2$O$_2$ is Src kinase dependent

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Keywords: Mitochondria Telomerase reverse transcriptase Src kinase Akt1 Endothelial cells H$_2$O$_2$

1. Introduction

The enzyme telomerase counteracts the shortening of telomeres, the physical ends of the chromosomes. Thereby it prevents the onset of replicative senescence and genetic instability (Blackburn, 2000; Buys, 2000; Collins, 2000; Hemann et al., 2001). Its catalytic subunit is the telomerase reverse transcriptase (TERT) (Collins, 2000). Several studies demonstrated that nuclear TERT increases cell survival and resistance against oxidative stress after short term incubation with different stimuli, which did not affect telomere length (Haendeler et al., 2003a,b; Rahman et al., 2005; Ramirez et al., 2003; Ren et al., 2001). Another unexpected finding was the observation that TERT is also localized in mitochondria (Ahmed et al., 2008; Haendeler et al., 2009; Santos et al., 2004, 2006). Since the circular mitochondrial DNA does not contain any telomeric repeats, mitochondrial TERT has to have also a telomere-independent role within mitochondria. However, the exact functions of TERT in mitochondria remain controversial. Santos et al. demonstrated in fibroblasts overexpressing TERT that it exacerbates oxidative injury (Santos et al., 2004, 2006). In contrast, Ahmed et al. showed in the same cells a protective role for mitochondrial TERT after oxidative stress (Ahmed et al., 2008). Furthermore, we recently revealed that endogenous, mitochondrial TERT protects mitochondrial DNA from damage and importantly contributes to the respiratory chain activity under basal conditions in endothelial cells as well as in human embryonic kidney cells (Haendeler et al., 2009). Besides this controversy, it is completely unknown how TERT is regulated in mitochondria. However, since changes in reactive oxygen species formation regulate nuclear TERT, one could speculate that also mitochondrial TERT is affected by oxidative stress. Moreover, there exists accumulating evidence that mitochondria are one compartment within the cell producing reactive oxygen species that contribute to aging processes, which has been demonstrated by several lines of evidence. Mice overexpressing mitochondrially targeted catalase show a significant increase in life span and a reduction in oxidative damage to DNA and consequently in apoptosis (Schriner et al., 2005). Moreover, overexpression of mitochondrially localized antioxidant enzymes lengths lifespan of Drosophila (Orr and Sohal, 1994; Ruan et al., 2002) and deletion of manganese superoxide dismutase in mice results in the age-related decline of mitochondrial function, culminating in increased apoptosis (Kokoszka et al., 2001). Thus, aging and apoptotic processes are associated with increased reactive oxygen species formation within the mitochondria. Because also TERT is involved in aging and apoptotic processes, we elucidated for the first time the regulation of mitochondrial TERT by oxidative stress and the role of the Src kinase therein.
2. Materials and methods

2.1. Cloning of expression vectors for mitochondrially targeted TERT

The N-terminal mitochondrial targeting sequence was amplified from pCMV/myc/mito (Invitrogen) using the primers mito-link for1 (5’-CAATGCTAGCCACCATGCAGTTCTGGACGCCG-3’) and mito-link rev1 (5’-GATGGATTCCAAGTTGGATCTTGGACGCCG-3’) in a standard PCR reaction. The resulting amplification product was cut with NheI and EcoRI inserted into hTERT expression vectors for TERT wild-type and TERT(Y707F), respectively (Haendeler et al., 2003a) and verified by sequencing.

2.2. Cell culture, transfection

Human embryonic kidney (HEK) cells were cultured in DMEM basal medium with 10% heat-inactivated fetal calf serum. Human umbilical vein endothelial cells (HUVEC) were cultured in endothelial basal medium supplemented with hydrocortisone (1 μg/ml), bovine brain extract (12 μg/ml), gentamicin (50 μg/ml), amphotericin B (50 ng/ml), epidermal growth factor (10 ng/ml), and 10% fetal calf serum. After detachment with trypsin, cells were grown for 24 h in basal medium with 10% heat-inactivated fetal calf serum. Human embryonic kidney cells as well as endothelial cells. Indeed, the Src kinase is localized in the mitochondria of these cell types (Figs. 1A and 2).

2.3. Cellular fractionation

Cellular fractionation and isolation of mitochondria was performed as described previously (Yang et al., 1997). To obtain total cell lysates, cells were lysed in RIPA-buffer as described previously (Haendeler et al., 2003a).

2.4. Immunoblotting

Immunoblotting was performed with antibodies directed against TERT (1:500, overnight, 4°C, Rockland), total Src (1:500, Invitrogen), phospho-SrcY418 (1:500, Invitrogen), phospho-SrcY29 (1:1000, Abcam), phospho-Akt1 (1:1000, Cell Signaling Technol.), Akt1 (1:1000, Cell Signaling Technol.), tubulin (1:5000, Sigma), Thioredoxin-1 (Trx-1) and translocase of inner membrane 23 (TIM23) were used to control for purity of the fractions. (B) Immunoblots with mitochondrial lysates from HUVEC were probed with antibodies against TERT, Akt1, Tim23 and Trx-1.

Fig. 1. Localization of TERT, Akt1 and Src kinase in mitochondria. (A) Mitochondrial and cytosolic fractions of HEK 293 cells were isolated as described in Section 2. Lysate proteins were resolved by SDS–PAGE, blotted onto PVDF membranes and the blots probed with antibodies against TERT, Src and Akt1. Antibodies against thioredoxin-1 (Trx-1) and translocase of inner membrane 23 (TIM23) were used to control for purity of the fractions. (B) Immunoblots with mitochondrial lysates from HUVEC were probed with antibodies against TERT, Akt1, Tim23 and Trx-1.

2.5. Statistics

Statistical analyses were performed with student’s T-test using winLSTAT 2008.

3. Results

3.1. TERT and the Src kinase are localized in mitochondria

We recently demonstrated that TERT is localized in mitochondria, where it protects mitochondrial DNA from damage and preserves mitochondrial functions (Haendeler et al., 2009). However, the regulation of mitochondrial TERT is completely unknown. From our studies of nuclear TERT in endothelial cells as well as in human embryonic kidney cells, we know that TERT is exported from the nucleus under conditions of oxidative stress in a Src kinase dependent manner (Haendeler et al., 2003a, 2004). Moreover, it has recently been demonstrated that the Src kinase is localized in mitochondria in rat brain and that the complexes of the respiratory chain are substrates for Src, indicating that respiratory chain activity is partially dependent on tyrosine phosphorylation (Arachiche et al., 2008). Therefore, we first investigated whether the Src kinase is present in mitochondrial fractions from human embryonic kidney cells as well as endothelial cells. Indeed, the Src kinase is localized in the mitochondria of these cell types (Figs. 1A and 2).

3.2. Mitochondrial Src kinase is activated by treatment with H2O2

Next, we determined whether treatment with H2O2 activates the Src kinase in mitochondria. Therefore, human endothelial cells were incubated with 200 μM H2O2 and active Src kinase was measured by immunoblot analysis of mitochondrial lysates. For that purpose immunoblots were performed with an antibody against active Src, phosphorylated at tyrosine 418 and an antibody against inactive Src, phosphorylated at tyrosine 529. The ratio between...
We previously demonstrated that nuclear TERT is complexed with Akt1 and Heat Shock protein 90 (Haendeler et al., 2003b). Disruption of this complex resulted in inactivation of Akt1, which subsequently led to a reduction of nuclear TERT and to induction of apoptosis (Haendeler et al., 2003b). Therefore, we next determined whether Akt1 is also localized in the mitochondria. Indeed, Akt1 was identified in mitochondria of HEK293 cells as well as endothelial cells (Fig. 1A and B). Since Akt1 is negatively regulated by increased oxidative stress in endothelial cells (Hoffmann et al., 2001), we hypothesized that incubation with $\text{H}_2\text{O}_2$ could also reduce active Akt1 in the mitochondria. To determine active Akt1, we measured phosphorylation of Akt1 on serine 473 in mitochondria of endothelial cells. Incubation with $\text{H}_2\text{O}_2$ reduced active Akt1 (Fig. 3A and B). These data suggest that mitochondrial TERT and nuclear TERT are regulated similarly.

3.4. Downregulation of mitochondrial TERT by $\text{H}_2\text{O}_2$ depends on tyrosine 707 in TERT and thereby on the Src kinase

Having demonstrated that exposure to $\text{H}_2\text{O}_2$ increases mitochondrial Src kinase activity and decreases mitochondrial Akt1 activity, we next wanted to investigate whether mitochondrial TERT is regulated by $\text{H}_2\text{O}_2$. Taking into account that TERT is exported from the nucleus (Haendeler et al., 2003a) and that after nuclear export, the TERT level is increased in the cytosol and in the mitochondria (Ahmed et al., 2008; Haendeler et al., 2003a), we decided to use TERT exclusively targeted to the mitochondria (mitoTERT) containing a myc-tag to prevent detection of endogenous TERT, which may be newly imported into these organelles. After overexpression of myc-tagged mitoTERT in endothelial cells and treatment with 200 $\mu\text{M} \text{H}_2\text{O}_2$, we measured the levels of mitochondrial TERT by immunoblot. Interestingly, $\text{H}_2\text{O}_2$ induced a significant reduction of TERT in the mitochondria (Fig. 4A and B). From our previous studies, we knew that tyrosine 707 in TERT is a putative Src kinase phosphorylation site (Haendeler et al., 2003a; Jakob et al., 2008). Mutation of tyrosine 707 to phenylalanine resulted in complete inhibition of nuclear TERT export triggered by the Src kinase. Therefore, we inserted a mitochondrial targeting sequence into the TERT(Y707F) expression construct to

![Fig. 2](image-url)  
**Fig. 2.** Src kinase is activated by $\text{H}_2\text{O}_2$ in mitochondria. HUVEC were treated with 200 $\mu\text{M} \text{H}_2\text{O}_2$ for 3–4 h ($\text{H}_2\text{O}_2$) or left untreated (co) and mitochondrial lysates prepared as described in Section 2. The proteins were resolved by SDS–PAGE and blotted onto PVDF membranes. Blots were probed with antibodies against active Src phosphorylated on tyrosine 418 (SrcY418-P), inactive Src phosphorylated on tyrosine 529 (SrcY529-P) and translocase of inner membrane 23 (TIM23). (A) Representative blot. (B) Blots were quantitated densitometrically and the levels of SrcY418-P and SrcY529-P normalized to TIM23 levels. The graph shows the ratio of active to inactive Src. Data are mean +/- SEM of 4 independent experiments. * Significantly different to control ($p < 0.05$).

![Fig. 3](image-url)  
**Fig. 3.** Akt1 is deactivated by treatment with $\text{H}_2\text{O}_2$ in mitochondria. HUVEC were treated with 200 $\mu\text{M} \text{H}_2\text{O}_2$ for 3–4 h ($\text{H}_2\text{O}_2$) or left untreated (co) and mitochondrial lysates prepared as described in Section 2. The proteins were resolved by SDS–PAGE and blotted onto PVDF membranes. Blots were probed with antibodies against active Akt1 phosphorylated on Serine 473 (P-Akt) and translocase of inner membrane 23 (TIM23). (A) Representative blot. (B) Blots were quantitated densitometrically and the levels of active Akt1 (P-Akt) normalized to TIM23 levels. Data are mean +/- SEM of 4 independent experiments. * Significantly different to control ($p < 0.05$).
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Akt1 and Src kinase are activated and TERT is exported from the nucleus, which results in the onset of replicative senescence and in increased apoptosis sensitivity (Haendeler et al., 2003a, 2004). Recent studies demonstrated a mitochondrial localization of TERT. However, the regulation of mitochondrial TERT is completely unknown. Several studies were undertaken to investigate, whether Akt1 and Src kinase are localized in mitochondria. Indeed, Akt1 and Src kinase were found to be localized in mitochondria of fibroblasts and rat brain, respectively (Antico Arciuch et al., 2009; Arachiche et al., 2008). Akt1 has been shown to cycle between the nucleus and the mitochondria dependent on the redox status of the cell (Antico Arciuch et al., 2009) and complexes of the respiratory chain have been demonstrated to be substrates of the Src kinase (Arachiche et al., 2008). Taking into account that mitochondrial TERT enhances respiratory chain activity and mitochondrial function (Haendeler et al., 2009) and the known regulation of TERT by the kinases Akt1 and Src in the nucleus as mentioned above, it is tempting to speculate that Akt1 and Src can also regulate mitochondrial TERT. Indeed, we also found the kinases Akt1 and Src in the mitochondria of primary human endothelial cells as well as human embryonic kidney cells (Figs. 1 and 2). Therefore, these kinases could also regulate mitochondrial TERT. Interestingly, H$_2$O$_2$ treatment led to a reduction in active Akt1, to an induction in active Src kinase and to a reduction in mitochondrial TERT levels. Thus, one may speculate that mitochondrial TERT is regulated in a similar fashion as nuclear TERT. This speculation is underscored by our finding that oxidative stress-induced downregulation of mitochondrial TERT (Fig. 4) like nuclear TERT (Haendeler et al., 2003a) depends on tyrosine 707. However, it has to be noted that export of nuclear TERT results in an increased amount of mitochondrial TERT (Ahmed et al., 2008). Taken these findings together, one has to suggest that oxidative stress initially leads to a reduction of TERT levels in the mitochondria, which is followed by a nuclear export of TERT and a concomitant mitochondrial import, but this is pure speculation and requires further experimental proof. However, the finding that an increased amount of TERT is found in the mitochondria after oxidative stress-induced nuclear export (Ahmed et al., 2008), suggests also that the mitochondrial Src kinase has to be inactivated at that time. Since we identified the tyrosine phosphatase Shp-2 as the counterplayer for Src dependent nuclear export of TERT (Jakob et al., 2008) and Arachiche et al. found that Shp-2 is also localized in the mitochondria of rat brain (Arachiche et al., 2008), it is tempting to speculate that Shp-2 inactivates the mitochondrial Src kinase.

In conclusion, our study demonstrates for the first time that the Src kinase and Akt1 are present also in the mitochondria of human endothelial cells and human embryonic kidney cells. Mitochondrial Src is activated, Akt1 is deactivated and mitochondrial TERT levels are downregulated by H$_2$O$_2$. This downregulation is dependent on a Src kinase dependent phosphorylation site, tyrosine 707, within TERT. Therefore, both mitochondrial and nuclear TERT might be regulated by the same mechanisms at least in primary human endothelial cells.

Acknowledgements

This work was supported by a grant from the Deutsche Forschungsgemeinschaft to J.H. (HA2868/3-2), by the Sonderforschungsrbung to J.H. (HA2868/3-2), by the Sonderfors-
chungsbereich 728 project B5 (to J.H.) and by the Bundesministerium fuer Bildung und Forschung (03NUK003C). We thank Diane Schmiegelt and Kerstin Kunze for expert technical assistance. The authors have nothing to disclose.

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Danksagung

Ich danke Frau PD. Dr. Jojo Haendeler für die freundliche Aufnahme in ihre Arbeitsgruppe, die Übergabe des interessanten Themas und die Unterstützung

Herrn Prof. Dr. William Martin danke ich für die Übernahme des Zweitgutachtens

Dr. Yogi Altschmied danke ich für die die kritische Durchsicht meiner Arbeit und für wertvolle Diskussionen

Für eine ausgezeichnete Zusammenarbeit danke ich der Arbeitsgruppe von Prof. Dr. Ulrich Laufs

Ebenso danke ich der Arbeitsgruppe von PD Dr. Klaus Unfried für die Zusammenarbeit und Unterstützung

Ich danke den Rolling Stones für Konzerte mit überraschenden Nebenwirkungen

Meiner Arbeitsgruppe sowie den „Schrödies“ und „Klotzies“ danke ich für das angenehme Arbeitsklima

Kerstin danke ich besonders für die Gesangseinlagen im Labor und lustige Diskussionen in der Küche

Meinen Eltern danke ich für die Ermöglichung des Studiums und meiner Schwester Tania danke ich für den Zuspruch während meiner Doktorandenzzeit

Ingeborg danke ich für aufmunternde Worte und klare Ansagen

Chaplin Schröder danke ich für die angenehme Gesellschaft beim verfassen der schriftlichen Arbeit und für seine zustimmenden Worte

Ein ganz besonderer Dank geht an meinen Partner Peet. Ihm danke ich für den Beistand in meiner „Doktorandenzzeit“, in der er meine schlechte Laune in den Tiefen ertrug und mir Mut zusprach, ebenso danke ich ihm für seine Diskussionsbereitschaft um Klarheit zu schaffen, wenn das Chaos Überhand nahm