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The Role of Annexin A1 in the Activation and Transport of

the EGF Receptor

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Die Bedeutung von Annexin A1 bei der Aktivierung und dem Transport des EGF-Rezeptors

I n a u g u r a l - D i s s e r t a t i o n

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vorgelegt von

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Declaration

I am making the declaration in lieu of oath that the here presented doctoral thesis is entirely my work. All sources, supports and biochemical tools prepared by third persons used for this work are indicated.

I also declare that this work, prepared in co-supervision, will only be presented at the Université Paris XI and at the Heinrich-Heine Universität Düsseldorf.

Düsseldorf, 13th of October 2003

Susanne Radke

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SUMMARY

ABBREVIATIONS

A1	ANX A1
AG1478	EGF receptor kinase inhibitor
ANX A1	annexin A1
COX	cyclo-oxygenase
EGF	Epidermal growth factor
EGF-R	EGF receptor
(E)GFP	(enhanced) green fluorescent protein
ERK	extracellular signal-regulated kinase
DAG	diacylglycerol
fig.	figure
fl.	full-length
GST	glutathione-S-transferase
hr, hrs	hour, hours
IP	immunoprecipitation
JNK	c-Jun N-terminal kinase
kDa	kilo Dalton
MAPK	mitogen-activated protein kinase
min	minute(s)
MVB	multi vesicular bodies
MW	molecular weight
pX	protein of X kDa
ppX	phosphorylated pX
phosphoproteins	phosphorylated proteins
PI	phosphoatidylinositol
PI-4,5 P_2	phosphoatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PL	phospholipase
PTB	phosphotyrosine binding
PTP	protein tyrosine phosphatase
RTK	receptor tyrosine kinase
SH	src homology
siRNA	silencing RNA
TGN	*trans*-Golgi network
wt	wild type

1. INTRODUCTION

Binding of cell activating signaling molecules such as hormones, cytokines and growth factors to their cell-surface receptors like ion channels, G-protein-coupled receptors and transmembrane receptor tyrosine kinases, triggers receptor activation. The cellular responses following receptor activation vary greatly, *i.e.* cell growth, and cell proliferation, programmed cell death and cell migration. Numerous proteins are implicated in the signal amplification. They are strictly organized in signal transduction cascades so as to achieve cell responses. Protein-protein interaction provokes activation or inactivation of the interaction partners and guarantees a highly regulated control of each step in the cascade. If this process gets out of control and cannot be stopped by cell protective mechanisms, physiological disorder can be a consequence, causing serious diseases.

After ligand binding, the activity of the receptor at the plasma membrane has to be controlled. One cellular mechanism used to determine signal intensity, duration and location is endocytosis (Sorkin 2000). The activated receptors, together with directly or indirectly interacting proteins can be targeted and concentrated in special membrane areas of specific lipid and protein composition – the clathrin coated membrane invaginations. Through a series of highly regulated biochemical events, a clathrin-coated vesicle is formed, and separated from the plasma membrane by budding which requires among numerous other proteins the GTPase dynamin (Schmid 1997). Internalization of membrane proteins into the cell can also be realized *via* a clathrin independent pathway, or *via* caveolae, a microdomain in the membrane, consisting of a special lipid and protein composition.

The clathrin-coated vesicle formed is uncoated shortly after budding and then transported to early endosomes where it can fuse with the endosomal target membrane. From the early endosomal compartment, cargo proteins can be recycled back to the plasma membrane, can be sorted to the perinuclear recycling compartment, or delivered to late endosomes. The trafficking from the early to the late endosomal compartment is thought to be a 'maturation' process accompanied by a change in biochemical protein composition, pH and morphology. Late endosomes often display a typical morphology of multivesicular bodies, which contain internal membrane structures. These structures appear to be formed by invagination of the limiting endosomal membrane.

Ligand activated and internalized receptors are deactivated by the separation of the ligand-receptor complex at low endosomal pH or by protein degradation after the transfer of the internalized receptor to lysosomes.

Figure 1: *Vesicle formation and intracellular vesicle trafficking*
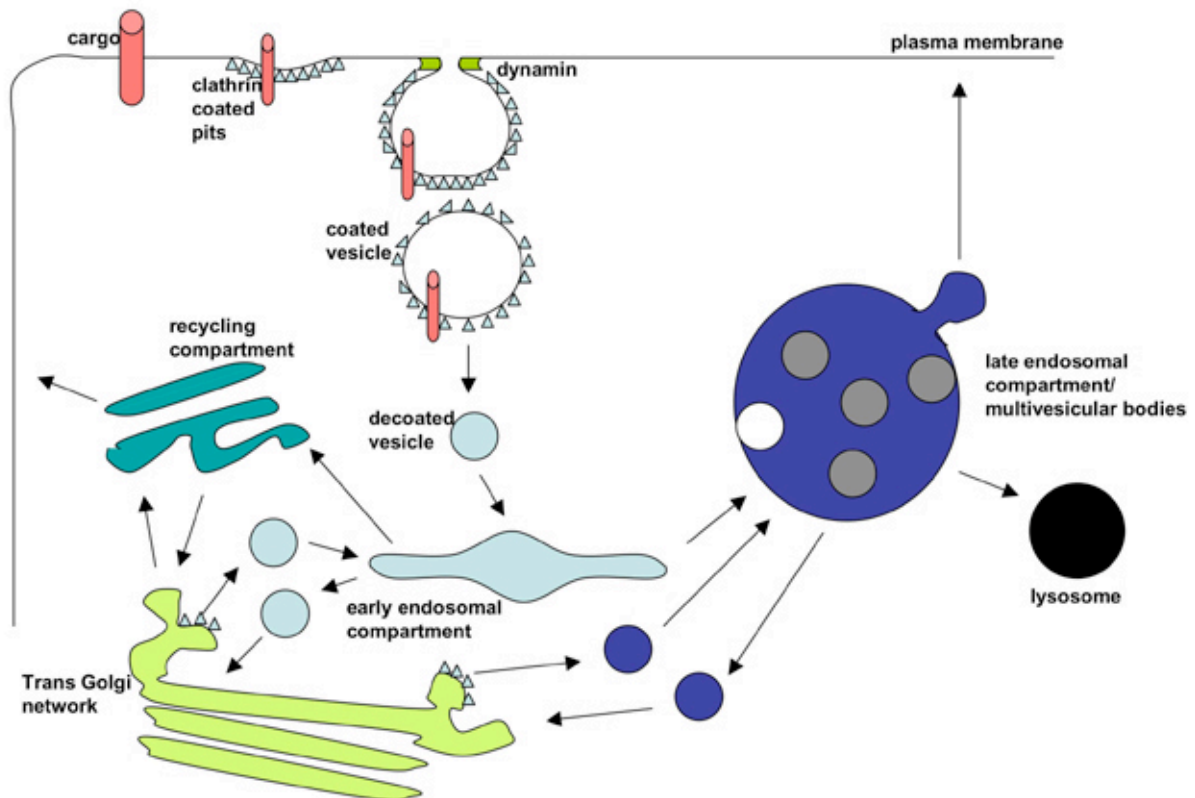
An important mediator of receptor activation and endocytosis is calcium. Receptor activation often triggers Ca^{2+}-influx from the extracellular milieu *via* Ca^{2+}-channels in the plasma membrane or from intracellular stores like the endoplasmic reticulum. Calcium plays an indispensable role in vesicle formation, membrane aggregation and in the activation of many proteins of signal transduction pathways including enzymes like protein kinase C, phospholipases A and C, calpain, proteins of the cytoskeleton or Ca^{2+}-binding proteins without kinase-activity that require Ca^{2+} for protein-protein or protein-membrane interaction such as calmodulins and annexins.

The following chapters are designated to give an overview of the interplay of the transmembrane receptor tyrosine kinase for the epidermal growth factor (EGF) and the Ca^{2+}/phospholipid binding protein annexin A1 during intracellular membrane transport.

1.1 The EGF receptor as a member of the transmembrane receptor tyrosine kinases superfamily

Numerous signal transduction pathways are regulated by protein phosphorylation and dephosphorylation. The transfer of the γ phosphate of ATP to hydroxyl groups of the amino acids serine, threonin or tyrosine on target proteins is catalyzed by protein kinases (Hunter 1998). Tyrosine kinases and their substrates play a prominent role in most fundamental cellular processes including the cell cycle, cell motility, cell survival, cell proliferation and differentiation and are important for the regulation of embryonic development and of many metabolic and physiological processes in a variety of tissues and organs (Schlessinger 2000 and 2002). Dysfunctions and aberrations in the activity of tyrosine kinases or their cellular localization can result in severe diseases such as cancer, diabetes, immune deficiencies and cardiovascular diseases (Blume-Jensen and Hunter 2001).

1.1.1 The structure of receptor tyrosine kinases

Tyrosine kinases are divided into transmembrane receptor kinases and into intracellular kinases without binding domain for extracellular ligands. Transmembrane receptor tyrosine kinases (RTK) comprise one large group of receptors that respond to polypeptide growth factors. Their structure consists of a usually glycosylated extracellular ligand-binding domain that is connected to the cytoplasmic domain by a single transmembrane helix. The cytoplasmic region contains a conserved protein tyrosine kinase core and additional regulatory sequences that are subjected to autophosphorylation and phosphorylation by heterologous protein kinases (Hunter 1998, Hubbard *et al.* 1998).

1.1.2 Activation of receptor tyrosine kinases

Several RTK ligands form homo- or heterodimers providing a simple mechanism for ligand-induced dimerization of receptor monomers. Dimerization is part of RTK activation followed by transphosphorylation by the intrinsic kinase (autophosphorylation). However, RTK monomers might also be in equilibrium with receptor dimers. Hence, ligand binding is not

absolutely necessary for receptor dimerization but stabilizes the dimer formation. The binding of specific RTK-ligands thus leads to stabilization of RTK dimerization, or of the conformational rearrangement of receptors and activation (Schlessinger 2000).

1.1.3 Activation of effector proteins and receptor tyrosine kinase signaling

Numerous proteins represent RTK-substrates. They are activated by three different mechanisms involving conformational change, translocation to the plasma membrane and tyrosine phosphorylation (Schlessinger 2000).

RTK autophosphorylation and the phosphorylation of other proteins create binding sites for proteins containing src-homology-2 (SH2) and phosphotyrosine-binding domains (PTB). The SH2- or PTB-domain presenting proteins are adaptor proteins that connect RTKs to other proteins *e.g.* Grb2 or enzymes *e.g.* src kinase, PLC-γ and PI-3 kinase (figure 2).

Figure 2: *Effector proteins of receptor tyrosine kinases*
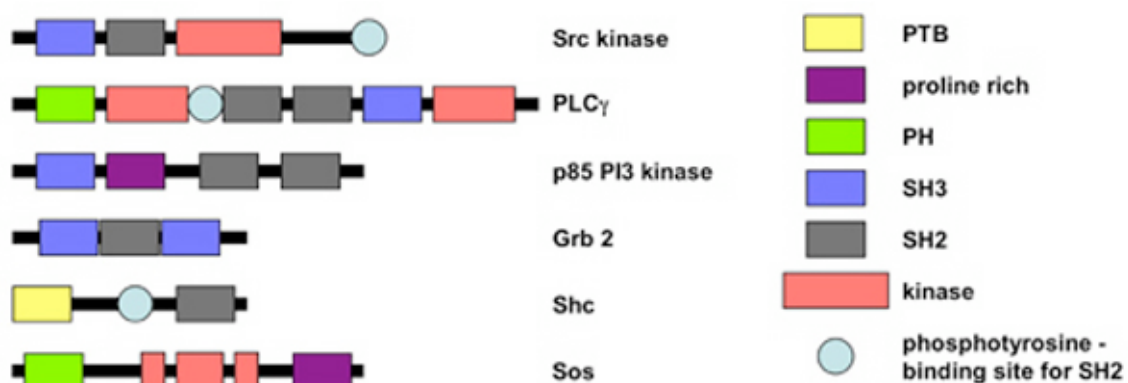
The adaptor protein Grb2 is implicated in the activation of the small G protein Ras by the exchange of GTP for GDP, which is effected by the exchange factor Sos. Sos forms complexes with Grb2 that can interact with RTKs *via* the SH2 domain in Grb2. The Grb2/Sos complex is translocated to the plasma membrane where Sos can interact with phospholipids *via* its PH domain and where it is close to its substrate Ras (Schlessinger 1994 and 2000, Pawson 1995, Bar-Sagi and Hall 2000). The Grb2/Sos complex can also be recruited to the plasma membrane by interaction with Shc, another adaptor protein that is able to interact with RTKs *via* its PTB domain (Margolis 1999). Activated Ras interacts with several effector

proteins like the Raf kinase, stimulating numerous intracellular processes. Activated Raf stimulates MAP-kinase kinase (MAPKK) by phosphorylating a key serine residue in the activation loop. MAPKK then phosphorylates a MAPK (ERK1/2, p38 or JNK) on threonine and tyrosine residues in the activation loop leading to its activation. Activated MAPK phoshorylates a variety of cytoplasmic and membrane linked substrates. In addition, MAPK is rapidly translocated into the nucleus where it phosphorylates and activates transcription factors (Karin and Hunter 1995, Hunter 2000). This highly conserved signaling cascade plays an important role in the control of metabolic processes, cell cycle, cell migration and cell shape, as well as in cell proliferation and differentiation (Davis 2000).

RTK-binding of enzymes *via* their SH2 domain can induce a conformational change that releases autoinhibitory effects, resulting in the stimulation of enzymatic activity (Schlessinger 2000). The intracellular tyrosine kinase src is activated when its SH2 domain binds to special tyrosine autophosphorylation sites of the tyrosine kinase PDGF receptor (Thomas and Brugge 1997, Xu *et al.* 1999). Similarly, binding of the regulatory subunit p85 of PI-3 kinase to the PDGF-R *via* a SH2 domain causes conformational changes that are transmitted to the catalytic subunit p110 followed by enhanced PI-3 kinase activity. At the same time this RTK binding provokes PI-3 kinase translocation to the membrane where it finds its substrates, the phospholipids phosphoatidylinositol-4-bisphosphate (PI-(4)P) or phosphoatidylinositol-4,5-bisphosphate (PI-$(4,5)P_2$).

Enzyme activation can result in the production of secondary messengers. PI-3 kinase phosphorylates PI-(4)P and PI-$(4,5)P_2$ in position 3 to PI$(3,4)P_2$ and PI$(3,4,5)P_3$. The produced second messengers can in turn recruit proteins representing special interacting domains like the PH domain for phosphoinositides, resulting in translocation to the membrane and thus indirect activation of effector proteins by RTKs.

PLC-γ hydrolyses PI$(4,5)P_2$ into the second messengers diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP$_3$). For its complete activation, PLC-γ is also membrane translocated by its PH domain and in addition tyrosine phosphorylated by RTKswhich is facilitated by the binding of PLC-γ to specific phoshotyrosines of the activated RTK *via* its SH2 domain (Falesca *et al.* 1998). By binding to specific intracellular receptors, IP$_3$ stimulates the release of Ca^{2+}. Ca^{2+} can then bind to calmodulin, which in turn activates a family of Ca^{2+}/calmodulin-dependent protein kinases. In addition, DAG and Ca^{2+} activate members of the

protein kinase C family. The second messengers generated by $PI(4,5)P_2$ hydrolysis stimulate a variety of intracellular responses in addition to phosphorylation and activation of transcriptional factors (Karin and Hunter 1995, Hunter 2000).

In conclusion, more then one mechanism can be necessary to activate effector proteins directly or indirectly by RTKs. Tyrosine phosphorylation and protein-protein or protein-lipid interactions give rise to a network of signal transduction cascades (only a few examples are described here), which can lead to altered gene transcription and other cellular responses.

1.1.4 Termination of receptor tyrosine kinase signaling

RTK activity must be tightly controlled and properly balanced in order to mediate their normal cellular and physiological functions. RTK activity can be shut off by several mechanisms including inhibition by phosphorylation, by dephoshorylation through phosphatases and by RTK degradation.

1.1.4.1 Receptor tyrosine kinase inactivation by phosphorylation

Phosphorylation of a special threonine by the serine/threonine protein kinase C (PKC) in the juxtamembrane domain of some RTKs including the EGF-R results in inhibition of the kinase activity and in strong inhibition of ligand binding to the extracellular ligand-binding domain (Cochet *et al*. 1984). Hence, phosphorylation by PKC seems to control receptor activity by a negative feedback mechanism.

1.1.4.2 Receptor tyrosine kinase inactivation by dephosphorylation

RTK activity termination by protein tyrosine phosphatases (PTP) seems to represent a very important mechanism as virtually all RTKs can be activated, even in ligand absence, by cell treatment with PTP inhibitors (Schlessinger 2000). This could mean that RTK activity is continually monitored by PTPs. Activity of most RTKs is positively regulated by one or several phosphotyrosine sites in the kinase activation loop. Dephosphorylation of this regulative site will inhibit RTK activity and terminate the RTK activity dependent downstream response.

1.1.4.3 Receptor tyrosine kinase inactivation by degradation

The end point of the endocytic traffic of activated RTKs is to undergo inactivation. This can occur by dissociation of the ligand at the acidic pH of endosomes and subsequent cell surface recycling or by receptor degradation in lysosomes (Di Fiore *et al*. 2001). The regulation of the direction of RTKs to lysosomes may affect the duration of the signaling.

1.1.4.4 Receptor tyrosine kinase ubiquitination

An additional regulatory mechanism by RTK ubiquitination has also been proposed. Protein ubiquitination is involved in selective proteasomal destruction of soluble proteins, but also implicated in multiple steps of the endocytic pathway ranging from the internalization reaction to the maturation of endosomes and lysosomal delivery (Schlessinger 2000). Both receptors and cytosolic endocytic proteins can be ubiquitinated. Impairment of their ubiquitination delays lysosomal degradation and may lead to prolonged receptor signaling.

1.1.5 The receptor tyrosine kinase superfamily

Based on structural homologies, the RTK superfamily is subdivided into different families (Fantl *et al*. 1993).

The insulin receptor (IR) family
The best-known members of this receptor family are the receptor for insulin, the receptor for IGF-1 (insulin-like growth factor) and the insulin-related receptor (IRR). Insulin receptors are heterotetramers consisting of two α- and two β-subunits linked by disulfid bridges. The ligand-binding site is formed by the α-chains that are linked *via* a disulfid bridge and present cystein-rich regions. Each β-chain contains a hydrophobic membrane spanning domain and a tyrosine kinase domain. Receptors of this family stimulate metabolic processes like glucose transport, glycogen synthesis, lipogenesis and lipolysis. Growth promoting effects include DNA synthesis, cell division and differentiation. The major IR substrate is IRS-1 but also the fatty acid binding protein, ecto ATPase and ERK are substrates for the insulin receptor (Sun *et al*. 1991, Roth *et al*. 1992).

Figure 3: *The receptor tyrosine kinase superfamily*
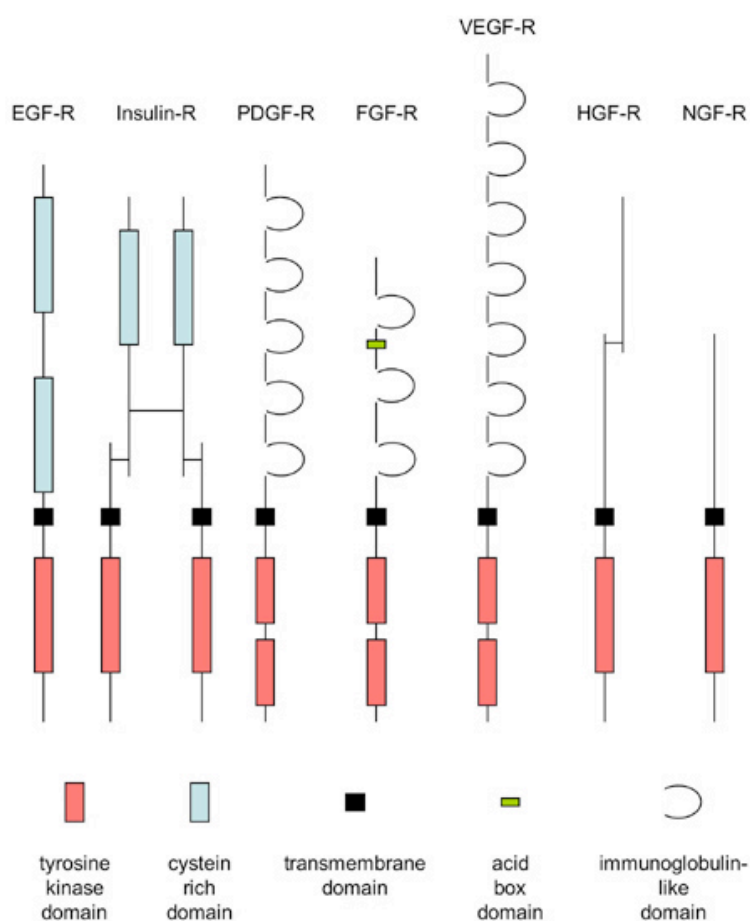
The PDGF receptor family

This receptor family includes the **p**latelet-**d**erived **g**rowth **f**actor α and β-receptors, the macrophage colony stimulating factor-1 receptor (MCSF-1) and the c-kit protein (Fantl *et al.* 1993). PDGF is a potent mitogen for smooth muscle cells, glial cells, oligodendrocyte progenitor cells, fibroblasts and selected types of endothelial cells.

At least three homo- or heterodimers can be formed by two different PDGF-monomers (AA, BB and AB). The PDGF α– and β-receptor can also create homo- or heterodimers. Characteristic of the PDGF-R are the pattern of expression in tissue and the ligand binding specificity. The AA-dimer only binds to the αα-receptor dimer, the AB-dimer to the αα- and αβ-receptor and the BB-ligand to all three possible receptor dimers. Due to the ligand-receptor combinations and differential expression the regulation by the PDGF receptor is very complex. The PDGF-R interacts with PI3-kinase, PLC-γ, GAP, Raf, and pp60c-src tyrosine kinase (Kaplan *et al.* 1990, Krypta *et al.* 1990, Kumjian *et al.* 1989, Meisenhelder *et al.* 1989, Molloy *et al.* 1989, Morrison *et al.* 1989).

The FGF receptor family

The **f**ibroblast **g**rowth **f**actor influences proliferation, differentiation and cell survival (Burgess *et al*. 1989, Gospodarowicz *et al*. 1986). FGFs also have the capacity to induce angiogenesis in a variety of organisms (Folkman *et al*. 1987). The FGF-family consists of at least 21 related growth factors. These include acidic and basic aFGFs and bFGFs, the products of the INT2- and the HST1-oncogene and the keratinocyte growth factor KGF. At least four FGF receptors exist, FGF-R 1-4, with three immunoglobulin-like (Ig) domains in the extracellular domain and a kinase domain that is divided into two subunits by a short insert of 14 amino acids. Between the Ig domains I and II is a short domain referred to as the acid box domain. Stimulation with FGFs leads to increased intracellular pH and Ca^{2+}-levels, increased PI turnover, and increased phosphorylation of a number of intracellular proteins (Burgess *et al*. 1989). FGF-R substrates include PLC-γ, Raf-1, ERK 1 and 2 and S6 ribosomal kinase (Burgess *et al*. 1990, Morrison *et al*. 1988).

The HGF receptor (MET) family

HGF was originally found as a **h**epatocyte stimulating **g**rowth **f**actor but is identical to the scattering-factor (SF) (Comoglio 1993). HGF triggers diverse responses in epithelial cells such as mitogenesis, dissociation of epithelial sheets and stimulation of cell motility (Tsarfarty *et al*. 1992). HGF binds to the met proto-oncogene. The extracellular domain of this receptor consists of two disulfide-linked subunits (50 kDa α- and 145 kDa β-subunit) which are formed from one precursor molecule by proteolytic cleavage. Receptor activation allows association with signaling molecules such as PLC-γ, GAP, src-related kinases and PI3-kinase (Bardelli *et al*. 1992).

The VEGF receptor family

VEGF (**g**rowth **f**actor for **v**ascular **e**ndothelial cells) receptors are expressed exclusively in endothelial cells (Peters *et al*. 1993). VEGF receptors possess seven Ig-like domains in the extracellular protein part and a cytoplasmic tyrosine kinase domain that is interrupted by a large kinase insert domain (de Vries *et al*. 1992). VEGF mediates mitogenesis in endothelial cells and stimulates an increase in vascular permeability presumably by affecting tight junctions between endothelial cells (Connolly 1991).

The neurotrophin receptor family

The neurotrophins are a family of ligands that play important roles in the growth, differentiation and survival of neurons. They also have the capacity to stimulate cellular proliferation in non-neuronal cell systems. At least five members of the neurotrophin family are studied, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4 and NT-5, which can bind to and activate the class of neurotrophin receptors called the Trk (A-C) proteins (Martin-Zanca *et al*. 1986, Middlemas *et al*. 1991, Klein *et al*. 1990, Lamballe *et al*. 1991). Effects of NGF stimulation are a rapid tyrosine phosphorylation and association of PLC-γ (Ohmichi *et al*. 1991, Vetter *et al*. 1991), membrane ruffling, increased intracellular Ca^{2+} levels, increased PI turnover, increased transcription of *c-fos* and *c-jun* and increased phosphorylation of ERK 1 and 2, S6 ribosomal kinase and Raf-1 (Halegoua *et al*. 1991, Zigmond *et al*. 1989, Boulton *et al*. 1991, Matsuda *et al*. 1987, Gomez *et al*. 1991). The cytoplasmic region of Trk proteins contains tyrosine kinase catalytic domains. The extracellular protein part of mammalian Trk proteins does not show any obvious structural elements.

1.1.6 The EGF receptor family

The EGF-R was the first receptor tyrosine kinase discovered (Carpenter *et al*. 1978). Activating mutations and overexpression of the EGF-R were implicated in a variety of cancers including mammary carcinomas, squamous carcinomas and glioblastomas as well as other malignant diseases such as brain, head, neck, pancreatic and colorectal tumors (Blume-Jensen and Hunter 2001, Yarden 2001).

The EGF-R is a large monomeric glycoprotein (1186 residues) that is composed like all RTKs of one ligand-binding domain, a transmembrane domain and one connected tyrosine kinase domain that is flanked by noncatalytic regulatory domains in the cytoplasmic part of the protein (Ullrich *et al*. 1984). Four members of the EGF-R family have been identified in vertebrates, namely EGF-R (HER1/ErbB1), ErbB2 (NEU/HER2), ErbB3 (HER3) and ErbB4 (HER4). The receptor monomers can form homo- or heterodimers and possibly higher-ordered oligomers after ligand activation. EGF-R ligands include the **e**pidermal **g**rowth **f**actor (EGF), the transforming growth factor α (TGFα), amphiregulin, betacellulin, epiregulin, heparin binding EGF, epigen and the neuregulins (Groenen *et al*. 1994, Yarden and Sliwkoski

2001). ErbB2 has no known ligand but seems to be the preferred partner for heterodimerization with other family members (Sundaresan *et al.* 1998).

1.1.6.1 Dimerization of the EGF-R

The extracellular portion of human EGF-R (residues 1-621) consists of the four subdomains L1, CR1, L2 and CR2 (Bajaj *et al.* 1987, Ward *et al.* 1995). Domains L1 and L2 are thought to form the ligand binding domain while other parts mediate receptor dimerization and interactions with other membrane proteins (Lax *et al.* 1989). Dimerization of EGF-Rs requires the binding of two molecules of monomeric EGF to two EGF-R monomers in a 2:2 EGF:EGF-R complex. There are two models as to how ligand induced EGF-R dimerization could occur. The first model describes the ligand-mediated mechanism, *i.e.* dimerization of the EGF-R (ErbB1) or heterodimerization of ErbB3 and ErbB4 with other family members is mediated by the bivalency of EGF (Gullick 1994). The second model is based on crystal structures of EGF-R in a complex with EGF or TGFα (Garrett *et al.* 2002, Ogiso *et al.* 2002). In both structures, each ligand molecule is bound exclusively to a single EGF-R monomer and dimerization seems to be mediated by receptor-receptor interactions (Lemmon *et al.* 1997). The EGF binding would induce a conformational change that exposes a receptor-receptor interaction site in the extracellular domain resulting in dimerization of two EGF-R monomers only when occupied by EGF.

All members of the EGF-R family present two homologous cystein-rich domains, the domains CR1 and CR2. Several functions have been proposed for these domains (Schlessinger 2002). CR1 could act as a scaffold that positions domains L1 and L2 in an orientation that generates a binding pocket for EGF and TGFα. Then again, interaction of CR1 and CR2 could have an autoinhibitory effect that reduces ligand binding as observed for ErbB3 (Cho and Leahy 2002). Following ligand binding and the release of the autoinhibition, CR2 may function as a spacer that controls the orientation of the extracellular domain relative to the transmembrane domain and the plasma membrane. Moreover, CR2 may represent an additional site of receptor-receptor interaction stabilizing the dimers formed by the dimerization loop of domain CR1. Last but not least, CR2 may mediate the formation of higher oligomerization states such as receptor tetramers.

1.1.6.2 EGF-R activation

Activation of RTKs is generally mediated by the phosphorylation of key tryosine(s) residues in the activation loop of the catalytic domain after ligand binding (Schlessinger 2002). In the inactive RTK, the activating loop adopts a configuration preventing access to ATP and substrates. Upon tyrosine phosphorylation the loop configuration changes and becomes more accessible resulting in enhanced kinase activity. However, mutation of a conserved tyrosine residue that might serve as an important autophosphorylation site in virtually all RTKs and which is also present in the activation loop of the EGF-R seems to be dispensable for protein tyrosine kinase activation and for signaling *via* the EGF-R (Gotoh *et al.* 1992). Hence, the activation loop of the EGF-R may not play the prominent role in autoinhibition as observed in the case of RTKs, or the inactive EGF-R is not as tightly autoinhibited. Indeed, ErbB2 shows kinase activity even in the absence of ligand stimulation.

1.1.6.3 Regulation of EGF-R activity by endocytosis

Endocytotic trafficking can regulate EGF-R signaling by binding of different receptor ligands that invoke different signaling potencies particularly in terms of their mitogenic potential (Reddy *et al.* 1998). This has been demonstrated using cells lacking EGF-R family members or cells that were stably transfected with the EGF-R (ErbB1) alone or in combination with either ErbB2 or ErbB3 (Lenferink *et al.* 1998, Waterman *et al.* 1998). The cells that expressed only ErbB1 were less proliferative in response to EGF than in response to TGFα. When either ErbB2 or ErbB3 was co-expressed, the cells became more responsive to EGF without altering their response to TGFα. The differences in mitogenic behaviour correlated with the increased recycling and decreased downregulation of ErbB1 homodimers that occur in the presence of TGFα or when ErbB2 or ErbB3 containing heterodimers are activated by either EGF or TGFα. Endosome acidification and the pH-dependent dissociation of receptor-ligand complexes are central in controling the sorting decision (Ceresa and Schmid 2000). TGFα dissociates from the receptor at lower pH than EGF suggesting that TGFα dissociation takes place in early endosomes which are involved in receptor recycling.
Studies with chimaeric EGF-Rs encoding the ligand-binding extracellular domain of ErbB1 and the intracellular domain of ErbB2 (Sorkin *et al.* 1993) or other ErbB family members (Baulinda *et al.* 1996) indicate that sorting signals in the ErbB1 cytoplasmic domain are required for receptor degradation. c-Cbl, a downstream substrate of numerous RTKs, is one

potential candidate molecule that could recognize sorting determinants in ErbB1 (Levkowitz *et al.* 1996). c-Cbl is recruited to endosomes in cells transiently expressing the lysosomal directed ErbB1 but not the recycling ErbB3. Hence, c-Cbl might be required for ligand-dependent ubiquitination of ErbB1 in endosomes targeting the receptor to the lysosomal degradative pathway.

Moreover, ErbB-2 shows a reduced internalization capacity compared to the EGF-R, indicating that heterodimerization with ErbB-2 decreases the receptor down-regulation (Lenferink *et al.* 1998).

1.1.6.4 EGF receptor membrane trafficking

Prior to ligand binding, 40-60!% of the total plasma membrane EGF-R population is associated with specialized plasma membrane microdomains known as caveolae (Anderson 1998, Smart *et al.* 1999) or other detergent-resistant, sphingolipid- and cholesterol-rich membrane domains. It is likely that the EGF-R population localized in these microdomains is not static but in equilibrum with receptors outside these specialized regions (Carpenter 2000). Caveolae are related to lipid "rafts" – also termed detergent-insoluble glycolipid-enriched complexes (DIGS). They are morphologically defined as 'omega'-shaped invagination of the plasma membrane. They contain a high concentration of gangliosides, sphingomyelin, cholesterol and in addition the caveolin proteins which function to establish and/or maintain caveolar structures (Ceresa and Schmid 2000). Their protein profile is specific and excludes most cell surface proteins. Only 30 membrane receptors, signaling molecules and membrane transporters are localized in caveolae which makes it to a microdomains with a very specific center of signaling activity (Anderson 1998).

The EGF-R localization to caveolae does not seem to be due to direct interaction with cholesterol as the EGF-R caveolae-association does not resist Triton X-100 extraction (Pike and Casey 1996). However, it has been reported that the EGF-R interacts directly with caveolin-1 and -3 (Couet *et al.* 1999) and that caveolin-1 is tyrosine phosphorylated in EGF-stimulated cells (Kim *et al.* 2000).

In contrast to other RTKs EGF stimulation provokes the migration of the EGF-R out of caveolae (Mineo *et al.* 1999). Certain requirements have been found for the EGF-R migration out of caveolae. The exit can be partially blocked by the src kinase inhibitor PP2 and

completely by the EGF-R kinase inhibitor AG1478, whereas active PKC seems to reduce the EGF-R trafficking away from caveolae.

Src kinase that has also been found in caveolae is known to be activated after EGF binding (Anderson 1998, Smart *et al.* 1999). As the EGF-R internalization rate is increased in cells that overexpress the src kinase and delayed in src-absence, it could be that src participates in EGF-R trafficking at the cell surface. The regulation could be realized by the src-dependent clathrin heavy chain phosphorylation on tyrosine 1477 that is thought to control the assembly of clathrin heavy and light chain needed to form the essential coated-pit clathrin triskelions components (Carpenter 2000).

Analysis of EGF-R mutants revealed that at least one autophosphorylation site between the residues 991 and 1022 is essential for the ligand-dependent caveolae exit.

Hence, the EGF-R might exit caveolae in its active state after dimerization and auto-phosphorylation and caveolae do not seem to mediate EGF-R internalization because in EGF stimulated cells the EGF-R exits this special microdomain and migrates toward clathrin coated pits. At least one SH2-containing protein seems to be implicated in the trafficking event.

The trafficking in direction to coated pits could be mediated by the clathrin adaptor protein AP-2 that is known to interact with the active EGF-R (Sorkin and Zastrow 2000).

Despite the fact that the active EGF-R leaves caveolae it has been shown that caveolae localized EGF-R participates in the activation signal transduction cascade and phospho-inositide signalling.

Activated ERK 1 and 2 have been found in caveolae and subsequently in the cytosol after EGF treatment, indicating that cytosolic active MAPK derives from caveolae (Furuchi and Anderson 1998). Surprisingly, the depletion of cellular cholesterol causes ERK-hyperactivation by EGF although the protein content of caveolae also must be disturbed. Cells with downregulated caveolin-1 showed a stronger MAPK activation by EGF and caveolin-1 overexpression seems to block EGF-dependent MAPK activation (Galbiati *et al.* 1998, Engelman *et al.* 1998) indicating that there is an interaction between MAP kinases and caveolin-1.

50!% of the phosphoatidylinositol 4,5-bisphosphate (PI-4,5 P_2) of the plasma membrane is concentrated in rafts and caveolae (Pike *et al.* 1996, Liu *et al.* 1998, Waugh *et al.* 1998, Liu *et al.* 1997). However, caveolar PI-4,5 P_2 can be extracted with Triton X-100 and is thus not part

of the detergent-resistant characteristics of this kind of microdomain. It seems that EGF activated PLC-γ hydrolyses its substrates preferentially from caveolae as EGF-R stimulation is followed by a decrease of caveolar PI-4,5 P_2, whereas no decrease in the non-caveolar level of PI-4,5 P_2 is measurable. The presence of PLC-γ was reported and also of other proteins of phosphoinositide signaling *e.g.* IP_3 receptors and certain PKC isoforms (Anderson 1998, Smart *et al*. 1999).

ErbB-2 is also present in caveolae and seems to exit more slowly than the EGF-R (Mineo *et al*. 1999). It is possible that heterodimerization of the EGF-R and ErbB-2 slows down the exit from caveolae and the endocytosis. As a consequence, the receptor dimer would stay longer in its active state at the membrane. However, neither EGF-R homodimers nor heterodimers have yet been shown in caveolar fractions.

In conclusion, the EGF-R is implicated in numerous signal transduction pathways and it has a great influence on cellular responses and activities. Many substrates have been found which are tyrosine phosphorylated in EGF stimulated cells, directly through the EGF-R or indirectly through tyrosine kinases downstream of the EGF-R. Among these substrates are members of the annexin family, in particular annexin A1 which has been shown mostly by *in vitro* assays to be an EGF-R substrate and which might be implicated in EGF-R activated protein cascades triggering *i.e.* cell proliferation.

1.2 Annexin A1 as a member of the annexin family

Annexin proteins comprise a large protein family with at least twelve members in vertebrates. They have also been found in non-vertebrates like insects, flatworms, roundworms including *C. elegans*, mycetozoa and fungi, plants and protests, showing that annexins represent an evolutionary conserved protein family.

When annexins were discovered in the late 1970s and early 1980s by research groups independent from one another, these proteins were given different names referring to their biochemical properties (table 1) such as synexin for granule aggregating protein (Creutz *et al.* 1978), chromobindin for chromaffin granule binding protein (Creutz *et al.* 1987), calcimedin for Ca^{2+} signal mediating protein (Moore *et al.* 1984), lipocortin for steroid-inducible lipase inhibitor (Flower 1986) and calpactin for Ca^{2+}-, phospholipid- and actin-binding protein (Glenney *et al.* 1987).

Later, a standardized nomenclature was introduced using the term "annex", which means bring/hold together. This term is justified by the definition of annexins which are able to connect themselves to negatively charged phospholipids *e.g.* phosphatidic acid, phosphatidyl-serin, phosphatidyl-inositol *via* bridges of calcium cations (Gerke *et al.* 2002). Indeed, these typical characteristics of annexins can cause membranes of neighboring biological structures to be brought more closely together and to be eventually connected, which could result in membrane aggregation or fusion.

1.2.1 The unique structure of the annexin family

The annexin structure is composed of two major parts: a highly conserved C-terminal protein core and an N-terminal domain that shows a great variety among the annexins – also named N-terminal peptide or tail.

The protein core is of a slightly curved structure with the calcium binding sites on the convex side. Hence, when annexins interact with lipids, the convex side of the protein is oriented to the membrane and its concave side where the N-terminal domain is located faces the cytosol. The N-terminal peptide is in this configuration accessible for cytoplasmic interaction partners.

Table 1: *The family of vertebrate annexins*

name	synonym	human gene symbol
annexin A1	cp35, cp37, p35, annexin!1/I, calpactin!II, chromobindin!9, EGF substrate, lipocortin!1/I, macrocortin!1, max, renocortin	ANX A1
annexin A2	p36, annexin!2/II, calpactin!I heavy chain, chromobindin!8, lipocortin!2/II, Locus 1B6, PAP-IV, Placental anticoagulant protein IV, pp60-src substrate, primer recognition protein!I, protein I	ANX A2
annexin A3	35-alpha calcimedin, annexin III, lipocortin III, PAP-III, placental anticoagulant protein III	ANX A3
annexin A4	32.5 kDa calelectrin, 35-beta calcimedin, annexin IV, endonexin I, lipocortin!IV, PAP-II, Placental anticoagulant protein II, placental protein 4-X, Protein II, Zap36	ANX A4
annexin A5	35-gamma calcimedin, 35 kDa calelectrin, anchorin CII, annexin V, calphobindin I, endonexin II, inhibitor of blood coagulation, lipocortin V, PAP-I, placental anticoagulant protein I, placental protein 4, vascular anticoagulant-alpha	ANX A5
annexin A6	67 kDa calcimedin, 67 kDa Calelctrin, p68, p70, 73k, annexin VI, calphobindin, chromobindin 20, lipocortin VI, protein III	ANX A6
annexin A7	annexin VII, synexin	ANX A7
annexin A8		ANX A8
annexin A9	annexin 31, annexin XXXI	ANX A9
annexin A10		ANX A10
annexin A11	annexin 11/XI	ANX A11
annexin A12	unassigned	
annexin A13	annexin 13/XIII	ANX A13

1.2.1.1 The Annexin protein core

The annexin core is made up of four or eight (ANX A6) highly structurally conserved segments; the annexin-specific repeats I-IV, each 70-80 amino acids in length. The core conformation represents a tightly packed disk due to the arrangement of five α-helices (A-E) in each repeat. Four helices are arranged vertically and the fifth helix horizontally at the concave side of the protein. The helices are connected by short turns or by longer loops composed of up to 12 residues. Some of the loops that are localized on the convex side of the core are ntegrated in the calcium binding mechanism.

The repeats are grouped in a cyclic formation that is stabilized by hydrophobic side-chain interactions starting with repeat I, followed by IV then repeat II and III. Due to this special arrangement of the four core segments, a pore is formed in the center of the protein that might

represent an ion conductance pathway for Ca^{2+}. Thus, the structure was the basis for the idea that annexins could have ion channel activity.

(Gerke and Moss 1997, Raynal and Pollard 1994)

1.2.1.2 Calcium binding sites

Two different novel Ca^{2+}-binding sites of type II and III have been found in annexins that are different from the well-known EF-hand motif (type I) (Weng *et al.* 1993, Jost *et al.* 1994). The EF-hand is structurally composed of a first α-helix (residues 1-10), a loop containing the residues that coordinate the calcium ion (residues 10-21) and a second α-helix (residues 19-29) motif, creating together the typical EF-hand helix-loop-helix motif. The second α-helix is almost oriented at a right angle to the first helix. Oxygens delivered by the amino acid residues of the loop, acidic amino acids such as aspartate or glutamate flanking the loop in position 10 and 21, carbonyl groups of the peptide backbone and bridging water molecules stabilize the binding of the Ca^{2+}-ion. The binding is reversible with a dissociation constant in the nanomolar to micromolar range. Type II and III Ca^{2+}-binding sites also show a helix-loop-helix motif but in their cases, the α-helices are not arranged at a right angle but almost parallel to one another. Another important difference between the EF-hand calcium-binding domain and the type II domain is that in the EF hand the Ca^{2+} is coordinated in a small 11-residue loop, whereas in the annexin Ca^{2+}-binding site of type II, the coordination of calcium is less tight and realized by two regions that are far apart in the primary amino acid sequence but close together in the three-dimensional folding forming the Ca^{2+}-binding site. Thus, the Ca^{2+} ion is not located inside the protein but on the protein core and needs more stabilization from outside in addition to water molecules *i.e.* by negatively charged phospholipids.

Three type II Ca^{2+}-sites located in the repeats I, II and IV of ANX A5 have been described first. They consist of a characteristic sequence (L,M)-K-G-X-G-T in the loop between the first and the second α-helices of one core-segment and one acidic residue that is located 38 residues downstream between the fourth and the fifth α-helices (Raynal and Pollard 1994). The structure of the Ca^{2+}-binding sites of type II in the repeats II, III and IV of ANX A1 resembles those of ANX A5: (E,M)-(K,R)-(G,R)-X-G-T-(38 residues)-(D,E). The type II Ca^{2+}-binding sites are also similarly organized in ANX A2.

A third type of Ca^{2+}-binding site (type III) has been found in ANX A1. Here three additional Ca^{2+}-binding sites have been characterized that also consist of a helix-loop-helix motif but the

cation is coordinated to two backbone carbonyl oxygens and one acidic side chain located nearby in the primary sequence (Raynal and Pollard 1994). The binding affinity of Ca^{2+} to type III sites is therefore lower than that to type II sites.

1.2.1.3 The N-terminal domain

The N-terminal peptides differ in length between 11 and 19 residues (annexin A3, 4, 5, 6, 10, 12 and 13), 33 and 42 residues (annexin A1 and A2) and 100 or more amino acids (annexin A7 and A11) (Gerke und Moss 1997) and also in sequence and structure among the annexins, which confers individuality and specificity to each annexin member. The N-terminal domain is thought to be the regulatory domain of the protein as within this region sites for posttranscriptional modifications including phosphorylation, transglutamination (ANX A1), S-gluthathiolation (ANX A2), N-myristoylation (ANX A13) and also sites for protein-protein interactions are located.

Moreover, the protein core characteristics concerning *i.e.* membrane binding and Ca^{2+}-affinity can be altered by way of a conformational change induced by the N-terminal domain (Gerke and Moss 1997). This has been demonstrated especially for ANX A1 and ANX A2 when removal of the N-terminal region resulted in an increased affinity towards Ca^{2+} and phospholipids (Powell *et al.* 1987, Ando *et al.* 1989, Liu *et al.* 1995).

Annexin phosphorylation has been observed frequently. Phosphorylation occurs on tyrosine, serine, threonine and even on histidine (ANX A1) residues by several kinases including the EGF-R, src kinase, Insulin-R, growth hormone receptor (GH-R), HGF-R, glucocorticoid receptor, MAP kinase, PI3-kinase, protein kinase C, VEGF-R, PDGF-R and Pyk-2 kinase (table 2).

The phosphorylation status can influence phospholipid binding characteristics as observed for ANX A1 and ANX A2. Phosphorylation of tyrosine-23 in ANX A2 decreased its affinity in liposome binding experiments (Powell *et al.* 1987) and phosphorylation of tyrosine-21 in ANX A1 increased its needs of Ca^{2+} for specific membrane binding (Futter *et al.* 1993).

Table 2: *Phosphorylation of annexins*

Annexin	phosphorylation site	involved kinase(s)	Reference
annexin 1	tyrosine-21	EGF-R pp60[c-src], pp50[v-abl] Ins-R	de Coupade *et al.* 2000 Rothhut 1997 Melki *et al.* 1994
annexin 1	tyrosine	HGF-R GH-R EGF-R	Skouteris *et al.* 1996 Salles *et al.* 1996 Futter *et al.* 1993 Pepinski 1991
annexin 1	serine	glucocorticoid-receptor, MAPK, PI3-kinase, Ca^{2+}- dependent PKC PKC	Solito *et al.* 2003 Katoh 2002, John *et al.* 2002
annexin 1	threonine-24, serine-27, serine-28	protein kinase C	Rothhut 1997 Ohnishi *et al.* 1995
annexin 1	serine-27		Kusumawati *et al.* 2001
annexin 1	histidine		Muimo *et al.* 2000
annexin 2	tyrosine-23	pp60[c-src] pp60[v-src] PDGF-R	Garver *et al.* 1999 Rothhut 1997 Hubaishy *et al.* 1995
annexin 2	tyrosine	Insulin-R	Zhao *et al.* 2003 Biener *et al.* 1996
annexin 2	serine-11		Liu *et al.* 2003,
annexin 2	serine-25	protein kinase C	Rothhut 1997 Liu *et al.* 2003
annexin 2	serine	protein kinase C	Delouche *et al.* 1997 Jost *et al.* 1996 Dubois *et al.* 1995
annexin 2		casein kinase I	Gao *et al.* 2000
annexin 4	threonine-6	protein kinase C	Kaetzel et al. 2001 Rothhut 1997
annexin 5	tyrosine	VEGF-R	Wen *et al.* 1999
annexin 6	serine, threonine		Moss *et al.* 1992
annexin 7		protein kinase C	Caohuy *et al.* 2002 Caohuy *et al.* 2001
annexin 7	tyrosine	PDGF-R, Pyk-2, src, EGF-R	Furge *et al.* 1999
annexin 11	serine, threonine	MAP kinase	Mizutani *et al.* 1993
annexin 11	tyrosine	PDGF-R, Pyk-2, src, EGF-R	Furge *et al.* 1999

1.2.2 Unique features in the annexin A1 structure

Many crystal structures of the protein cores of several annexins are solved including ANX A1, 2, 3, 4, 5 and 6 (www24.brinkster.com/annexins/structures/structures.asp).

In the case of ANX A1 it was also possible to get the crystal structure of the full-length protein (Rosengarth *et al.* 2001).

Certain ANX A1 characteristics require the N-terminal domain such as its capability to aggregate model membrane vesicles and chromaffin granules (secretory vesicles from adrenal medulla) in the presence of Ca^{2+}, whereas the membrane binding is mediated *via* the core domain (de la Fuente *et al.* 1995, Wang *et al.* 1994). Therefore, a chimera consisting of the core domain of ANX A5 and the N-terminal domain of ANX A1 is capable of membrane aggregation while ANX A5 is not able to induce membrane or vesicle aggregation alone (Bitto and Cho 1998, Andree *et al.* 1993).

Modification of the N-terminal domain by phosphorylation of tyrosine-21 changes the Ca^{2+}-requirements for membrane binding of ANX A1 and the protein also becomes more sensitive for proteolytic cleavage of its N-terminal peptide (Futter *et al.* 1993, Schlaepfer *et al.* 1987, Sheets *et al.* 1987).

Therefore, the complete structure is of special interest when considering the behavior of ANX A1.

The first ANX A1 structure was published lacking the 32 amino acids of the N-terminal domain (Weng *et al.* 1993). Afterwards, the crystal structure of the S100A11 dimer in complex with a part of the N-terminal peptide of ANX A1 comprising the residues 2-12 could be solved (Réty *et al.* 2000). In 2000, the crystal structure of full-length ANX A1 was solved in the absence of Ca^{2+} (Rosengarth *et al.* 2001). It was shown that the ANX A1 N-terminal domain consists of two α-helices. The first helix (residue 2-12) is connected to the core domain by a flexible linker. It replaces helix D of repeat III that is implicated in calcium binding. The replaced helix is unwound into a flap. It has been suggested that this structure in the absence of Ca^{2+} represents the inactive conformation of ANX A1. Next, the full-length ANX A1 protein was crystallized in the presence of Ca^{2+} and its structure solved (Rosengarth *et al.* 2003). This time the D-helix was back in place for Ca^{2+} coordination while parts of the now exposed N-terminal peptide were disordered. The assumption has been made that due to a Ca^{2+}-driven conformational switch of the core domains of ANX A1, the N-teminal peptide was expelled from the core domain and the helix D can be rewound and take its place in the core domain.

Figure 4: *ANX A1 structure*

A: Amino acid sequence of human Annexin A1

```
1    mamvseflkq awfiene eqe yvqtvk sskg gpgsavspyp tfnpssdvaa lhkaimvkgv
61   deatiidilt krnnaqrqqi kaaylqetgk pldetlkkal tghleevvla llktpaqfda
121  delraamkgl gtdedtliei lasrtnkeir dinrvyreel krdlakdits dtsgdfrnal
181  lslakgdrse dfgvnedlad sdaralyeag errkgtdvhv fntiltnrsy pqlrrvfqky
241  tkyskhdmnk vldlelkgdi ekcltaivkc atskpaffae klhqamkgvg trnkalirim
301  vsrseidmnd ikafyqkmyg islcqailde tkgdyekilv alcggn
```

phosphorylation sites - N-terminal domain - α-helice of the N-terminal domain

repeat I - repeat II - repeat III - repeat IV - D loop

B: ANX A1 structure lacking the N-terminal domain in the presence of Ca^{2+} (Weng *et al.* 1993)
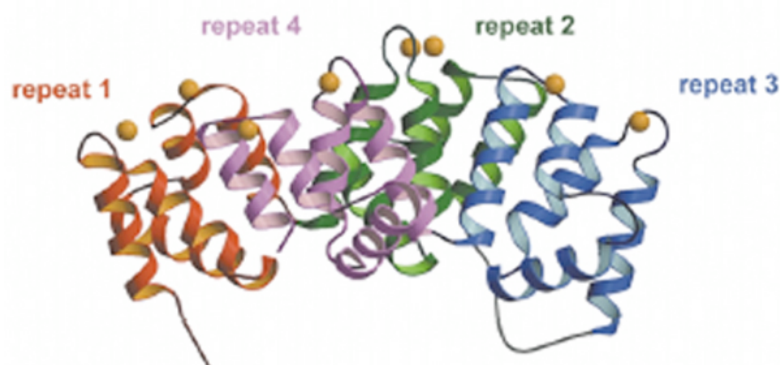
C: ANX A1 structure in the absence of Ca^{2+} (Rosengarth *et al.* 2001)
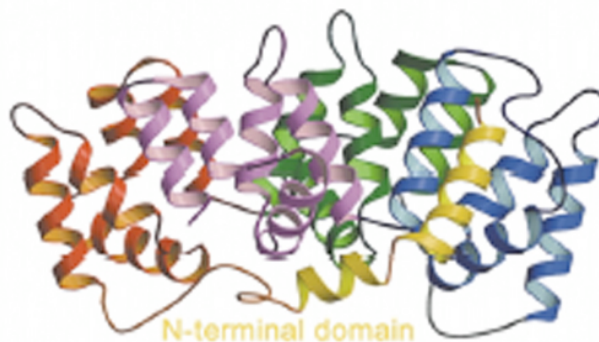
D: Conformational switch of the N-terminal peptide and the core domain driven by Ca^{2+} (Rosengarth *et al.* 2003)
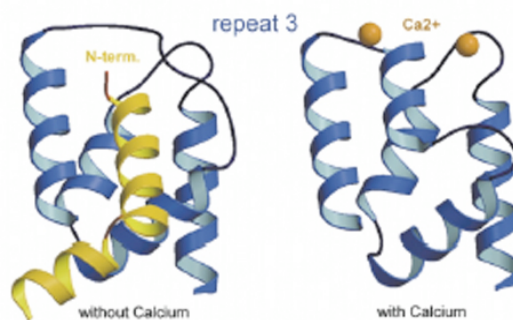
The structures are copied from Rosengarth *et al.* 2003.

1.2.3 Biochemical characteristics of annexins

Annexins are targeted to different cellular membranes and therefore show different subcellular localization (Gerke and Moss 1997 and 2002). Hence, it seems that annexins have different affinities for special membrane components or specific compositions. Often two annexin-pools are present in the cell: one bound to a membrane subpopulation and the other present in the cytosol. A Ca^{2+}-dependent dynamic exchange between both pools has been proposed (Gerke and Moss 2002).

ANX!A1 has been localized to the plasma membrane of neutrophils, BHK and MDCK cells, at phagosomal structures in neutrophils and at endosomal (early and/or late) structures in BHK, Hela and NIH3T3 cells (Diakonova *et al.* 1997, Futter *et al.* 1993, Seemann *et al.* 1996, Ernst *et al.* 1991, Kaufman *et al.* 1996, Rescher *et al.* 2000).

ANX!A2 has been identified as a constituent of the submembranous cytoskeleton (Gerke and Weber 1984), bound to the plasma membrane in HepG2 and HeLa cells and to early endosomes in BHK, MDCK, J774 cells (Emans *et al.* 1993, Harder *et al.* 1993, 1997). In addition, it has been found on vesicles of the secretory transport route of *i.e.* adrenal chromaffin cells (Creutz *et al.* 1987, Nakata *et al.* 1990) and in phagosome preparations from J774 macrophages (Desjardins *et al.* 1994).

ANX!A3 has been localized to the plasma membrane and to membranes of intracellular granules and phagosomes of neutrophils and monocytes/macrophages (Diakonova *et al.* 1997).

ANX!A4, which is abundantly expressed in epithelial cells has been observed at the plasma membrane and connected to endocytic structures in J774 and BHK cells (Massey *et al.* 1991, Mayran *et al.* 1996, Diakonova *et al.* 1997, Jost *et al.* 1997).

ANX A5 has been identified at the plasma membrane and at endocytic membranes of a variety of cells (Diakonova *et al.* 1997) and also associated with the endoplasmic reticulum in glial cells (Giambanco *et al.* 1993) and with the sarcoplasmic reticulum of skeletal muscle (Spreca *et al.* 1992).

The localization of ANX!A6 seems to be differently regulated depending on the cell type. It is found on the plasma membrane of a variety of cells including lymphocytes (Owens *et al.* 1984) and hepatocytes (Weinman *et al.* 1994, Tagoe *et al.* 1994), on the sarcoplasmic reticulum of skeletal muscles (Hazarika *et al.* 1991). ANX A6 has also been localized to phagosomes of J774 macrophages (Desjardins *et al.* 1994) and to endosomal membranes of

rat liver (Jäckle *et al.* 1994) but is not enriched in the endosomal fraction of BHK cells (Seemann *et al.* 1996).

ANX A7 has been found in the cytosol of adrenal chromaffin cells, of cultured muscle cells and on the plasma membrane and on transverse tubules in striated muscles (Kuijpers *et al.* 1992, Selbert *et al.* 1995).

ANX A11 has been detected in the nucleus in an N-terminal domain dependent manner (Mizutani *et al.* 1992, Mizutani *et al.* 1995, Tomas *et al.* 2003) but also in the cytosol of neutrophils from where it can trans-locate to the membrane of neutrophil granules (Sjolin *et al.* 1996).

Last but not least ANX A13 has been found at the plasma membrane of enterocytes (Wice and Gordon 1992) as well as at exocytic carrier vesicles from MDCK cells (Fiedler *et al.* 1995).

Annexin binding to negatively charged phospholipids is in most cases Ca^{2+}-dependent and mediated *via* the conserved protein core. However, the calcium-requirements of annexins vary from sub-micromolar and a few micro-molar Ca^{2+} (ANX!A1 and A2) to ten to more than 100!μM Ca^{2+} (ANX A4) for half-maximal binding (Raynal and Pollard 1994). Annexin membrane binding is often accompanied by a Ca^{2+}-dependent aggregation of membrane surfaces. The mechanism as to how membrane aggregation is provoked by annexins is yet to be completely understood.

Due to their nature all annexins are able to interact with membranes *via* their protein core and they could be capable of committing interactions *via* their N-terminal peptide at the same time with cytosolic proteins, other membrane proteins, other members of the annexin family, proteins of the cytoskeleton or even membrane lipids (Rosengarth *et al.* 2001, Gerke and Moss 2002). This could trigger off certain biological processes such as translocation of the annexin or the approach of two neighboring membrane structures.

There are indications that some annexins *e.g*. ANX A1 possess more then one phospholipid binding site. It has been found that ANX A1 that was initially bound to liposomes at relatively low Ca^{2+}-concentration was able to aggregate additional liposomes when the Ca^{2+}-concentration was increased (de la Fuente *et al.* 1995). A co-aggregation between both liposome populations was demonstrated, indicating a bivalent binding mechanism of ANX A1. Thus, membrane aggregation could be due to a second phospholipid binding-site in ANX A1. Other findings uncovered the possibility that annexins self-associate *via* their N-terminal domain suggesting that the dimers formed are capable of linking membranes (Creutz 1992,

Raynal and Pollard 1994). A third possiblity is that annexins need other components to provoke membrane fusion as their fusogenic activity is very slow in a limited *in vitro* system consisting of liposomes, annexin and Ca^{2+} (Creutz 1992). The missing components in that system could be interaction partners *e.g.* proteins of the S100 family.

1.2.4 S100-proteins as annexin interaction partners

S100 proteins represent a multigenic family of 20 known small Ca^{2+}-binding proteins (10-12!kDa) of the EF-hand type. They are composed of two EF-hands separated by a short linker and flanked by unique extensions at the C- and N-terminal extremity (Schäfer and Heizmann 1996, Heizmann *et al.* 2002). S100 proteins are implicated in the Ca^{2+}-dependent regulation of many intra- and in some cases in extracellular processes including protein phosphorylation, enzyme activity, cell proliferation, neoplastic transformation, differentiation, dynamics of cytoskeleton constituents, structural membrane organization, intracellular Ca^{2+}-homeostasis, inflammation and protection from oxidative cell damage (Donato 1999). S100 proteins form anti-parallel bound homodimers (in some cases heterodimers), which can allow the cross-bridging of two homologous or heterologous target proteins in a Ca^{2+}-dependent manner. Ca^{2+}-binding seems to provoke a conformational change in S100 proteins and thus the exposure of binding domains for their interaction partners (Donato 2001). High target-protein specificity has been described among S100 proteins suggesting that this is the way how this protein family is implicated in numerous different cell activities.

At least ANX A1, ANX A2 and ANX A11 interact with the S100 proteins S100A11, S100A10 and S100A6 respectively. The interaction domain for the S100 proteins is located in the N-terminal domain within the first 14 or 13 residues of ANX A2 or ANX A1 respectively. The binding domain for ANX A1 in S100A11 is located in its C-terminal extension (Seemann *et al.* 1996). The phosphorylation of ANX A1 by protein kinase C is inhibited *in vitro* when ANX A1 and S100A11 are allowed to interact (Naka *et al.* 1994). It has also been shown that ANX A1 and S100A11 are detected at early endosomal membranes and that the recruiting of S100A11 even depends on ANX A1 (Seemann *et al.* 1997). These findings suggest an interaction between ANX A1 and S100A11 that could be of importance for physiological functions of both proteins.

ANX A2 forms heterotetramers with its ligand of the S100 protein family (Annexin $A2_2S100A10_2$). Present in this complex Annexin A2 shows different behavior concerning

intracellular localization and biochemical properties (Thiel *et al*. 1992). Hence, S100A10 seems to have a regulative effect on ANX A2.

1.2.5 Functions of Annexins

Although the whereabouts of annexins have been known for 25 years and intensive work has been carried out, the specific physiological functions of most annexin members are still not clear. It is possible that each family member could have its own physiological function(s) due to the specificity given by the N-terminal domain and by the finding that annexins are differentially expressed.

Dreier *et al*. (1998) performed a detailed histochemical study of the expression of ANX A1, 2 and 4 in a broad variety of human tissue. They found distinct expression patterns of the three annexins. ANX A4 was detected in epithelial tissue of the respiratory and digestive tract, of the liver and pancreas, female and male reproductive tract, the urinary system, endocrine organs, nerve tissue and the skin. ANX A2 was especially found in epithelia of the respiratory tract, pancreas, urinary system, blood vessels and heart. Only ANX A1 was found in blood cells including lymphocytes, granulocytes and monocytes. It was also expressed in the respiratory tract, lymphatic tissue, urinary system and in nerve tissue. ANX A1 was not detected in the hepato-pancreatic system or in the digestive tract including stomach, duodenum, jejunum and ileum.

Within another study concerning ANX A1 expression in liver tissue using hepatectomy techniques and a transgenic mouse developing constitutively a hepatocarcinom, it was observed that ANX A1 was expressed during liver development in the embryonic mouse (de Coupade *et al*. 2000). ANX A1 expression decreased after birth, remaining absent in the adult liver. However, when hepatectomy was performed or the hepatocarcinom grew, ANX A1 was re-expressed. Interestingly, in both cases ANX A1 appeared tyrosine phosphorylated in parallel to EGF-R activation.

Thus, it is possible that annexins could be involved in aspects of differentiation and/or physiological functions of special organs.

The *in vitro* functions of annexins that could be determined so far are mostly linked to the regulation of membrane-membrane and membrane-cytoskeleton contacts such as membrane aggregation and fusion processes, blood coagulation, traffic including endocytosis and

exocytosis and ion channel activity. There are indications that annexins are involved in cell proliferation, differentiation and programmed cell death (apoptosis). As some annexins are substrates for certain kinases like the EGF-R, src and protein kinase C, they seem to play a role in signal transdution events. Last but not least, one of the most studied roles of ANX A1 is the anti inflammatory effect.

As far as ANX A1 is concerned it has been shown that it is implicated in the control of cell growth (Croxtall *et al*. 1992), differentiation (Violette *et al*. 1990), signal transduction and arachidonic acid release (Croxtall *et al*. 2000, Pepinsky *et al*. 1991) and intracellular vesicle trafficking (Meers *et al*. 1992, Futter *et al*. 1993, Diakonova *et al*. 1997).

1.2.6 Annexin A1 functions

1.2.6.1 Annexin A1 in inflammation processes

Glucocorticoid steroids are known to control and to reduce the severity of a potentially self-damaging inflammatory response (Weismann *et al*. 1964). They represent potent suppressors of inflammatory and allergic pathologies. Glucocorticoids act by altering gene expression of many cellular proteins, thereby provoking a biological change as well as a change in the activity of the target cell. One of the glucocorticoid-induced biomolecules that showed $cPLA_2$-inhibitory (cytosolic phospholipase A_2) activity was ANX A1 *alias* lipocortin-1 (Flower *et al*. 1979).

Additional inducers of ANX A1 synthesis have been discovered, including dexamethasone (synthetic derivate of glucocorticoid), cAMP (Antonicell *et al*. 2001), phorbol 12-myristate 13-acetate (PMA) (Solito *et al*. 1998) and interleukin-6 (Solito *et al*. 1998). The transcriptional activation of ANX A1 by PMA and IL-6 involved C/EBPß or the C/EBPß-like transcriptional factor respectively.

$cPLA_2$ is involved in cleavage of arachidonyl containing phosphatides in the cell. Arachidonic acid is the most abundant substrate for the cyclo oxygenase (Cox) mediated generation of prostaglandin E_2 that provokes many of the symptoms in inflammation including hyperalgesia (Ferreira *et al*. 1973) and fever (Milton *et al*. 1971, Flower *et al*. 1972). Although ANX A1 and other annexins have been described on cPLA2 inhibition, the inhibition is in most cases indirect by substrate depletion *i.e*. occupation of the $cPLA_2$-substrate at the membrane (Davison *et al*. 1990, Comera *et al*. 1990). This caused doubts as to the specificity and the physiological relevance of the effect, although it has also been shown that the fourth domain

of the protein core of ANX A1 contains a specific cPLA2 binding site (Kim *et al.* 1994, Kim *et al.* 2001).

Most likely the anti-inflammatory activity of ANX A1 is due to the regulation of leukocyte migration toward the site of inflammation (Teixeira *et al.* 1998).

In this anti-inflammatory process, ANX A1 is externalized out of circulating neutrophils and monocytes and proteolytically cleaved (Goulding *et al.* 1990, Francis *et al.* 1992, Perretti *et al.* 1996). The mechanism of ANX A1 secretion is not known yet. Localized on the plasma membrane of the blood cells, ANX A1 is capable of inhibiting leucocyte transmigration. This inhibition is already executed by ANX A1 N-terminal derived peptides (Perretti *et al.* 1995). Several studies have shown that ANX A1 regulates neutrophil extravasation by binding to the formyl peptide receptor (FPR) (Walther *et al.* 2000, Perretti *et al.* 2001, Rescher *et al.* 2002, Gavins *et al.* 2003).

In order to analyze the ANX A1 function *in vivo,* an ANX A1 knock out mouse was generated (Hannon *et al.* 2003, Roviezzo *et al.* 2002) and cell lines derived from this knock out mouse were established (Croxtall *et al.* 2003).

There was no obvious difference in physical appearance or behavior between the ANX A1-/-, ANX A1-/+ or ANX A1+/+ mice. All animals were healthy and fertile. However, differences in some biochemical and physiological parameters were observed *e.g.* a change in the expression of ANX A2, ANX A5 and ANX A6. This effect was thought to be due to reciprocal regulation between annexin members as observed before (Vishwanath *et al.* 1993, Newman *et al.* 1997). The expression of cPLA$_2$ and COX-2 was also upregulated in lung and thymus tissue. When inflammatory diseases such as mouse paw carrageenin-induced edema or zymosan-induced peritonitis were induced, the ANX A1-/- mouse showed a different phenotype compared to the wild type mouse (Hannon *et al.* 2003). Enhanced sensitivity to the stimuli and an increase in neutrophil migration was observed. In addition, treatment with dexamethasone was visibly less effective.

A lung fibroblast cell line established from the ANX A1 knock out mice shows different morphology as compared to the wild type (wt) cells. ANX A1-/- cells have spindle-shaped appearance and they accumulate intracellular organelles. As in the ANX A1 knock out mouse, the expression of some annexin family members was altered in these cells and COX-2, cPLA$_2$, sPLA$_2$ were also overexpressed. When ANX A1-/- cells are stimulated by fetal calf serum the release of arachidonic acids and prostaglandin E$_2$ was more pronounced than in wt cells. This effect was insensitive to dexamethasone (Croxtall *et al.* 2003). In contrast to wt

cells, stimulation of growth, cell cycle progression and expression of cell cycle-associated intermediates including cyclin D1, ERK2 and the phosphorylation of JNK1 was also not inhibited by dexamethasone in the ANX A1 knock out cell line showing that ANX A1 could represent a mediator of glucocorticoid action that leads to cell proliferation (Croxtall *et al.* 2003).

1.2.6.2 Annexin A1 and signal transduction

As mentioned before in chapter 1.2.1.3, ANX A1 represents a substrate for numerous different protein kinases including the EGF receptor and the HGF receptor, the cytosolic tyrosine-kinase src and also serine/threonine kinases including protein kinase C.

As ANX A1 has been implicated in a variety of cellular functions including anti inflammatory effects, signal transduction, proliferation, differentiation, apoptosis, intracellular trafficking, different findings suggest that the ANX A1 phosphorylation status of the different phosphorylation sites is implicated in the decision, which ANX A1-activity is triggered.

ANX A1 phosphorylation by protein kinase C seems to be important for the regulation of phospholipid-vesicle aggregation (Johnstone *et al.* 1993) and for the association of ANX A1 with phagosomal protein complexes (Kusumawati *et al.* 2001). In both cases ANX A1 activity is inhibited by phosphorylation.

In contrast, tyrosine phosphorylation of ANX A1 by the HGF-receptor might have an influence on HGF stimulated cell proliferation of A549 lung carcinoma cells (Skouteris *et al.* 1996). It has been postulated that the cell proliferation is promoted by the release of prostaglandin E_2. Similar to these findings, tyrosine phosphorylated ANX A1 seems to be necessary for the transduction of a positive growth signal from the EGF receptor to $cPLA_2$ in the mouse hepatocyte cell line mhAT (de Coupade *et al.* 2000). This would mean that ANX A1 would have an activating and not inhibitory effect on $cPLA_2$ when acting like an adaptor protein between the EGF-R and cytosolic proteins.

There are more observations connecting ANX A1 to hepatocyte proliferation processes. Being absent from healthy adult hypatocytes (Dreier *et al.* 1998), ANX A1 has been found in proliferating or regenerating hepatocytes (De Coupade *et al.* 2000, Masaki *et al.* 1994 and 1995). It was even overexpressed at both the transcriptional and translational levels and tyrosine phosphorylated in tumorous and nontumorous regions of human hepatocellular carcinoma (Masaki *et al.* 1997). Hence, the hypothesis has been made that ANX A1 is

implicated in the malignant transformation process leading to human hepatocellular carcinoma (Masaki *et al.* 1997).

Another study concerning ANX A1 in signal transduction was performed using the macrophage cell line RAW 264.7 expressing ANX A1 at either low or increased levels (Alldridge *et al.* 1999). In cells with high ANX A1 expression levels, the MAPkinase ERK was constitutively active, whereas in cells with low ANX A1 level, ERK showed prolonged acitvity after LPS stimulation. The acitivity of the MAPkinases p38 and JNK remained unaffected. This study showed that ANX A1 might be implicated in a signal transduction cascade upstream of the MAPkinase ERK. A potential interaction partner of ANX A1 could be Grb2 (Alldridge *et al.* 1999).

1.2.6.3 Annexin A1 and intracellular trafficking

Participation of annexins in membrane transport or intracellular trafficking has been described by several research groups (Gerke and Moss 2002). ANX A1 has been found in association with endosomal membranes (Rescher *et al.* 2000, Seemann *et al.* 1996, Futter *et al.* 1993). In addition, ANX A1 was localized to cytoskeletal filaments (Croxtall *et al.* 1998, Traverso *et al.* 1998, Kusumawati *et al.* 2000). These findings, together with the biochemical observation that ANX A1 can aggregate and fuse membranes suggest that ANX A1 does play a role in vesicle formation and intracellular trafficking of vesicular structures along cytoskeletal filaments.

Groups who have studied the processing of the internalized EGF-R found out that ANX A1 might be implicated in EGF-R trafficking (Futter *et al.* 1993). They described first that efficient degradation of the EGF-R requires the EGF-R kinase in its active state and/or that the phosphorylation of a special substrate could be necessary for its sorting towards the lysosmal compartment (Felder *et al.* 1990). Indeed, within the search for substrates of the still active EGF-R in late endosomes, so called multivesicular bodies (MVB), ANX A1 was found to interact with the EGF-R and to be tyrosine phosphorylated (Futter *et al.* 1993). In addition, a change in the Ca^{2+}-requirements for ANX A1 was determined. Non-phosphorylated ANX A1 was able to bind membranes at low Ca^{2+}-concentration, whereas the phosphorylated form needed an elevated Ca^{2+}-concentration for its binding to MVB membranes. Therefore, it was hypothesized that ANX A1 could represent the required substrate for lysosomal sorting and that due to its characteristics in interacting with membranes, it could favour inward-

vesiculation of the EGF-R towards the center of MVBs. In contrast to membrane proteins that are localized on the surface of MVBs and that might be destinated to recycle back to the plasma membrane, inward vesiculation of the EGF-R would trigger its complete degradation in lysosomes.

Endosomal localization of ANX A1 is also seen in live cells or revealed by ANX A1-GFP fusion proteins expressed in HeLa cells (Rescher *et al.* 2000).
A different localization of GFP-tagged full-length ANX A1 and core ANX A1 has been observed. Full-length ANX A1-GFP was localized at early endosomal membranes and hardly at all at late endosomal membranes in unstimulated HeLa cells, whereas the ANX A1 core-GFP chimera was mainly localized at late endosomal structures. This showed on the one hand that the core already presents an interface for specific localization and on the other hand that the N-terminal peptide might direct the protein's fine-localization (Rescher *et al.* 2000). By using the ANX A1-constructs containing a point-mutation in each Ca^{2+}-binding domain of type II in repeats 2, 3 and 4, respectively, it has been shown that the intact Ca^{2+}-binding domain of repeat 2 is indispensable for ANX A1 membrane binding to endosomal structures.

Taking into account the data on ANX A1 localization in endosomal membranes and its EGF-R mediated phosphorylation in MVBs, a model has been developed (Gerke and Moss 2002). As it is known that ANX A1 targets S100A11 to early endosomes, it is possible that ANX A1 is localized at the endosomal membrane as a heterodimer with its interaction partner. An as yet putative $S100A11_2$/ANX $A1_2$-hetero-tetramer could be formed and this entity could support inward budding. Closely localized EGF-R would phosphorylate ANX A1, which makes it more susceptible for proteolytic cleavage between the N-terminal peptide and the protein core. Cleavage would release a $S100A11_2$/N-terminal peptide$_2$ and could complete the inward vesiculation with the EGF-R and the ANX A1 core as vesicle content inside the maturating multivesicular body.

Figure 5: *Possible implication of ANX A1/S100A11 in endosomal inward vesicularisation*
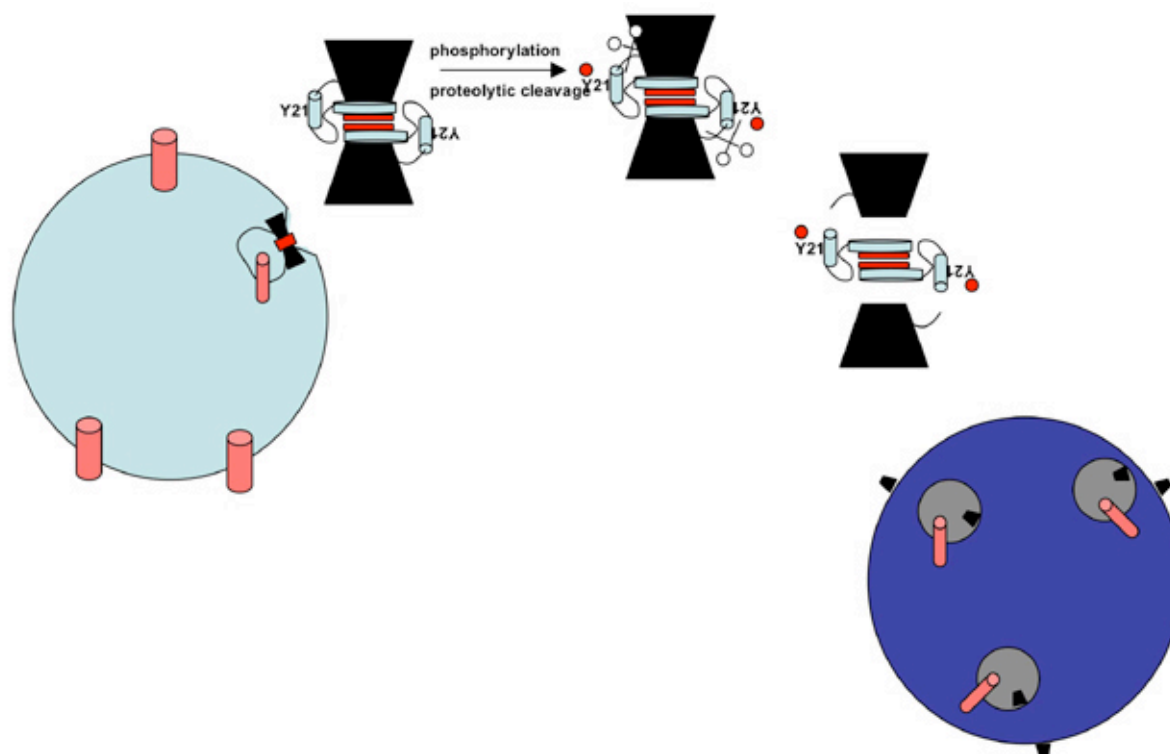
1.2.7 Annexin A1 and pathological disorder

ANX A1 appears dysregulated in several diseases and therefore seems to play a role in these pathological disorders including a wide range of inflammatory diseases and cancers, especially prostate and lung cancer, hepatocarcinoma, esophageal carcinoma and cancer of the mammary gland (table 3).

It is not yet fully understood as to whether the dysregulation of ANX A1 is the origin or an effect of the disorder. The effects on ANX!A1 differs between the disorders. In prostate, esophageal and tabacco induced oral cancer, the ANX A1 protein expression is down-regulated. In parallel to the ANX A1 downregulation, COX-2 and cPLA$_2$ are upregulated, which could mean that the missing anti-inflammatory effect of ANX A1 is one reason for the disease.

In some disorders such as rheumatoid arthritis and a special kind of lung cancer, auto-antibodies directed against ANX A1 have been detected which cause a decrease of the ANX A1 anti-inflammatory activity.

In human breast cancer ANX A1 appears to be overexpressed as it does in hepatocarcinoma where it is also tyrosine phosphorylated. In these cases it is possible that ANX A1, which is

known to play a role in cell proliferation and differentiation, is a part of the machinery that drives cells to uncontrolled cell proliferation.

Table 3: *Participation of ANX A1 in pathological disorders*

disorder	effect on ANX A1	secondary effect	Reference
tobacco induced inflammation and oral cancer	loss of anti-inflammatory activity due to cleavage of the N-terminal peptide	upregulation of the pro-inflammatory COX-2	Vishwanatha *et al*. 2003
esophageal squamous cell carcinoma	- downregulation of ANX A1 expression - translocation from the plasma membrane to the nuclear membrane	upregulation of the expression of COX-2 and cPLA$_2$	Zhi *et al*. 2003 Paweletz *et al*. 2000 Liu *et al*. 2003
prostatic intraepithelial neoplasia and prostate cancer	downregulation on protein and mRNA level		Kang *et al*. 2002 Paweletz *et al*. 2000
lung adenocarcinoma, squamous cell lung carcinoma	occurrence of ANX A1 autoantibodies		Brichory *et al*. 2001
mammary adenocarcinoma/ breast cancer	upregulated ANX A1 protein level		Pencil and Toth 1998 Ahn *et al*. 1997
human hepatocellular carcinoma	enhanced expression, tyrosine phosphorylation		Masaki *et al*. 1996
Rheumatoid Arthritis	occurence of ANX A1 autoantibodies		Podgorski *et al*. 1992
Multiple sclerosis (MS)	association of ANX A1 with active MS lesions, expression of ANX A1 in MS plaques		Probst-Cousin *et al*. 2002

1.3 The aim of the work

Annexins represent a large family of evolutionary highly conserved proteins. Their structure composed of a unique protein core containing novel Ca^{2+}-binding sites and a very variable N-terminal domain is well documented. Numerous roles in physiology have been proposed for annexins such as regulation of cell proliferation, differentiation, apoptosis, cell trafficking and inflammation and participation in signal transduction processes. ANX A1, in particular has been implicated in inflammatory processes, in special signal transduction events including MAP kinase cascades, and in intracellular trafficking of ligand bound EGF-R.

The EGF-R is a very potent transmembrane receptor kinase known to participate in many cellular activities that control the expression of numerous proteins or transcription factors, cell growth, proliferation, development and cell motility. Deregulated EGF-R is found in many pathologies like a variety of tumours. The activation, internalization, trafficking, sorting, deactivation and degradation of the EGF-R are tightly controlled. Like other transmembrane kinases, EGF-R endocytosis not only triggers degradation by lysosomal enzymes but also regulates their activity by specific localization and by bringing together interaction partners.

ANX A1 represents a potential *in vivo* substrate for the EGF-R and both proteins can be present in endosomal structures. In addition, ANX A1 has been implicated in EGF stimulated proliferation processes. However, as of yet, no careful documentation of the *in vivo* phosphorylation of ANX A1 following EGF-R activation has been carried out. Moreover, it had not been established where phosphorylation would occur within the cell and whether ANX A1 directly participates in intracellular trafficking of the activated EGF-R.

These questions were addressed by choosing two principal cell systems. One cell system was defective in clathrin-dependent internalization mechanisms and the other was the ANX A1 knock out cell line. How the localization and the phosphorylation status of both proteins changed when the cells are EGF stimulated was investigated. A possible influence on EGF-R internalization, trafficking and degradation when ANX A1 was absent was also investigated.

The hypothesis in the beginning of the study was with regard to the existing literature that ANX A1/EGF-R-interaction would take place in late endosomal structures and thus ANX A1 tyrosine phosphorylation with the consequence that in clathrin-dependent internalization deficient cells, EGF-dependent ANX A1 phosphorylation could not occur. It was also thought that the activity of the EGF-R might be prolonged and its degradation retarded in the absence of ANX A1.
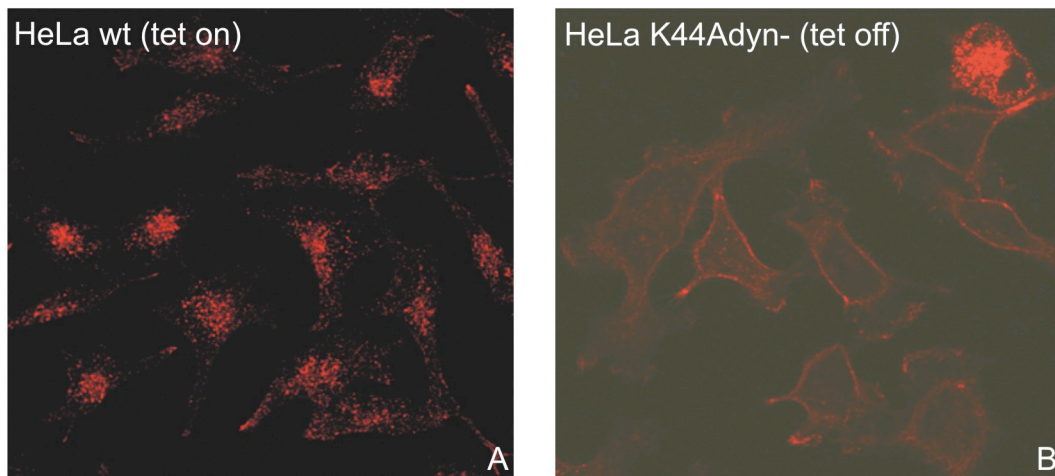
Figure 6: Transferrin uptake in HeLa wt and K44Adyn- cells

HeLa wt (A) and HeLa K44Adyn- cells (B) cultivated in the presence or absence of tetracyclin respectively (tet on/off) were serum starved over night and TexasRed®-conjugated transferrin was then internalized for 20 min.

2. RESULTS

Two cell lines were used to study the role of ANX A1 in activation and transport of the EGF receptor. The first represented a modified cell line of the human epithelial HeLa cell line that was conditionally and specifically defective in clathrin-dependent receptor-mediated endocytosis (Vieira *et al*. 1996). The conditional defect in endocytosis was imposed by the inducible expression of the lysin-44!$\rightarrow$!alanin-44 (K44A) mutant form of dynamin, a guanosine triphosphatase that is required for clathrin-coated vesicle formation. The expression of the non-functional dynamin mutant was repressed in the presence of the antibiotic tetracycline (tet on) and allowed in the absence of tetracycline (tet off).

In the following text, the cells overexpressing non-functional dynamin were distinguished from HeLa wt cells as HeLa K44Adyn-. So as to visualize the inhibition of clathrin-dependent receptor internalization in HeLa K44Adyn- cells, serum starved wt and K44Adyn- cells were incubated for 20!min with fluorescent transferrin and the transferrin uptake of the cells was analyzed by confocal microscopy (fig.6). In HeLa wt cells, the transferrin was internalized and detected inside the cells (fig.6A), whereas it remained on the cell surface of the endocytosis defective HeLa K44Adyn- cells (fig.6B). Approximately 10-20!% of the cells cultivated in the absence of tetracycline did not overexpress the dynamin mutant, *i.e.* showed transferrin internalization.

The second cell line JaCro derived from lung tissue of the ANX A1 knock out mouse. This fibroblastic cell line JaCro ANX A1-/- showed an altered morphology characterized by a spindle-shaped appearance and an accumulation of intracellular organelles compared to the control ANX A1+/+ cell line JaCro wt (Croxtall *et al*. 2003). JaCro ANX A1-/- cells were described to overexpress cyclo-oxygenase!2 (COX!2) and cytosolic and secretory phospholipase A_2 (PLA_2) in comparison to JaCro wt cells. They exhibit an exaggerated release of eicosanoids, which was insensitive to dexamethasone inhibition. JaCro ANX A1-/- cells were also observed to be insensitive to the dexamethasone inhibition of proliferation and serum-induced progression in the cell cycle and the associated expression of extracellular signal-regulated kinase 2 (ERK2), cyclin-dependent kinase 4 (cdk4) and COX 2 (Croxtall *et al*. 2003).

The JaCro cell line was especially applied to study EGF receptor internalization and deactivation processes in the presence and absence of ANX A1 (chapter 2.2).

A

Protein solution containing recombinant ANX A1-GST

↓

Affinity Chromatography GST-Trap/ Glutathione Sepharose

↓

Gel Filtration

↓

Cation exchange Chromatography

↓

Purified ANX A1

B

BP - bacterial pellet
TPS - Total protein solution
GSTE - GST-Trap elute
GSTCM - GST-Trap column material
GFF - Gel filtrated fraction
ECE - elute of the cation exchanger

C

Anti ANX A1 (r656)

Anti ANX A1 N-terminal peptide (TDL)

D

Anti ANX A1 (Sidney)

Figure 7: Annexin A1 protein purification and antibody production

Expression of recombinant ANX A1-GST was induced in BL21(DE3) *E.coli.* Soluble proteins were applied to Glutathione-Sepharose. Cleavage by thrombin separated ANX A1 from GST. After gel filtration, ANX A1 was further purified by cation exchange chromatographie (A). Samples taken after each purification step were separated by SDS-PAGE and stained with Coomassie blue (B).
Purified ANX A1 was applied to immunoblotting using a polyclonal ANX A1 full-length (r656) and a monoclonal ANX A1 N-terminus antibody (TDL) (C).
(A1 ctrl. - purified ANX A1 obtained from Andreas Wilbers, ZMBE Muenster)
The anti ANX A1 serum Sidney was obtained after rabbit immunization with the purified ANX A1 was tested by immunoblotting of HeLa wt total cell lysate (D).
(* 1 min ** 15 min exposition)

2.1 Interaction and transport of ANX!A1 and the EGF-R in HeLa cells

2.1.1 Characterization of ANX A1 antibodies

2.1.1.1 Production of a new polyclonal ANX A1 antibody

For the production of a new antibody directed against the ANX!A1 protein, recombinant ANX A1 was expressed as a GST (glutathion-S-transferase) fusion protein in the IPTG inducible *E. coli* system BL21(DE3) and purified. The ANX!A1 purification was realized in three steps of different chromatographic techniques (fig.7A). After extracting the bacteria, the soluble proteins were injected into a glutathion-sepharose containing chromatography column (GST-Trap) in order to retain the proteins that interacted with glutathion like the GST coupled to ANX A1 (first step). The ANX!A1 fusion protein contained a thrombin cleavage side between ANX A1 and the GST so that thrombin incubation of the column content provoked the liberation of ANX A1 from the GST and its elution, whereas the GST remained bound to the column material (fig.7B, lanes GSTE and GSTCM). In order to perform further purification by cation chromatography (third step), the salt content of the GST-Trap elute was altered by gel filtration (second step). The filtrated fraction (lane GFF) was applied to the cation exchanger and two concentrated proteins of apparent molecular masses 35 and 37!kDa (lane ECE) were eluted at the end of the last purification step. The elute of the cation exchanger (ECE) was analyzed by immunoblotting using the ANX!A1 antibodies r656, a polyclonal rabbit antibody, directed against the full-length ANX A1 protein, and TDL, a monoclonal mouse antibody, directed against an unknown epitope of the N-terminal domain (fig.7C). The monoclonal anti-peptide antibody did not detect ANX A1 in the ECE-fraction, whereas the r656 antibody detected one main protein band that was inferior to the molecular weight of ANX!A1 in the control solution previously purified by Andreas Wilbers (ZMBE Münster).

The ECE-fraction was injected as immunogen in two rabbits, called Sidney and Atlanta, to produce polyclonal anti ANX!A1 sera. One of the sera (Sidney) was tested on cell lysates of HeLa cells (fig.7D). One protein band was decorated corresponding in molecular weight to that of ANX!A1 (lane 1). Only after longer exposition times, additional protein bands appeared with weaker intensities compared to the principal signal.
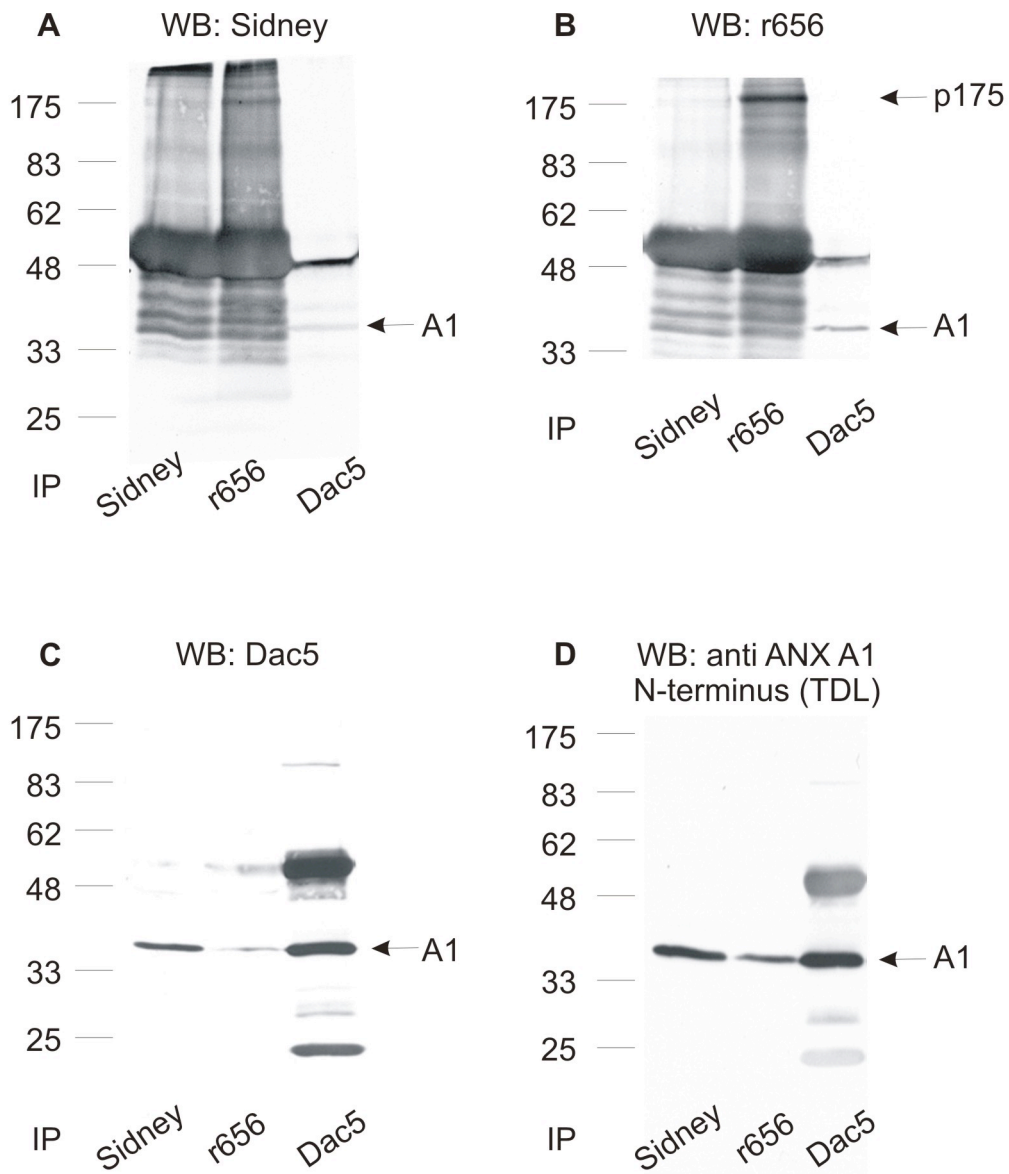
Figure 8: Immunoprecipitation of ANX A1

Total cell lysate of HeLa wt cells was incubated with the ANX A1 antibodies Sidney (polyclonal), r656 (polyclonal) and Dac5 (monoclonal) and protein A sepharose to perform ANX A1-immunoprecipitation (IP).

Precipitated proteins were applied to immunoblotting using the same antibodies and in addition the monoclonal ANX A1 N-terminal peptide antibody from Transduction laboratories (TDL).

2.1.1.2 Application of ANX A1 antibodies

In addition to the Sidney antibody four different ANX A1 antibodies were used in this study. They are listed in table 4.

Table 4: *Annexin A1 antibodies*

Antibody	polyclonal	monoclonal	Immunogen	Epitope(s)
Sidney	X		ANX A1 full-length	ANX A1 full-length
r656	X		ANX A1 full-length	ANX A1 full-length
DE99603	X		N-terminus	N-terminus
Dac5		X	ANX A1 full-length	not known
TDL (Transduction Lab.)		X	ANX A1 full-length	N-terminus

Sidney, r656 and Dac5 were tested for use in immunoprecipitation experiments (fig.8). Immunoblotting of the precipitated proteins was performed with the same antibodies and, in addition, with the commercial anti N-terminal peptide antibody TDL from Transduction Laboratories. When ANX A1 was precipitated and immunoblotted with Sidney (fig.8A) or r656 (fig.8B), the background in the molecular weight range from 30 to 50 kDa was too strong to identify the ANX A1 protein. Only when precipitation was performed with the monoclonal antibody Dac5 were Sidney and r656 able to detect a protein band at the molecular weight of ANX!A1. In addition, r656 appeared to interact with proteins of the molecular weight of the EGF-R (175!kDa). The immunoblots probed with Dac5 (fig.8C) and TDL (fig.8D) showed a low background, especially when ANX!A1 was precipitated with Sidney. This test showed that Sidney and Dac5 were useful antibodies to concentrate ANX!A1 by immunoprecipitation.

The peptide antibody DE99603 was also tested for immunoprecipitation applications, but revealed a high background (data not shown).

Sidney purified by protein A-, or by specific antigen affinity chromatography and the N-terminal peptide antibodies DE99603 purified by antigen affinity chromatography and TDL were used for immunofluorescence studies in HeLa wt cells (Fig.9).

All antibodies revealed a punctual staining in the cytoplasm except for Sidney when purified by the more specific method that labeled the plasma membrane when the cellular monolayer

had reached a high density (fig.9A) but also when the cells were more separate (not shown). The protein A-purified antibody Sidney labeled granular structures throughout the cytosol (fig.9B) and in addition big concentrated structures close to one side of the nucleus that became visible at special levels in the cell by confocal microscopy (fig.9C). In order to know ascertain if this labeling was ANX A1-specific or not, the protein A purified immunoglobulin-solution was incubated with purified, full-length ANX!A1 protein before it was added to the fixed and permeabilized cells (fig.9D). The microscopical analysis showed that the cytosolic punctual staining disappeared completely after the protein competition but that the concentrated structures next to the nucleus were still detectable – hence most likely representing non-specific signals.

The antibody DE99603 labeled fine, single vesicular structures that were distributed evenly in the cytosol (fig.9E). The monoclonal antibody TDL labeled the plasma membrane in addition to the granular structures that contrasted less with the background than the vesicles labeled by DE99603 (fig.9F).

In the following co-localization study, the ANX!A1 antibodies Sidney and DE99603 were mainly applied.
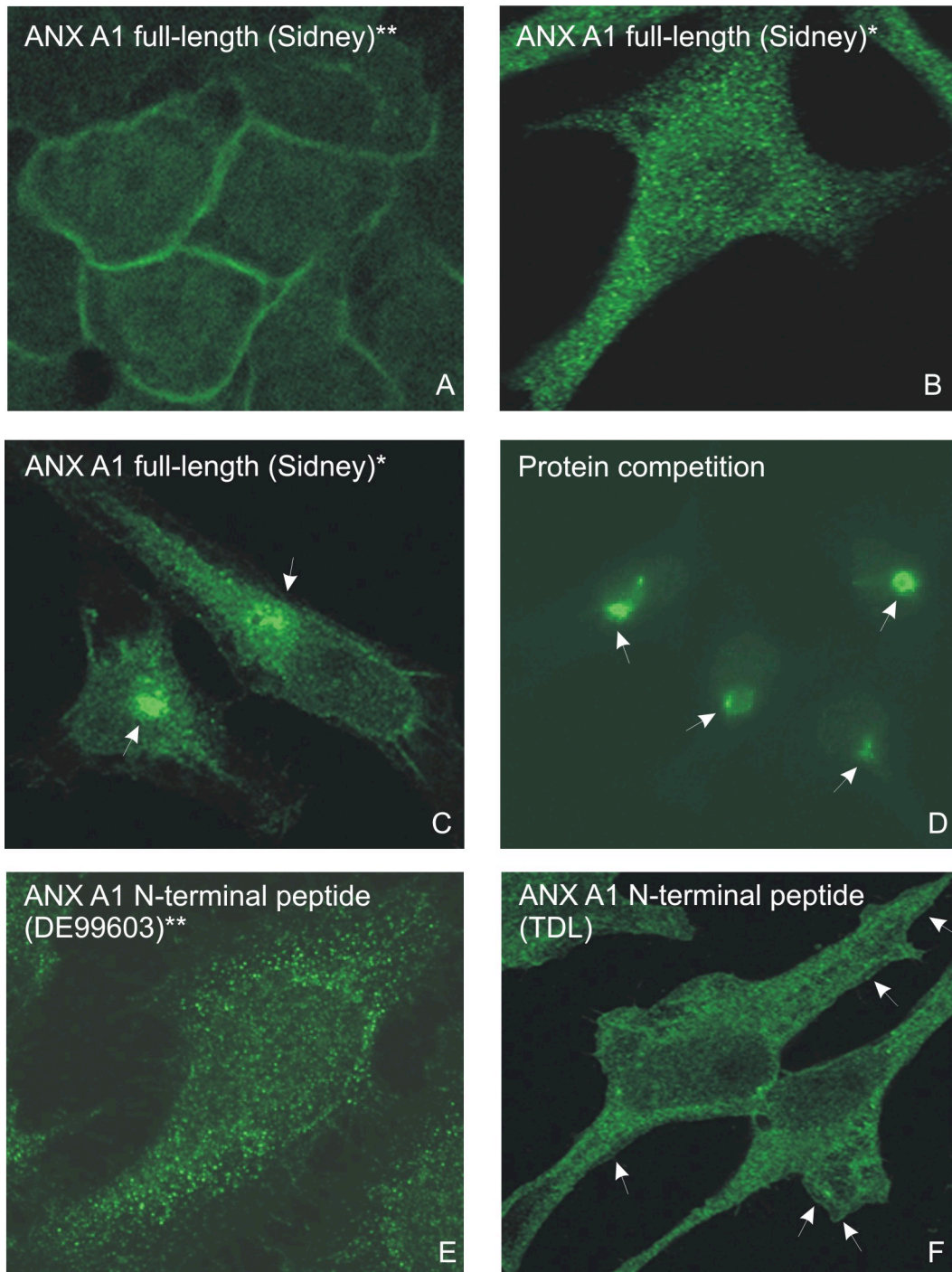
Figure 9: ANX A1 localization in HeLa wt cells

HeLa wt cells were fixed using PFA, permeabilized with saponin and incubated with three different ANX A1 antibodies, two N-terminal peptide antibodies, TDL from Transduction Laboratories (monoclonal) (E), DE99603 (polyclonal) (F) and the ANX A1 full-length antibody Sidney (polyclonal) (A-D).
The ANX A1 full-length antibody was incubated with purified ANX A1 protein prior to the immunostaining to detect unspecific labeling (D).
* serum was purified by protein A or
** specific antigen affinity chromatography.
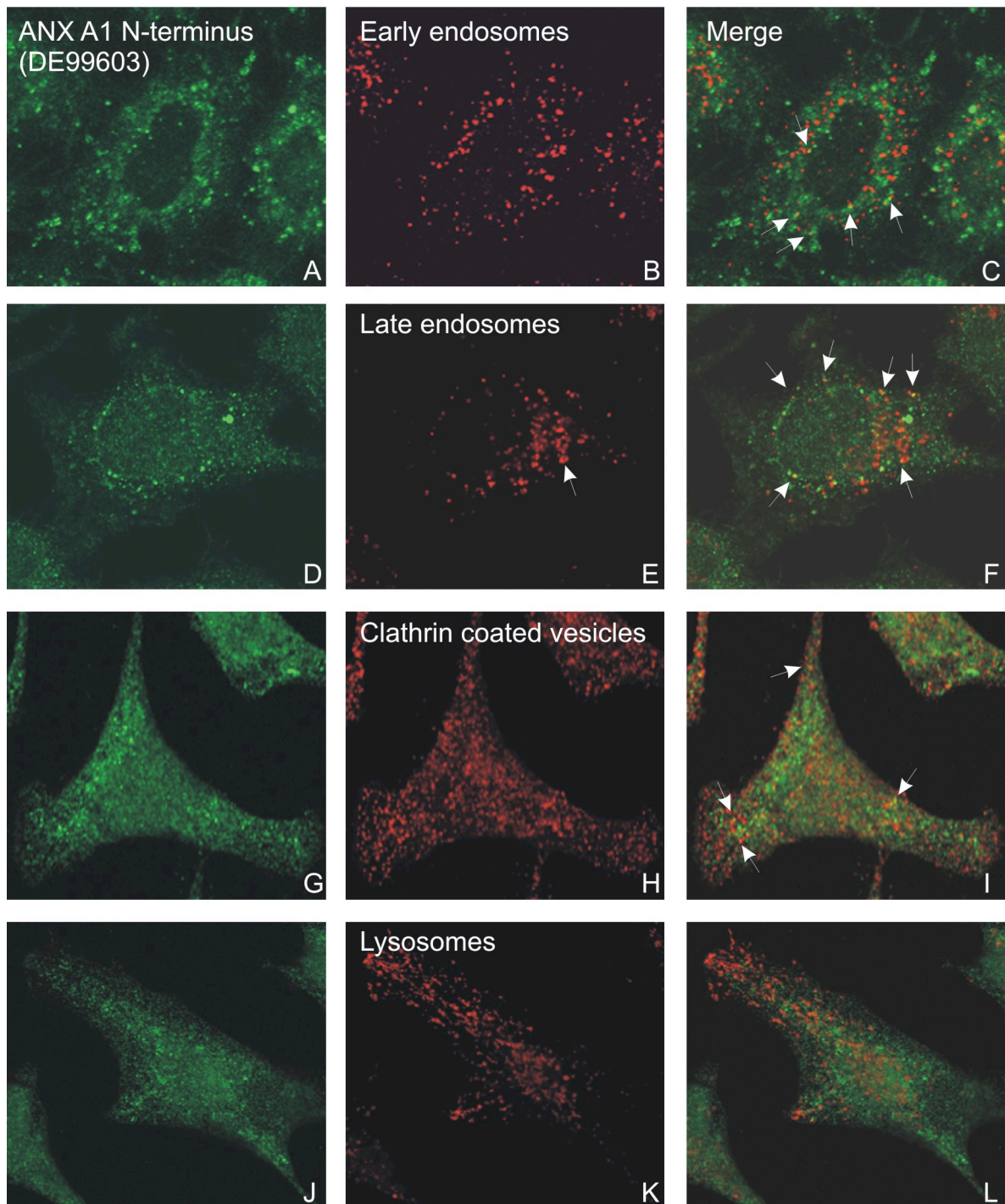
Figure 10: Co-labeling of ANX A1 terminal peptide and endosomal structures

HeLa wt cells were fixed with PFA (4%) and permeabilized using saponin (0.05%). The ANX A1 N-terminal peptide antibody DE99603 was purified by antigen affinity chromatographie before it was used for immunofluorescence studies.
Antibodies against the endosomal markers early-endosomal-antigen1 (EEA1), lysobisphosphatidic acid (LBPA), clathrin and lamp 1 were applied to label early endosomes (A-C), late endosomes (D-F), clathrin coated vesicles (G-I) and lysosomes (J-L) respectively.

2.1.2 Localization of endogenous ANX A1 in HeLa wt cells

2.1.2.1 Co-localization of ANX A1 with endosomal structures

So as to describe the fine vesicular structures stained by the ANX A1 N-terminal peptide antibody DE99603, a double-labeling analysis in fixed HeLa wt cells was performed. Antibodies directed against the known marker proteins early-endosomal-antigen 1 (EEA1), lysobisphosphatidic acid (LBPA), lamp!1 and clathrin were applied to label early endosomes, late endosomes, lysosomes and clathrin coated vesicles, respectively (Fig.10).

The DE99603 labeling and that of early endosomes resulted in both cases in staining of small vesicles that were distributed throughout the cells (fig.10A&B). The superposition of the labeled structures showed few co-localizing vesicles indicated by arrows (fig.10C).

The late endosomal structures stained by the LBPA antibody seemed to be localized more regionally on one side of the nucleus and closer to the nucleus compared to the DE99603-staining. Some vesicles were larger and small hollow spaces became visible (fig.10D&E). Co-labeling of LBPA and the ANX A1 N-terminal domain was again very limited (fig.10F).

The clathrin staining revealed numerous vesicles present in the whole cytosol. In a very few cases, co-localizations of clathrin-coated and ANX!A1-positive structures were found (fig.10G-I).

No co-staining of ANX A1 N-terminal domain and lamp!1 was detected (fig.10J-L).

2.1.2.2 ANX A1 localization at the cytoskeleton

The immunofluorescence data shown in figures 9 and 10 gave the impression that the ANX!A1-positive vesicles were not randomly distributed in the cytoplasm, but that the orientation of the vesicles to each other was regulated. Therefore, the localization of ANX!A1 with regard to the cytoskeleton was studied next.

The cytoskeletal proteins α-tubulin, F-actin, α-cytokeratin and α-vimentin were labeled in parallel with the DE99603 (fig.11) or the Sidney staining (fig.12).

Tubulin (fig.11/12B) and cytokeratin (fig.11/12E) seemed to be present in the whole cell, whereas vimentin (fig.11H) presence was more limited in specific regions of the cell at one side of the nucleus. The vesicular structures labeled by the peptide antibody were spread out in the cytoplasm as observed previously (fig.11A, D&G). The superposition of the staining of

the three cytoskeletal proteins and ANX A1 showed that some of the vesicles appeared to reside in close proximity of the filaments stained.

Similar results were obtained using the antibody Sidney directed against the full-length ANX A1. Some of the ANX!A1 positive vesicles present throughout the whole cell seemed to be localized along the filaments of tubulin and cytokeratin (fig.12A-F). Hardly any co-localization of ANX A1 and F-actin was detected (fig.12G-I).
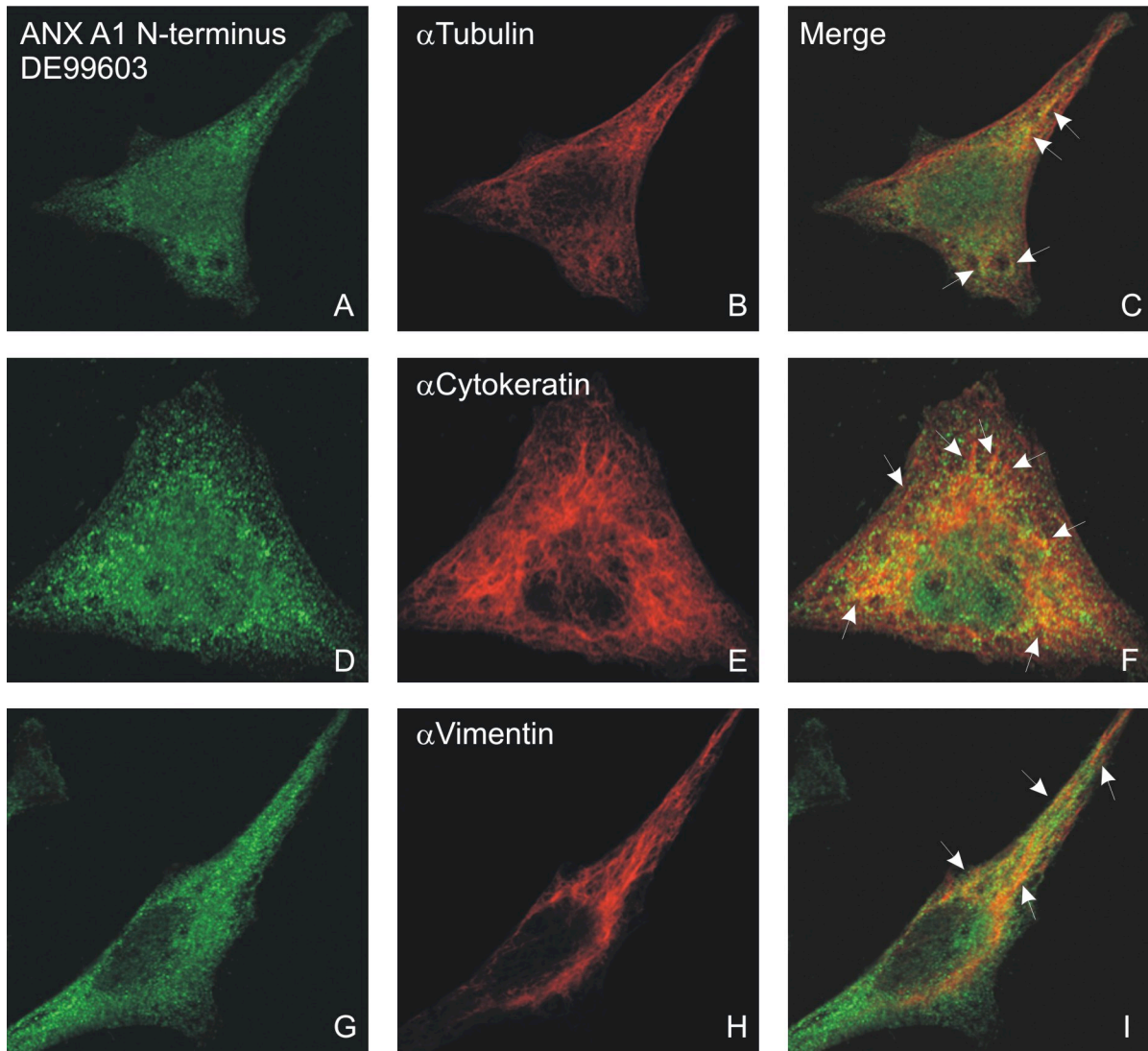
Figure 11: Co-labeling of DE99603 and antibodies directed against proteins of the cytoskeleton in HeLa wt cells

HeLa wt cells were fixed with PFA (4%) and permeabilized using saponin (0.05%). The ANX A1 N-terminal peptide antibody DE99603 was purified by antigen affinity chromatographie before its use in the immunofluorescence studies. Different antibodies of cytoskeletal proteins were applied, anti α-tubulin (A-C), anti α-cytokeratin (D-F) and anti α-vimentin (G-I).
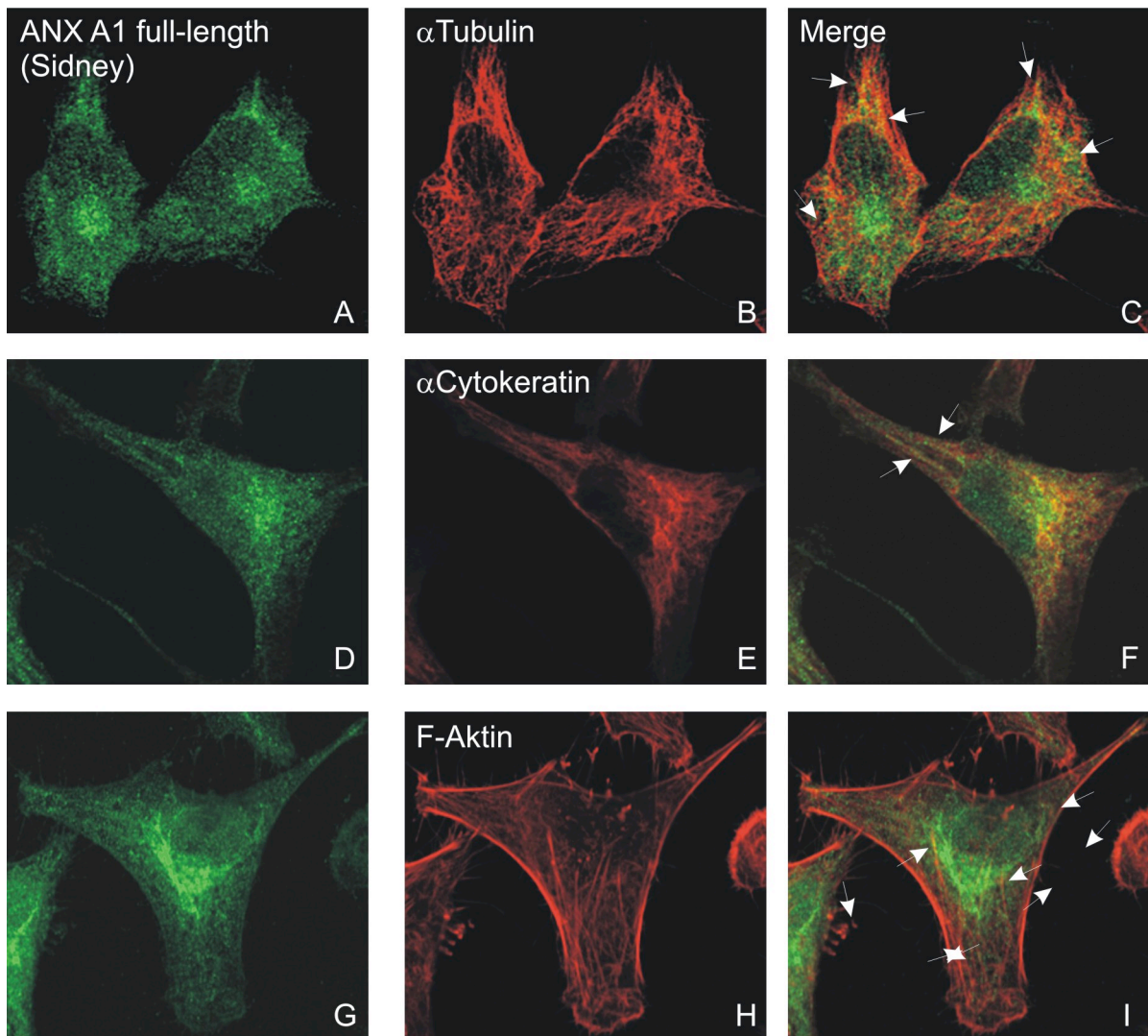
Figure 12: Co-labeling of full-length ANX A1 and proteins of the cytoskeleton

HeLa wt cells were fixed with PFA (4%) and permeabilized using saponin (0.05%). The full-length ANX A1 antibody Sidney was purified by protein A-chromatography before its use for immunofluorescence studies.

The same α–tubulin and α–cytokeratin antibodies as in figure 11 were used to stain the cytoskeleton. In addition, phalloidin-TRITC was applied to label actin (G-I).

2.1.3 Expression and co-localization of ANX A1-GFP fusion proteins in HeLa wt cells

The constructs encoding porcine ANX!A1!full-length and the core attached at its carboxyl terminus to the bright green variant of the green fluorescent protein (EGFP) were used for the following study. The protein sequence of the core construct started with the phenylalanine residue in position 39 (Rescher *et al.* 2000).

HeLa wt cells were transfected with the described plasmids and the vector without insert. The green fluorescent protein alone was visible in the whole cell including the nucleus (fig.13A). The ANX A1 full-length fusion protein and the core without N-terminal domain were detected in parts of the cytoplasm bound to granular structures close to the nucleus (fig.13B&C).

2.1.3.1 Localization of ANX A1-GFP in HeLa wt cells

Co-localization studies were performed with full-length ANX A1-GFP expressing HeLa wt cells using different markers for the following intracellular organelles: mitochondria (Mito-Tracker), trans Golgi network (anti TGN 46), lysosomes (DQ-BSA), late endosomes (LBPA antibody) and clathrin coated vesicles (clathrin antibody) (fig.14). The double labeling of ANX A1 and mitochondria or the trans Golgi network revealed differently stained structures and no co-localization was detected (fig.14A-F). Likewise, the staining of lysosomes in the presence of ANX A1-GFP uncovered only some double-labeled vesicles in the cytoplasm (fig.14G-I). Co-localizations of ANX!A1 positive vesicles with late endosomes were not detected (Fig.14J-L). However, ANX A1-GFP was found partially co-localized with clathrin in the cytosol and at the plasma membrane (Fig.14M-O).
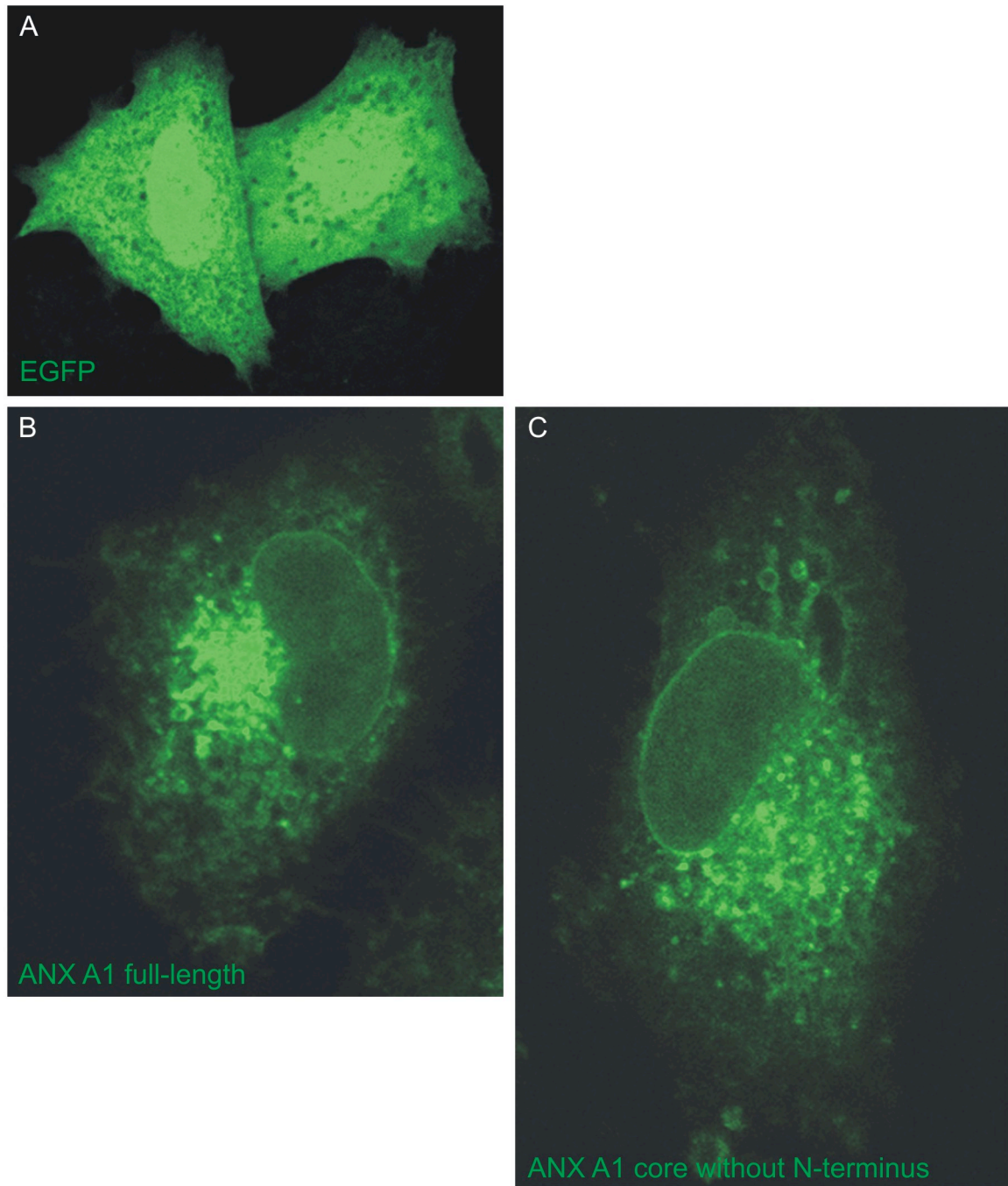
Figure 13: Annexin A1-GFP Fusion proteins

Two ANX A1-GFP constructs encoding full-length or the core without N-terminal domain fused to the enhanced green fluorescent protein (EGFP) were transfected. HeLa wt cells were transfected by the calcium/phosphate method with the empty vector (A) or one of the constructs (B and C).

In order to analyze if ANX A1-GFP was localized at early endosomal structures or in the recycling compartment, transfected cells were incubated for 5 or 30 min with Texas-Red® conjugated transferrin (fig.15A-F). At both incubation periods, ANX A1-GFP was detected in the cytoplasm at vesicular structures and also bound to the plasma membrane. It would appear that more green fluorescent vesicles were found closer to the nucleus at 30 min than at 5 min transferrin stimulation. After 5 min transferrin, numerous ANX A1-GFP positive vesicles co-localized with transferrin containing vesicles. These co-labeling events were detected throughout the cytoplasm. At 30 min, double-labeled vesicles were still found in the cytoplasm. It would appear as well that most of the labeled vesicular structures were more concentrated and closer to the nucleus.

So as to analyze if the N-terminal tail played a specific role for the ANX A1-localization in the endosomal and the recycling compartment, the study was repeated with cells transfected with the core-GFP construct (fig.15G-L).

The ANX A1 core was expressed inside the cell bound to vesicular structures as observed previously. The results of the transferrin study were very similar at 5 min of transferrin incubation to those obtained with full-length ANX A1-GFP. The core-GFP positive vesicles localized with the transferrin containing endosomes. When the cells were treated for 30!min with transferrin (fig.15J-L), transferrin-containing endosomes and core-GFP colored vesicles were still distributed in the cytosol.
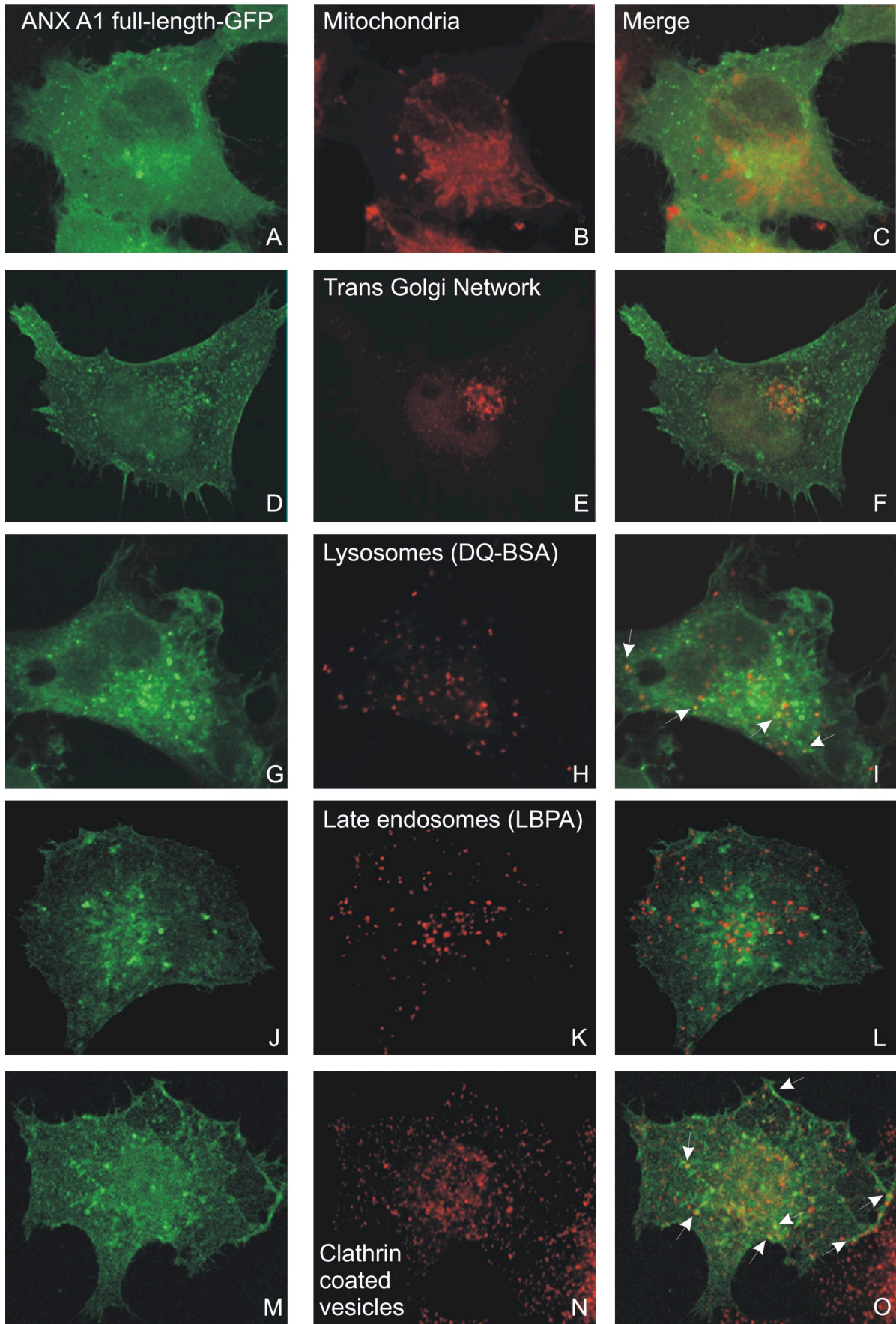
Figure 14: Localization study of ANX A1-GFP in HeLa wt cells
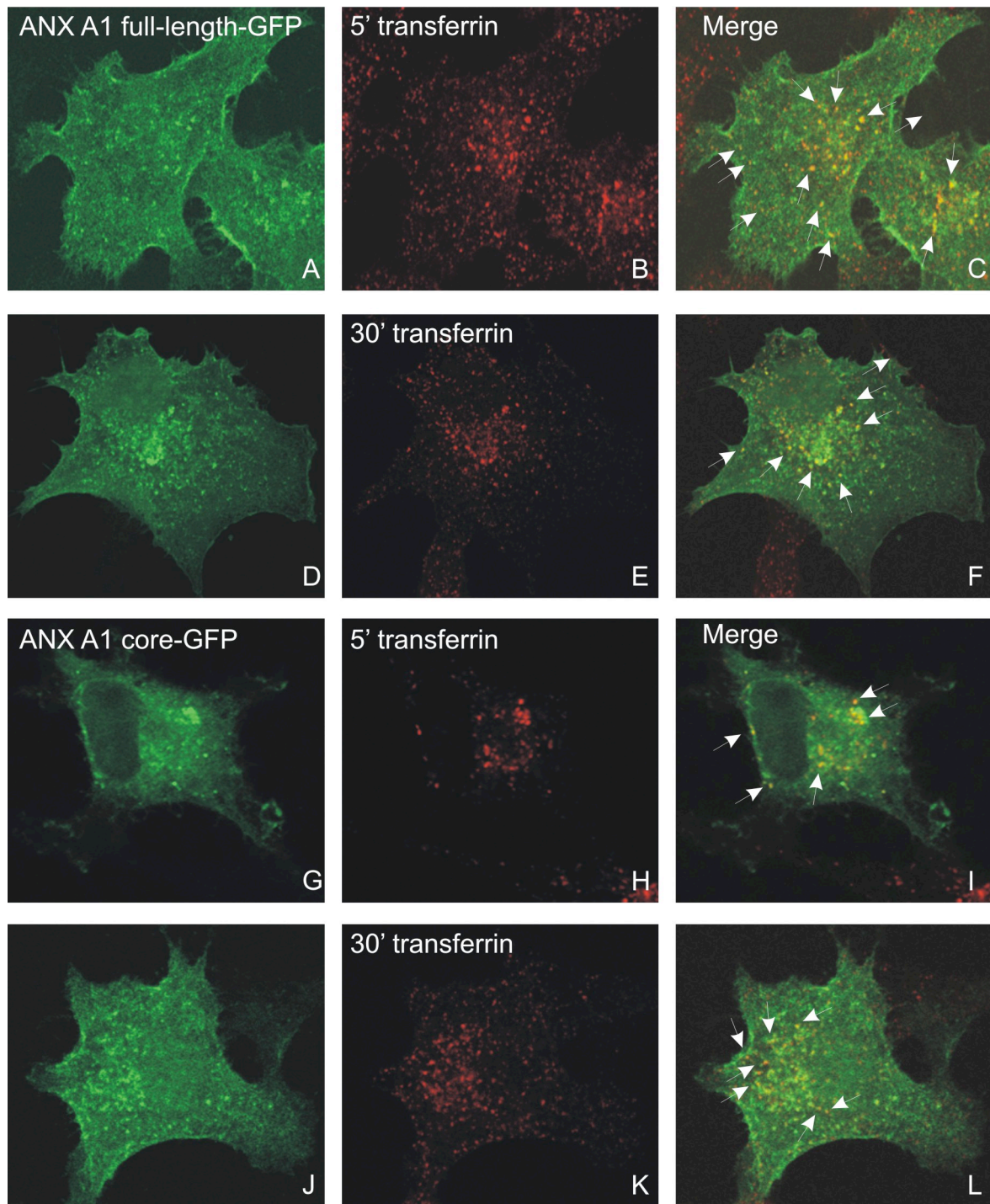
Figure 15: Transferrin uptake in ANX A1 full-length-GFP and core-GFP transfected HeLa wt cells

HeLa wt cells were transfected with the plasmids panx a1 full-length-GFP (A-D) and pcore-GFP (G-L) by the calcium-phosphate method.The cells were starved and then incubated with transferrin-TexasRed® for 5 min to label early endosomes (A-C and G-I) and for 30 min to label early endosomes and the recycling compartment (D-F and J-L).
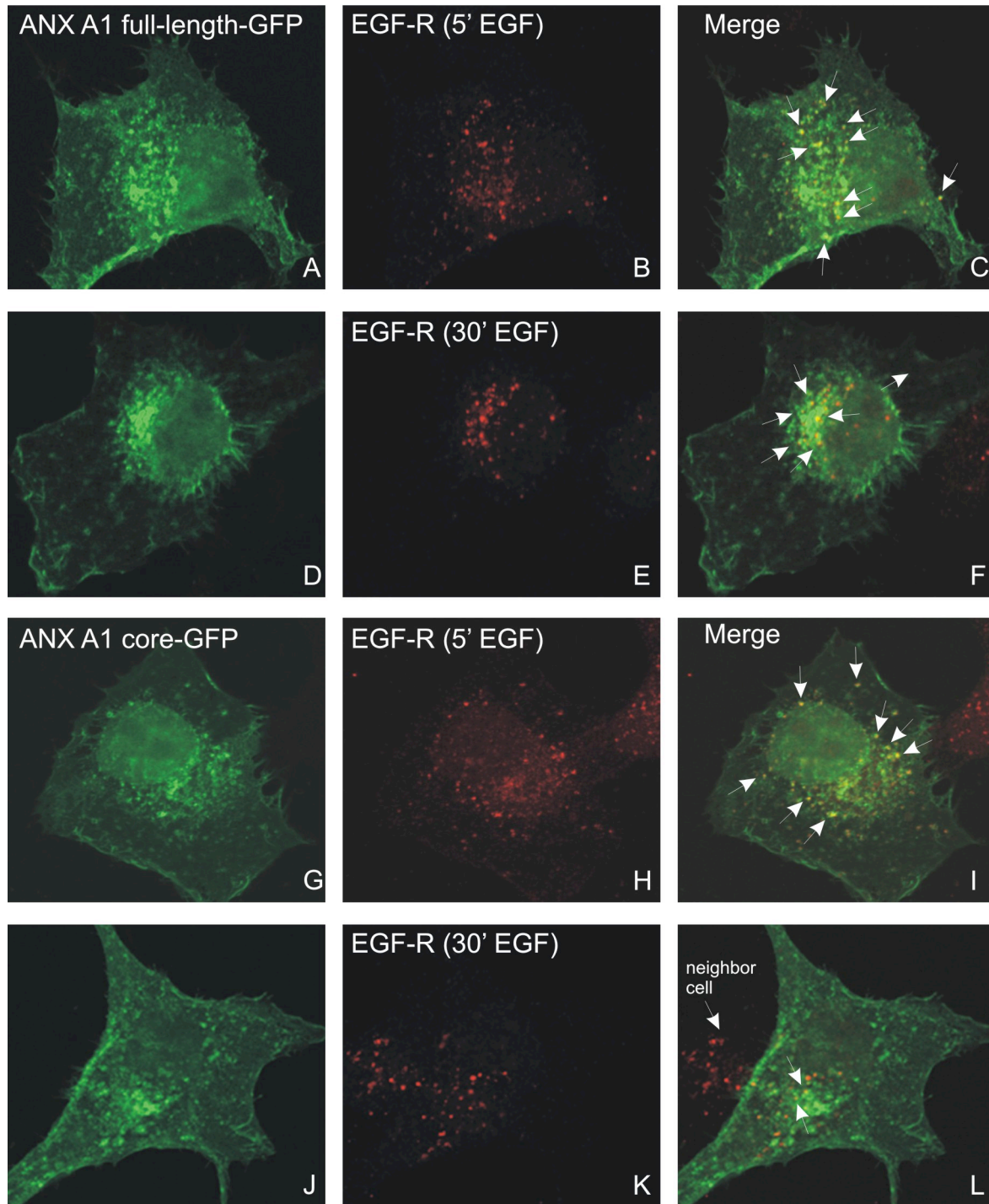
Figure 16: Co-localization study of the EGF-R and ANX A1 full-length-GFP or core-GFP in EGF stimulated HeLa wt cells

HeLa wt cells were transfected with panx a1 full-length-GFP (A-D) and pcore-GFP (G-L) by the calcium-phosphate method.The cells were starved and then incubated with EGF for 5 min (A-C and G-I) and for 30 min to follow the EGF-R transport to early and late endosomes.
After the EGF stimulation, the cells were fixed and permeabilized with a combined PFA/Triton X-100 protocol in the presence of calcium. The EGF-R was labeled with a polyclonal antibody.

2.1.4 Co-localization of ANX A1 and the EGF receptor in EGF stimulated HeLa wt cells

After having studied the localization of ANX A1 compared to the transferrin cycle, the presence of ANX A1 with or without its N-terminal domain during intracellular transport of the EGF-R was analyzed.

Hela wt cells expressing the ANX!A1!full-length- or the core-GFP fusion protein were serum starved over night and then EGF stimulated for 5 and 30 min (fig.16). At 5 min EGF stimulation, ANX A1 and its core alone were bound to vesicular structures in the cytoplasm (fig.16A&G). The fusion proteins were also detected in parts of the plasma membrane. The EGF-R was also present in or bound to vesicles, which were localized in similar cytoplasmic regions where ANX A1 was present (fig.16B&H). Indeed, co-localizations on the described vesicular structures of the EGF-R and both ANX A1-GFP fusion proteins were found (fig.16C&I).

After 30 min EGF, the ANX A1-GFP- and also the EGF-R-colored structures appeared compacter in the perinuclear region than at 5!min EGF stimulation (fig.16D&E). Here, almost all EGF-R-positive endosomes co-localized with vesicles containing ANX A1 full-length GFP (fig.16F).

The localization of the core-GFP positive vesicles did not seem to change within 30!min EGF stimulation (fig.16J). The EGF-R localized at 30 min in vesicles closer to the nucleus than at 5!min (fig.16K). In this part of the cell co-localizations of the EGF-R and the core were found (fig.16L).

The same experiments were performed with HeLa cells defective in clathrin-dependent endocytosis. Using these cells, the EGF-R or the transferrin receptor remained at the plasma membrane, so that receptor containing endosomes could not be detected in the cytoplasm and also no co-localization of ANX A1-GFP fusion proteins with transferrin or the EGF-R (not shown).

Figure 17: Presence of ANX A1-GFP fusion proteins in EGF-R clusters

HeLa wt cells were transfected with six different GFP constructs, the empty vector pEGFP (A, B), four ANX A1-GFP constructs, panx a1 full-length-GFP (C-G), pcore-GFP (H-L), panx a1 full-lengthY21A-GFP (M-O), panx a1 full-lengthY21E-GFP (P-R), and two GFP-constructs of two different annexin members, panx a3-GFP (S, T) and panx a6-GFP (U-V).

The transfected cells were serum-starved over night and first incubated with an EGF-R antibody directed against the extracellular domain and then either incubated immediately with the fluorescent dye coupled secondary antibody (cluster) followed by fixation or first fixed and permeabilized by the combined PFA/Triton X-100 method before incubated with the secondary antibody (control).

Untransfected cells were also used. In this case, the cells were treated by the combined PFA/Triton X-100 method after the EGF-R clustering. ANX A1 was labeled applying the monoclonal ANX A1 N-terminal peptide antibody TDL (W-Y).
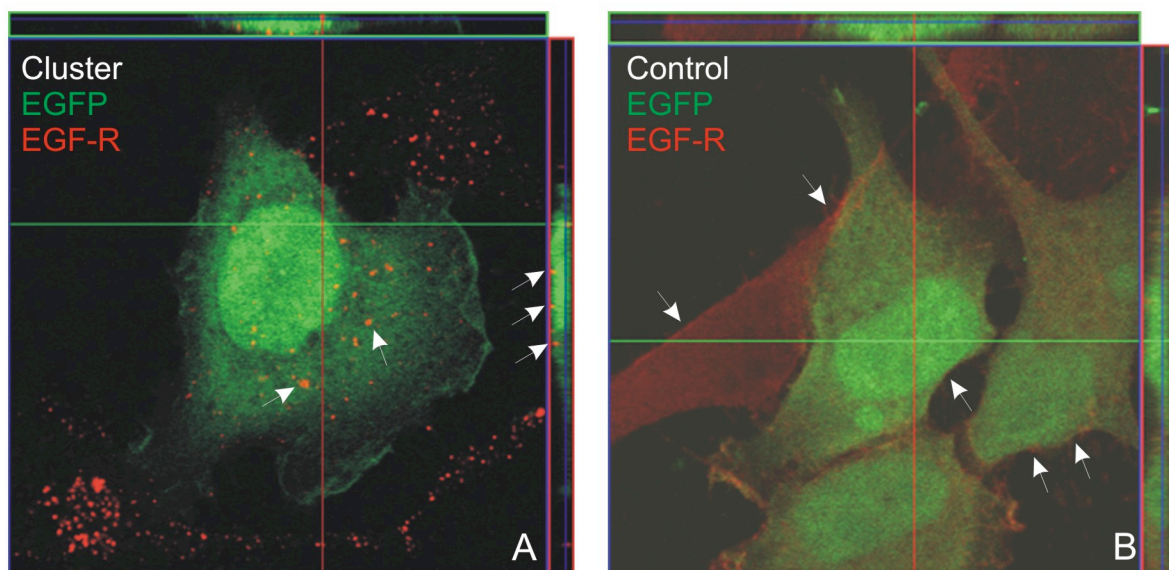
Figure 17 A and B: EGF- R cluster formation in HeLa wt cells expressing the green fluorescent protein

2.1.5 Localization of ANX!A1!wt and ANX!A1!mutants in EGF receptor clusters

Next, the ANX A1 distribution was analyzed, following EGF-R clustering in the plasma membrane. For this, the serum-starved cells were first of all incubated for 15!min with an antibody directed against the external domain of the EGF-R that was able to compete with EGF for the binding domain. The cells were then directly, without fixation or any other treatment, incubated with the secondary antibody that was linked to a red fluorescent dye. Only after the second antibody incubation, the cells were fixed with PFA without permeabilization step. In controls, cells were fixed and permeabilized between the antibody incubations so as to prevent clustering.

Cells transfected with different ANX A1-GFP constructs were used for this study. In addition to the ANX A1 full-length- and core-GFP encoding constructs, two mutants fused to GFP were expressed in HeLa wt cells (Judith Austermann 2003). Each contained a point mutation at position 21, the phosphorylation site for the EGF-R in one case tyrosine 21 was replaced by alanine (ANX!A1!Y21A), in the other by glutamate (ANX!A1!Y21E) (fig.17).

To begin with clustering was realized in cells transfected with the empty pEGFP vector (fig.17A). The expression of the green fluorescent protein alone was diffuse in the cytosol, but mainly in the nucleus. The EGF-R clusters were visible as small red spots mainly on the surface of the cell, especially visible in the third dimension of the confocal image. The control experiment showed that the EGF-R did not form clusters, but remained at the plasma membrane when cell fixation was carried out before the secondary antibody incubations (fig.17B).

Cells that expressed ANX!A1!full-length-GFP showed more clusters of the EGF-R that also appeared to be bigger in size (fig.17C) than in EGFP-expressing cells. The image of the cell with ANX!A1-GFP containing intracellular vesicles appeared as seen previously. The ANX!A1-positive vesicles were localized especially in a region on one side of the nucleus and they did co-localized with the EGF-R clusters, which had also been formed mostly in this perinuclear compartment. As visible in the third dimension and in the image gallery, the clusters seemed to be internalized into the cell, but in some cases keeping contact with the plasma membrane (fig.17D). The control experiment showed that the clusters were only formed when the fixation step had taken place after the incubation with the secondary antibody (fig.17E-G).
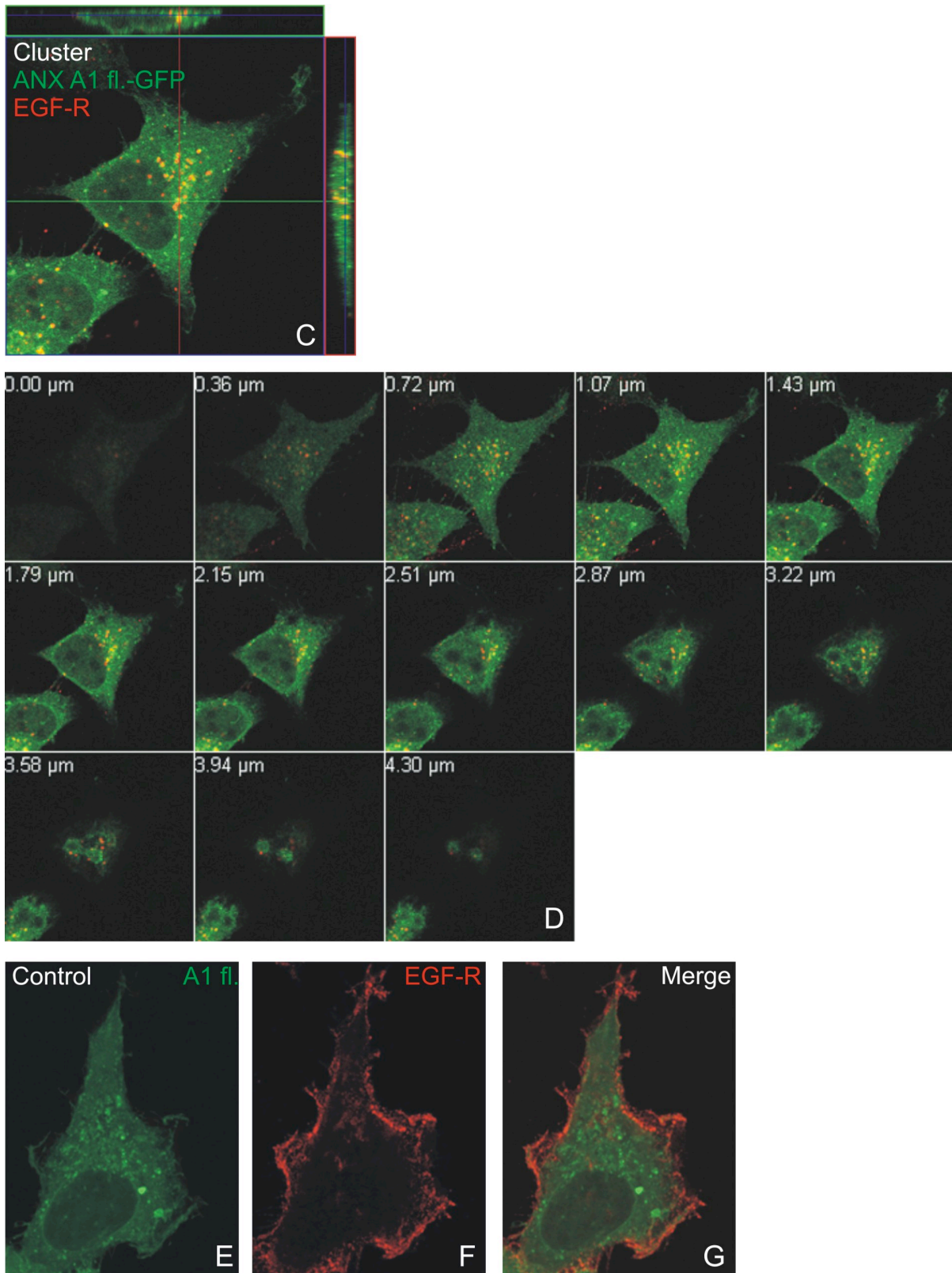
Figure 17 C-G: Presence of ANX A1 full-length-GFP in EGF-R clusters
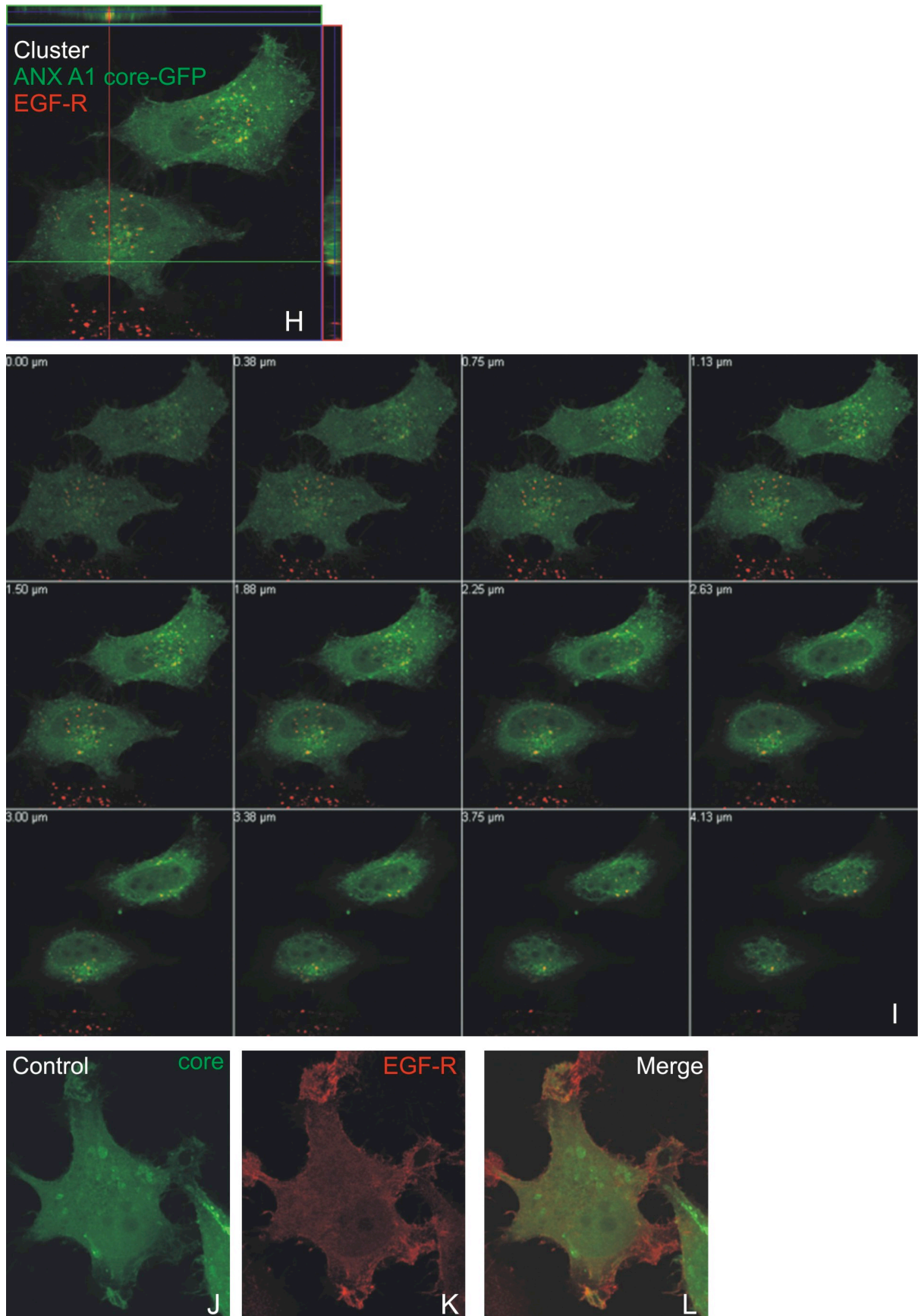
Figure 17 H-L: Presence of the ANX A1 core-GFP in EGF-R clusters
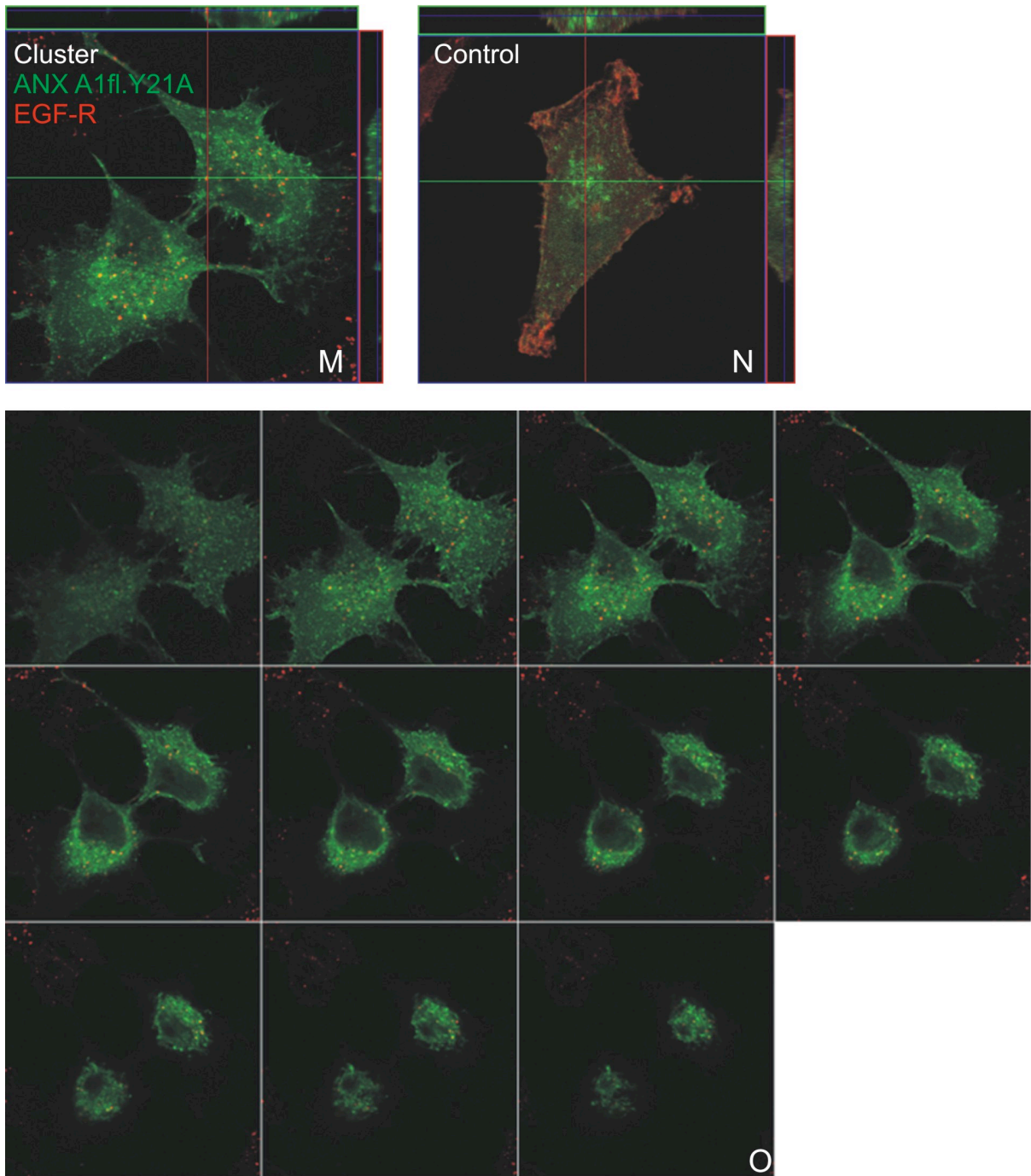
Figure 17 M-O: Presence of ANX A1 full-length Y21A-GFP in EGF-R clusters

Less numerous smaller and more cytosolic distributed clusters of the EGF-R were found in cells expressing ANX!A1 core-GFP without the N-terminal domain (fig.17H&I). Few co-localizations of the ANX A1 core with the clustered EGF-R were found. The double colored clusters seemed also to internalize into the cell. The control performed in the presence of core-GFP showed no EGF-R clustering (fig.17J-L).

The results obtained with the two ANX!A1 mutants differed from each other. Small EGF-R clusters were distributed evenly over the cell in ANX!A1!Y21A-GFP expressing cells (fig.17M). In contrast, when the ANX!A1!Y21E-GFP was expressed (fig.17P), the clusters seemed to be localized in a more perinuclear manner concentrated on one side of the nucleus similar to what was seen in cells expressing wildtype ANX!A1. In each case, co-localization of the GFP fusion proteins with EGF-R clusters was detected. However, the co-localization events appeared more often with the tyrosine(21)glutamate mutant. Especially in cells expressing the ANX!A1!Y21E-GFP, the GFP- and EGF-R containing clusters were also internalized inside the cells, visible in the image gallery (fig.17O&R) when at almost all levels the co-labeling effect was observed.

In order to examine if other members of the annexin family behaved in a similar manner to ANX A1 upon EGF-R clustering, HeLa wt cells were transfected with ANX!A3- and ANX!A6-GFP encoding plasmids (fig.17S-V). Both fusion proteins were expressed in the cytoplasm bound to vesicular structures that were distributed throughout the cell. ANX!A6 was, in addition, localized at the plasma membrane. Similar to ANX A1, ANX A6-positive vesicles were more concentrated in a cytosolic region close to the nucleus. Although the localization of ANX A1, A3 and A6 had seemed to be comparable so far, differences became visible when the EGF-R clustering method was applied. The concentration of the EGF-R resulted in small clusters comparable to the clusters observed in the cells transfected with the empty control vector. The clusters remained on the cell surface and neither ANX A3- nor ANX A6-GFP did co-localize with the EGF-R.

The final cluster experiment was performed with non-transfected cells and the localization of the endogenous ANX A1 was visualized using N-terminal peptide antibody (TDL) (fig.17W-Y). Following clustering of the EGF-R both the receptor and endogenous ANX A1 co-localized in perinuclear structures. The clusters had sizes similar to those seen in ANX A1-wt- and the ANX!A1!Y21E-GFP-expressing cells and they had most likely been internalized.
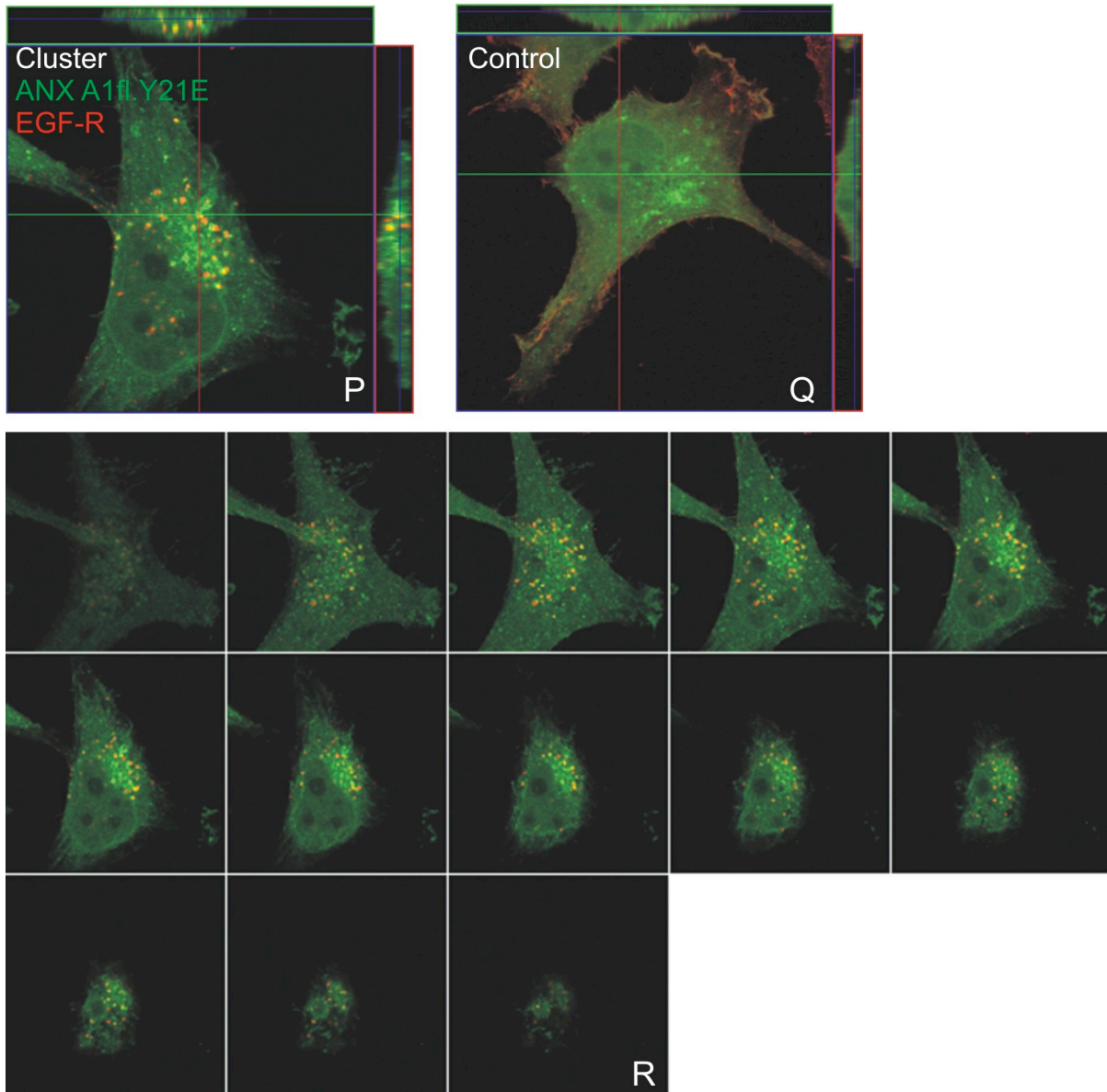
Figure 17 P-R: Presence of ANX A1 full-length Y21E-GFP in EGF-R clusters

Figure 17 S-T:
Presence of
ANX A3-GFP
in EGF-R clusters

Figure 17 U-V
Presence of
ANX A6-GFP
in EGF-R clusters

Cluster
ANX A1N-terminus
EGF-R

W

Control

X

Y
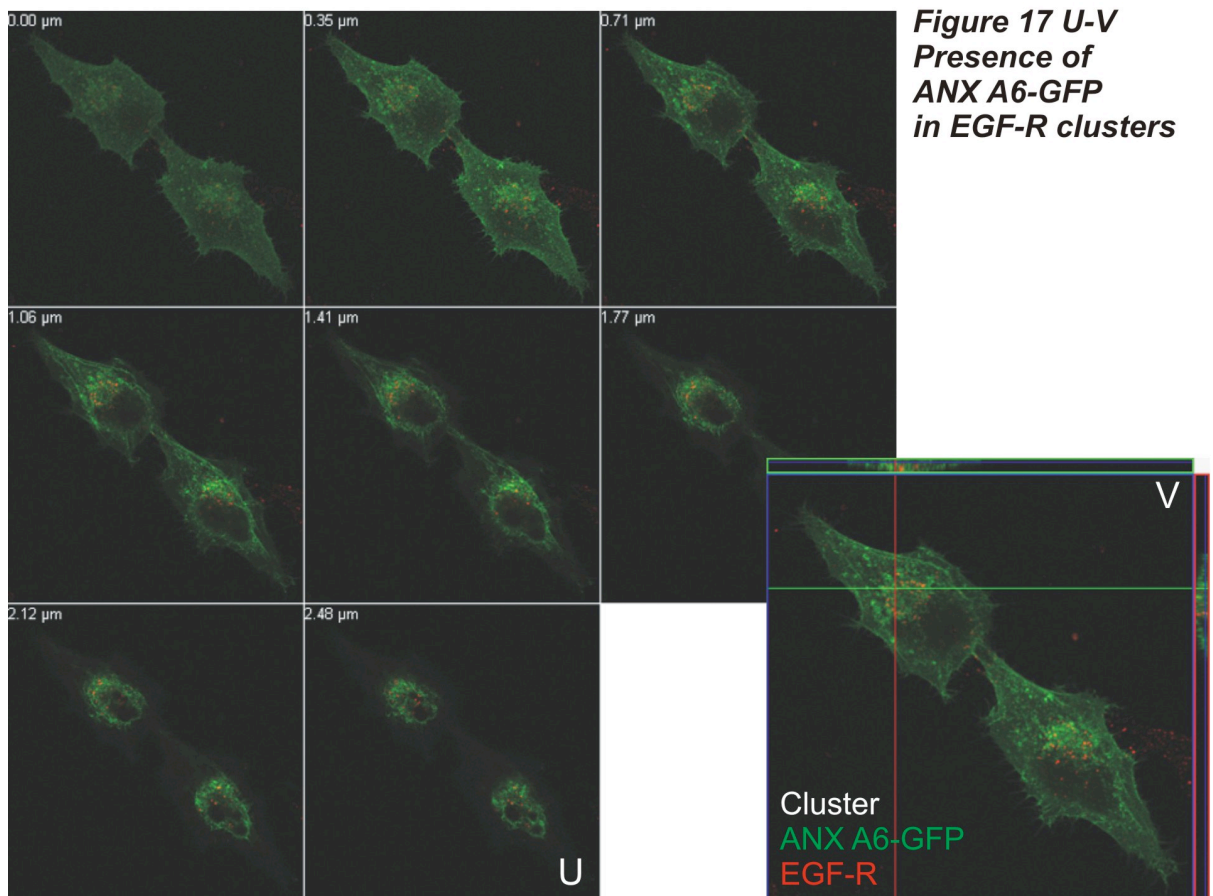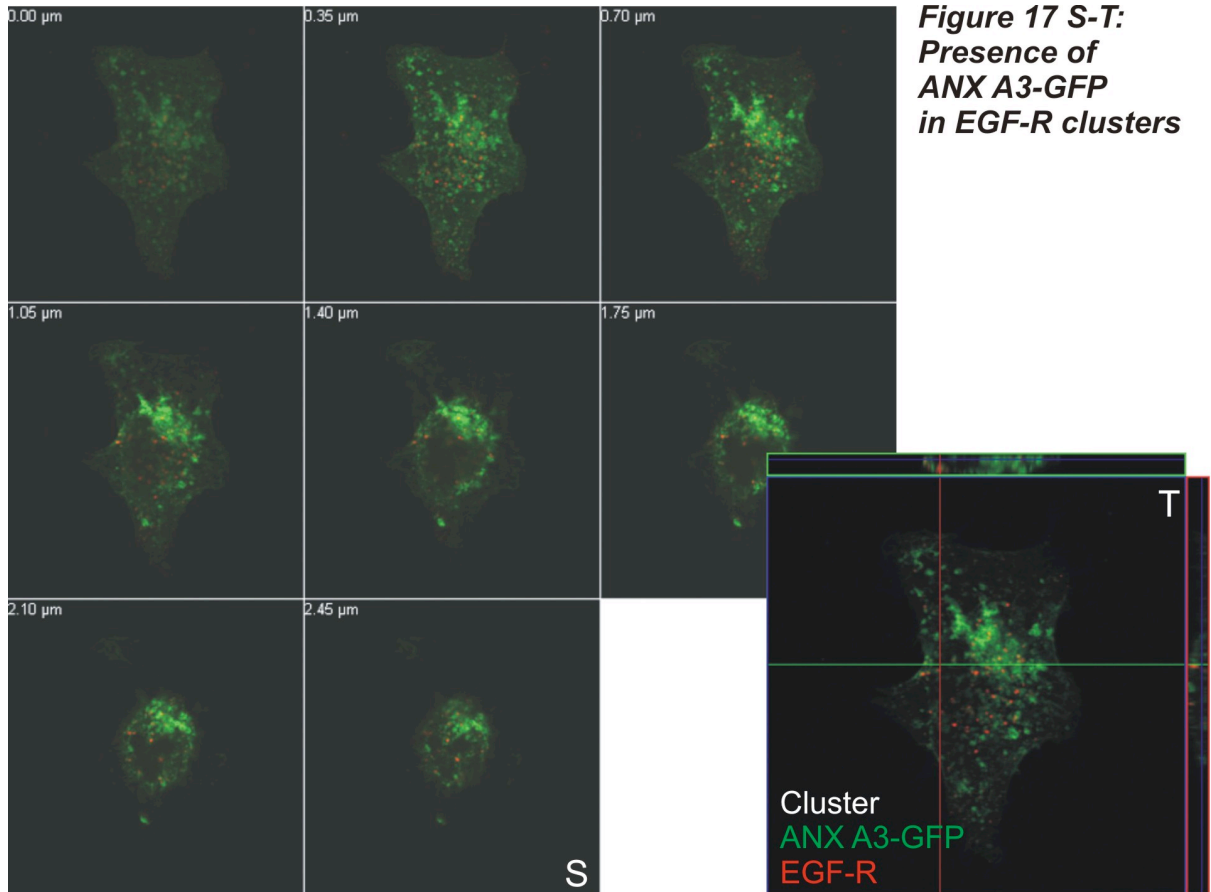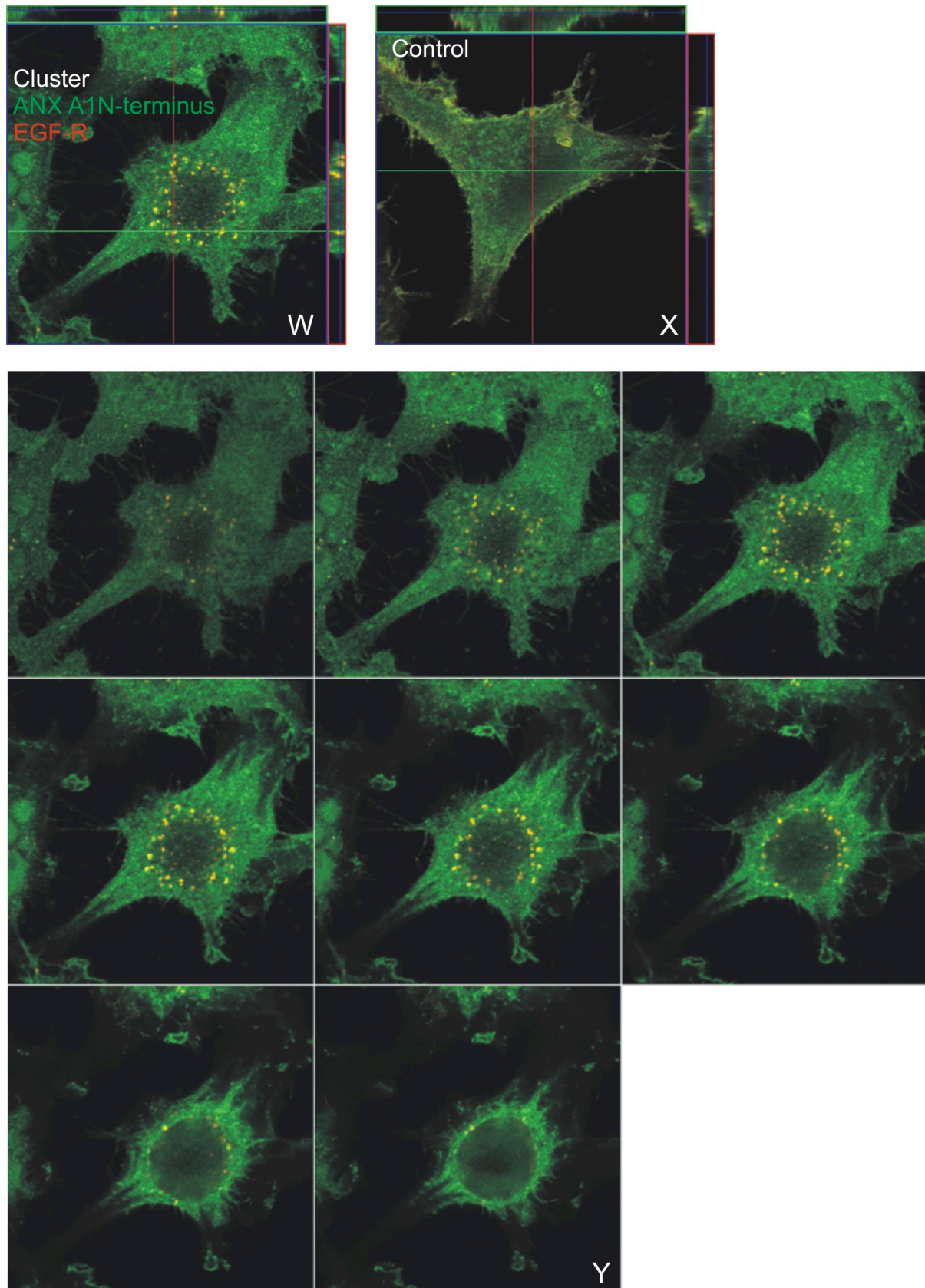
Figure 17 W-Y: Presence of the endogenous ANX A1 in EGF-R clusters

2.1.6 EGF dependent ANX A1 phosphorylation

Based on the observation that ANX A1 and the EGF-R localized to the same cellular compartment in EGF stimulated HeLa wt cells, the question as to whether and how ANX A1 is phosphorylated by the EGF receptor related to the internalization of the receptor was addressed next. For that reason, HeLa cells overexpressing the non-functional dynamin mutant were used in parallel to the wildtype cells.

2.1.6.1 Search for phosphorylated ANX A1 in total cell lysate

HeLa wt and HeLa K44Adyn- cells were serum starved over night and EGF stimulated for 1 to 60 min. Total cell lysate was prepared and the ANX A1 presence in all samples was verified by immunoblotting with the polyclonal ANX A1 antibody Sidney (fig.18A). The quantity of ANX A1 was equal at all stimulation times in cells with functional or non-functional clathrin dependent endocytosis machinery.

The same samples were used to compare EGF dependent protein tyrosine-phosphorylation in wt and K44Adyn- cells (fig.18B). Proteins at the apparent molecular mass of the EGF-R (175!kDa) were strongly phosphorylated after 1 min stimulation in both cell types. In the cells without functional dynamin dependent internalization machinery, the degree of p175 phosphorylation remained almost unchanged from 5 min until 60!min stimulation, whereas in the HeLa wt cell lysate, the decrease continued until 60!min EGF.

In the lower molecular weight range, at least three protein bands at 35, 38 and 45 kDa were detected that were phosphorylated to a lower degree, or not at all, in K44Adyn- cells. pp45 appeared after 15 EGF stimulation only in wt cells. p38 was phosphorylated already at the short stimulation time of 1 min and disappeared within 15 min. In contrast, the maximum of p35-phosphorylation was found at 15!min EGF. Afterwards, pp35 almost disappeared in the next 15 min.

A

WB anti ANX A1 full-length

HeLa wt cells / total lysate   HeLa K44Adyn- cells / total lysate

EGF   -   1   5'   15'   30'   60'   -   1'   5'   15'   30'   60'

B

WB anti phosphotyrosine

HeLa wt cells / total lysate   HeLa K44Adyn- cells / total lysate

EGF   -   1   5'   15'   30'   60'   -   1'   5'   15'   30'   60'
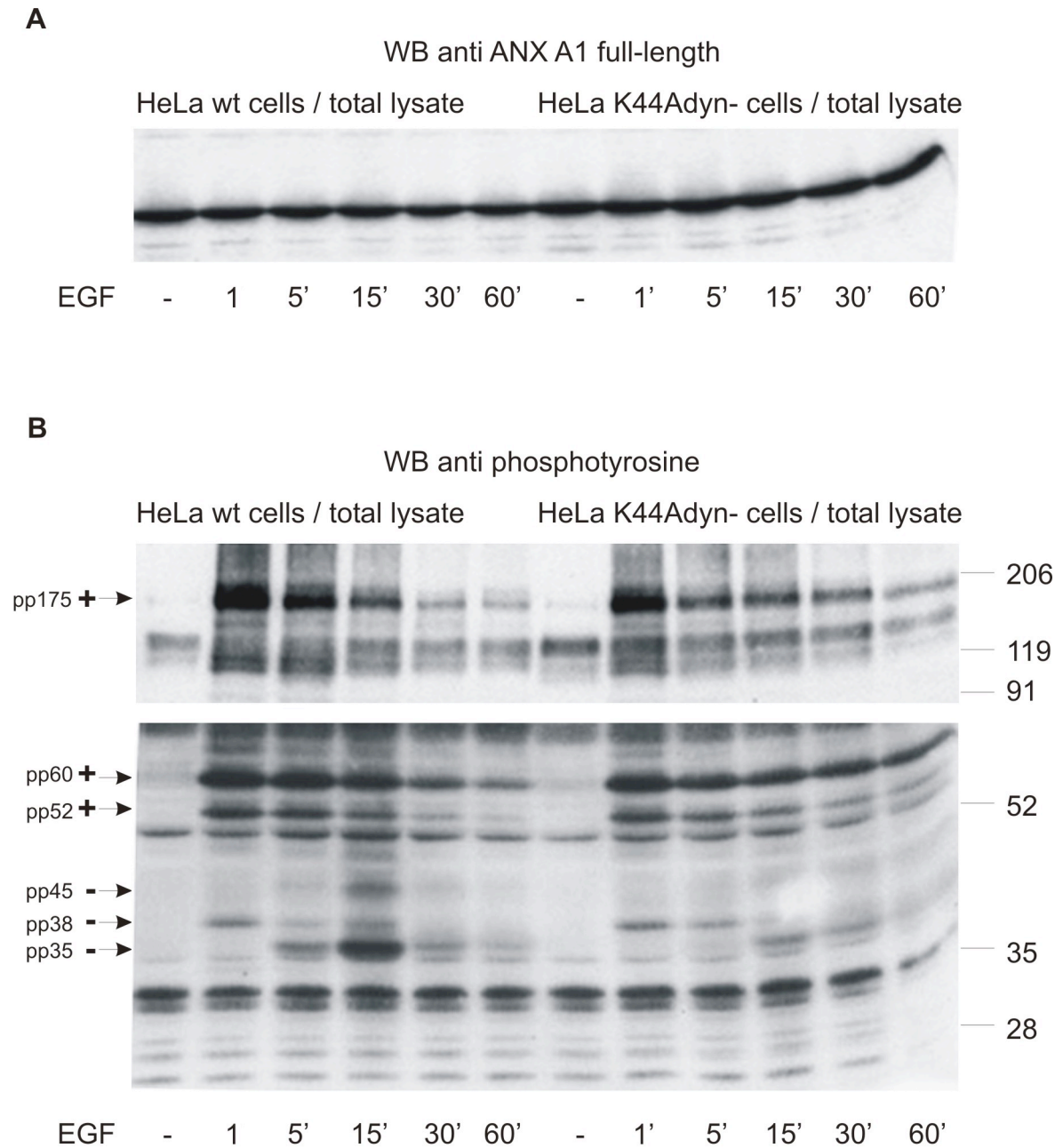
Figure 18: Presence of ANX A1 and protein phosphorylation in EGF stimulated HeLa wt and K44Adyn- cells

HeLa wt and K44Adyn- cells were EGF stimulated as indicated. Total cell lysate was prepared, the same amounts of protein were separated by SDS-PAGE and immunoblotting was performed using the ANX A1 antibody Sidney (A) and anti-phosphotyrosine antibody (B). Arrows mark proteins that were differently phosphorylated in K44Adyn- compared to wt cells.
(**+** means hyperphosphorylated or more stably phosphorylated proteins within the time course and **-** hypophosphorylated in dyn- cells compared to wt cells)

The question as to whether pp35 and/or pp38 included ANX A1 was further addressed by performing two-dimensional electrophoresis with isoelectric focusing (IEF) in the first dimension. Total cell lysates of non-stimulated HeLa wt and K44Adyn- cells or of cells stimulated for 15 min with EGF were prepared with high urea buffer and subjected to two-dimenstional gel separation (chapter 5.4.7). The separated phosphorylated proteins (phosphoproteins) were analyzed by immunoblotting with the anti-phosphotyrosine antibody (fig.19). The same membranes used for the phosphotyrosine blots were stripped and the presence of ANX A1 and the number of its isoforms were elucidated by anti-ANX A1 blotting.

Signals of phosphoproteins were detected in the lysates of unstimulated HeLa wt and also K44Adyn- cells (fig.19A&C) mainly in the area between pH 6 and 8. After 15!min EGF stimulation, phosphoproteins appeared at more acidic pH 4-6 than without EGF stimulation (spots 1-5). More phosphoproteins were also detected in the neutral range. The molecular weights of most of the detected phosphoproteins were between 45 and 83!kDa. Few EGF-dependently phosphorylated proteins were found in the range of 30 to 40!kDa (fig.19B&D).

ANX A1 was detected in all cell lysates, wt or K44Adyn-, stimulated or unstimulated cells (fig.19E-H). However, in wt cells the number of ANX!A1 isoforms was higher than in K44Adyn- cells. The main ANX A1 signals in lysate of non-stimulated cells of both cell types was found between pH 6 and 7 (fig.19E&G). The signals in the acidic pH range became more intense after 15!min EGF stimulation of HeLa wt but not of K44Adyn- cells. The most acidic ANX A1-spot was apparent at pH 4.

The experiment was repeated after treating the cells with an inhbitior of the protease calpain II so as to study its effect on protein phosphorylation and on the isoelectric behavior of ANX A1 isoforms (calpain is known to cleave the ANX A1 N-terminus) (Fig.20). Fewer phosphoproteins were detected in lysates of stimulated or unstimulated HeLa wt and K44Adyn- cells when the calpain inhibitor was applied (fig.20A-D). The presence of ANX A1 isoforms was unchanged in unstimulated cells of both cell types and in stimulated HeLa K44Adyn- cells (fig.20 E, G, H). The appearance of numerous ANX A1 isoforms after EGF stimulation was less evident in calpain inhibitor treated HeLa wt cells. The ANX A1 signal at pH 4 was hardly detected (fig.20F).
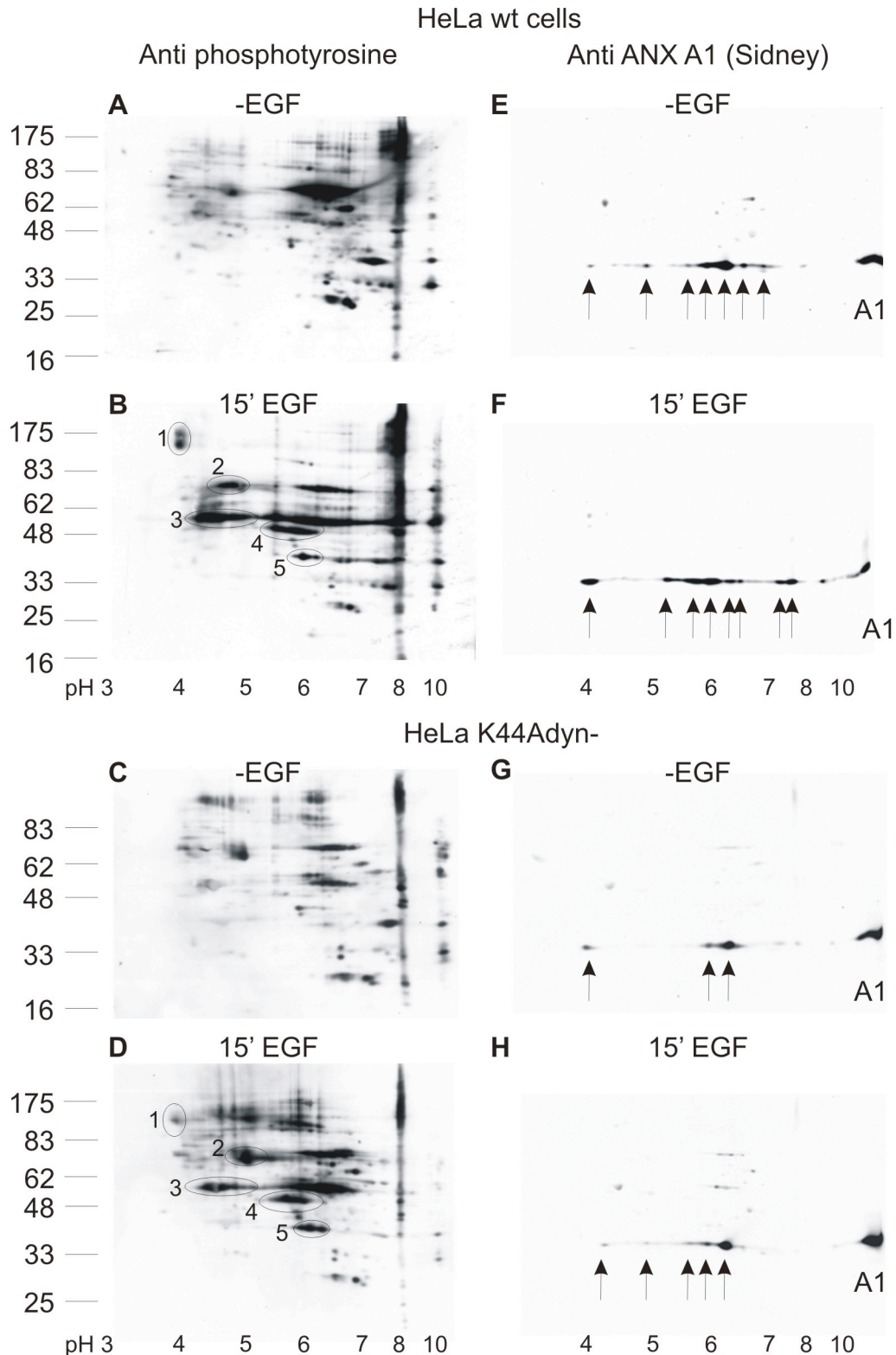
Figure 19: Immunoelectric focusing (IEF) of phosphorylated proteins and ANX A1 in total cell lysate from EGF stimulated HeLa wt and K44Adyn- cells

HeLa wt (A, B, E, F) and HeLa K44Adyn- cells (C, D, G, H) were serum starved over night before EGF stimulated for 15 min. Total cell lysates were prepared and two dimensional electrophoresis was performed followed by immunoblotting. Membranes were decorated with phosphotyrosine (A-D) or ANX A1 antibodies (E-H).
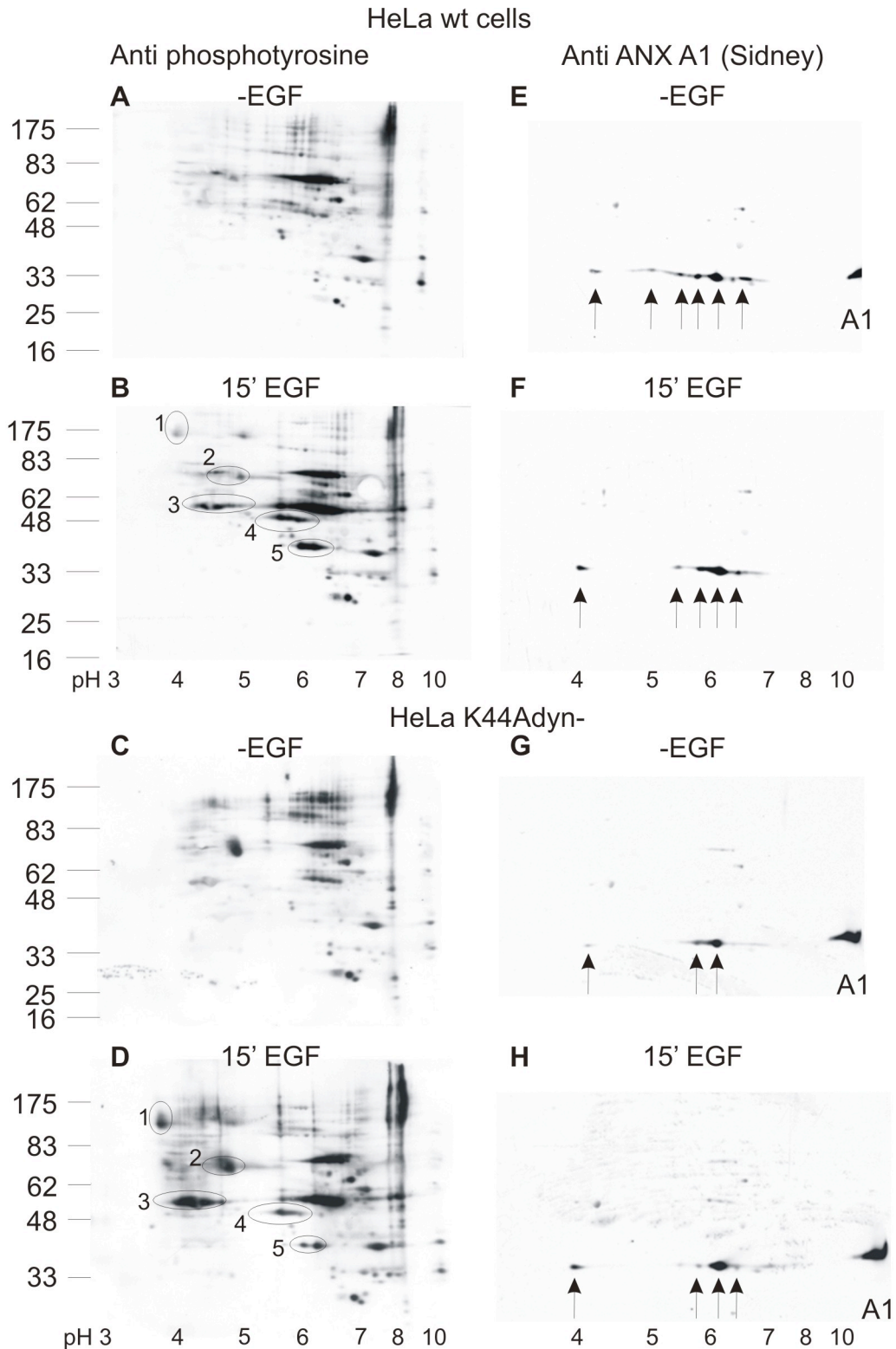
Figure 20: The effect of calpain inhibitor on phosphorylated proteins and ANX A1 in total cell lysate from EGF stimulated HeLa wt and K44Adyn- cells

HeLa wt (A, B, E, F) and HeLa K44Adyn- cells (C, D, G, H) were serum starved over night. Previously to the EGF stimulation, the cells were incubated with a calpain II inhibitor. Total cell lysates were prepared and IEF was performed followed by immunoblotting. The membranes were decorated with phospho-tyrosine (A-D) or ANX A1 antibody (E-H).

IP: ANX A1 (DAC 5) - WB: Anti phosphotyrosine

A HeLa wt   B wt + AG1478   C K44Adyn-

pp175

pp70

pp40

pp37

175
83
62
48
33
25

EGF - 1' 15' 30' 60'   - 1' 15' 30' 60'   - 1' 15' 30' 60'

IP: ANX A1 (DAC 5) - WB: Anti ANX A1 (DAC5)

D HeLa wt   E wt + AG1478   F K44Adyn-

A1

62
48
33
25

EGF - 1' 15' 30' 60'   - 1' 15' 30' 60'   - 1' 15' 30' 60'
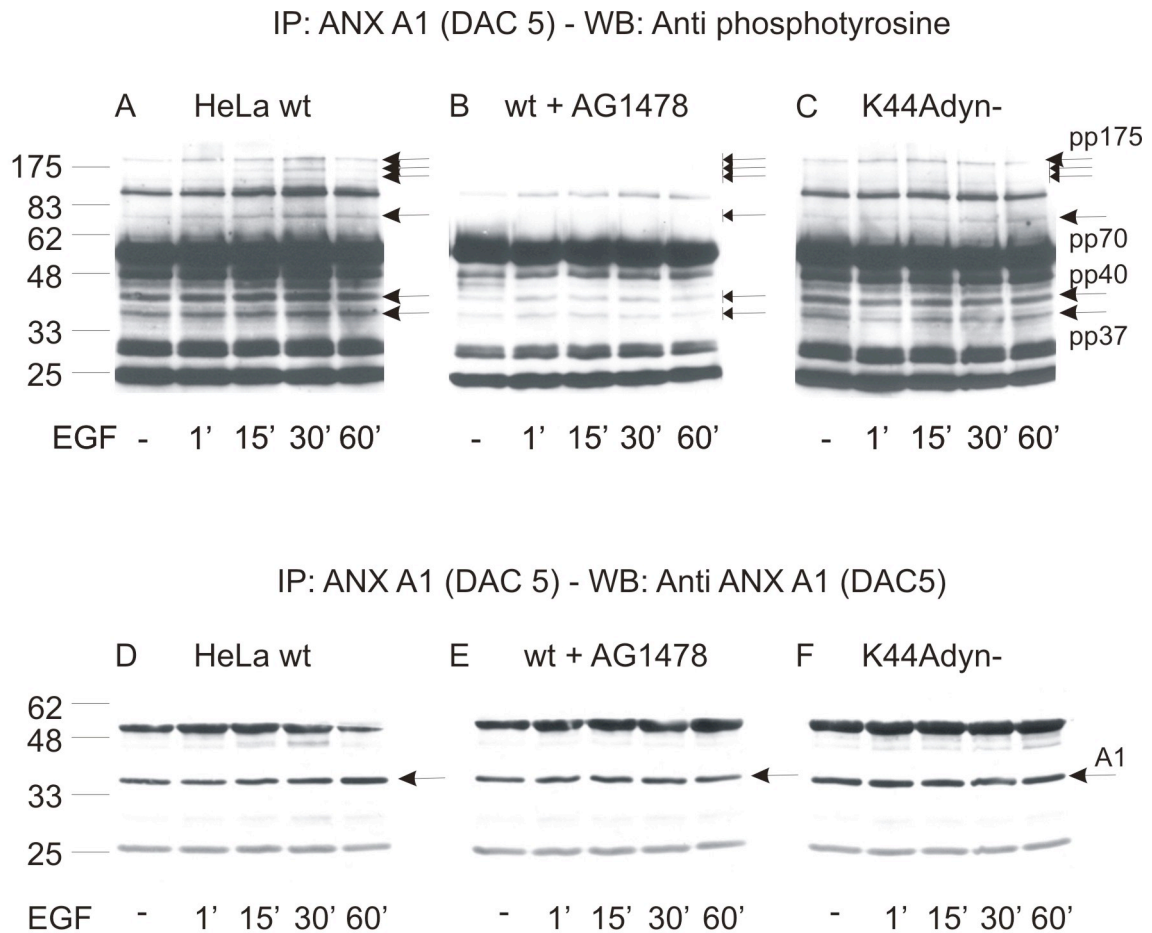
Figure 21: ANX A1 phosphorylation in EGF stimulated HeLa cells

HeLa wt (A and D) and HeLa K44Adyn- cells (C and F) were serum starved over night before EGF stimulated for 1 to 60 min. In addition, HeLa wt cells were pre-incubated with 15 μM of the EGF-R kinase inhibitor AG1478 for 15 min (B and E).

Total cell lysates were prepared and immunoprecipitation using the monoclonal ANX A1 antibody Dac5 was performed. The membranes were either decorated with phosphotyrosine antibodies (A-C) or with Dac5 (D-F).

2.1.6.2 Interaction of ANX A1 with phosphorylated proteins

ANX A1 phosphorylation and its interaction with proteins phosphorylated upon EGF stimulation were studied in HeLa wt and K44Adyn- cells following an immunoprecipitation strategy. Parallel to this, HeLa wt cells were treated with the irreversible EGF-R kinase inhibitor (AG 1478) for 15 min before EGF stimulation began.

ANX A1 was precipitated with the monoclonal ANX A1 antibody Dac5 (Fig.21). The phosphorylation status of ANX A1 and of co-precipitated proteins was analyzed by immunoblotting using anti-phosphotyrosine antibodies (fig.21A-C). In the precipitates made of Hela wt and K44Adyn- cells, four phosphoproteins at 37, 40, 70 and 175!kDa were detected that were not or less intensely phosphorylated in the samples of cells treated with AG1478. The phosphorylation of p70 and p175 was more intense following EGF stimulation, maximum at 30 min, whereas, the signal intensities of the phosphorylated p37 and p140 remained stable during the time period and phosphorylation was already seen in non-stimulated cells. With an inferior molecular weight (MW) to p175, bands of phosphorylated proteins appeared only in inhibitor untreated HeLa wt cells but not in cells with non-functional clathrin-dependent endocytosis machinery.

The immunoblots were also decorated with the antibody Dac5 used for the ANX A1-precipitation (fig.21D-F). Equal amounts of ANX A1 were precipitated at all times in both cell types with or without EGF-R kinase inhibitor. Superposition of the phosphotyrosine- and ANX!A1-blots showed a possible match of pp37 and the ANX A1-protein band revealed by Dac5.
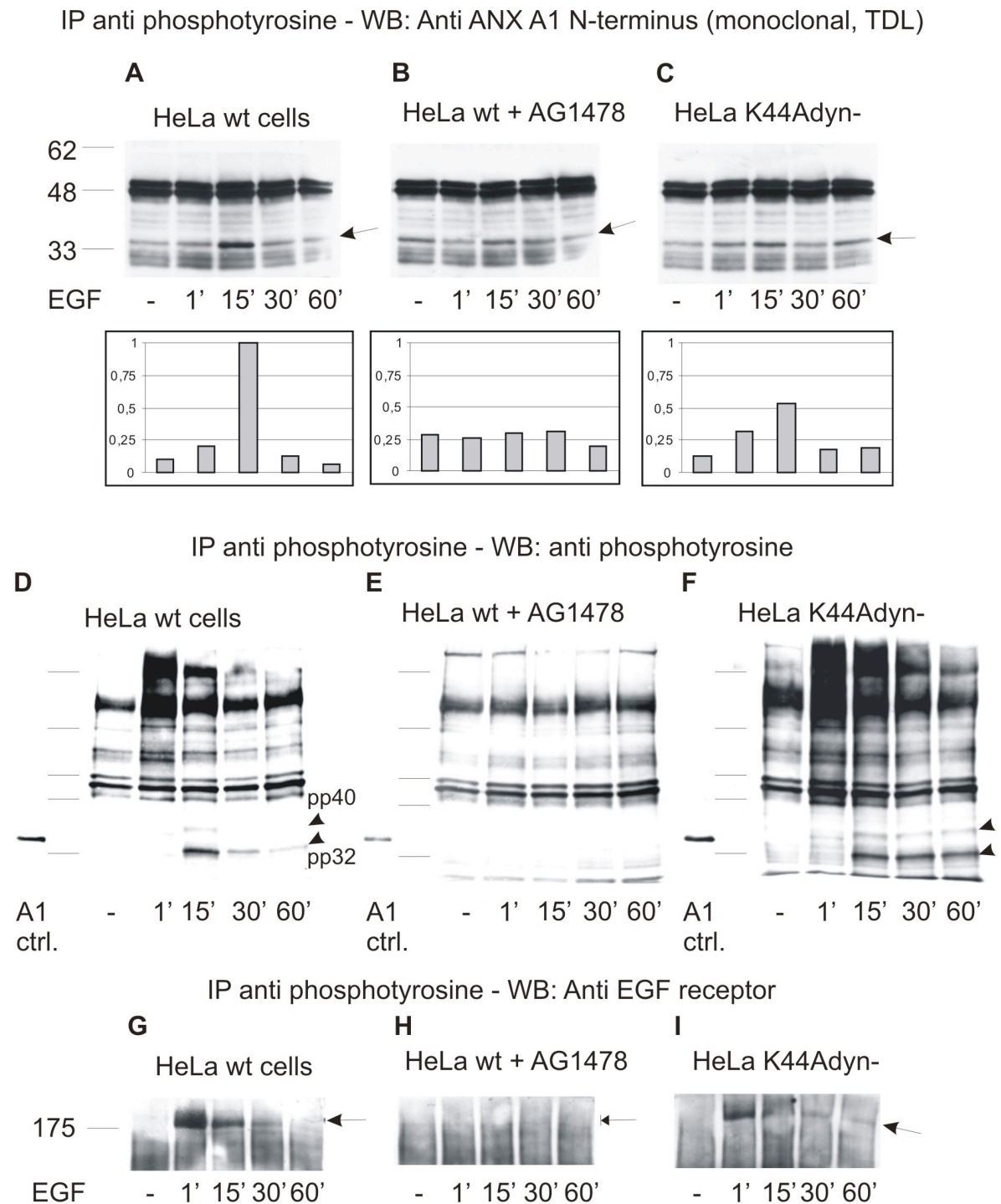
Figure 22: Phosphorylation of ANX A1 and the EGF-R in EGF stimulated HeLa cells

HeLa wt (A, D and G) and HeLa K44Adyn- cells (C, F and I) were serum starved over night before EGF stimulated for 1 to 60 min. In addition, HeLa wt cells were pre-incubated with 15 μM the EGF-R kinase inhibitor AG1478 for 15 min (B,E and H). Total cell lysates were prepared and immunoprecipitation using phosphotyrosine anti-body was performed. The membranes were either decorated with ANX A1 (A-C), phosphotyrosine (D-F) or with EGF-R antibodies (G-H).The phosphoprotein bands marked by arrows in A-C were quantified and represented in the diagramms below. (A1 ctrl. - purified ANX A1 obtained from A. Wilbers, ZMBE Muenster)

For the following immunoprecipitation experiment, the use of the antibodies was inversed. The phosphoproteins were precipitated applying phosphotyrosine antibodies and the immunoblots were decorated either with the monoclonal antibody directed against the ANX A1 N-terminal domain (TDL), anti-phosphotyrosine or EGF-R antibodies (fig.22). TDL immunoblots showed a protein band at the MW of ANX A1 in all samples (fig.22A-C). In the blot of the EGF-R inhibitor-treated Hela wt cells, the intensitiy of this band was relatively stable as shown in the diagram below that represents the quantification of the ANX!A1-band (fig.22B). In contrast, the result obtained using HeLa wt cells showed that more ANX A1 was precipitated with the phosphotyrosine antibody at 15 min EGF stimulation than at shorter or longer stimulation periods (fig.22A). This effect was weaker in K44Adyn- cells but here the highest intensity of the ANX A1-band was also reached at 15 min (fig.22C).

The anti-phosphotyrosine antibodies detected phosphorylated proteins between 30 and 50!kDa only in AG1478-untreated cells (fig.22D-F). Two phosphoprotein bands were visible after 15!min EGF stimulation at a MW of 33 kDa and 40!kDa, which was smaller or larger compared to the ANX A1 control. The intensity of these bands remained stable in K44Adyn- cells, whereas the phosphorylation degree of both bands decreased between 15 and 60!min in HeLa wt cells.

The EGF-R antibody was detected only in the samples of inhibitor-untreated cells proteins at the MW of the EGF-R that precipitated with the phosphotyrosine antibody (fig.22G-I). The highest signal intensity was detected at 1!min EGF stimulation and decreased in wt and K44Adyn- cells within the kinetic. At 60!min there was still a weak signal detectable in the K44Adyn-, but not in the HeLa wt cells.

A IP: ANX A1 (Sidney) - WB: Anti phosphotyrosine

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	
																pp175
175																
83																
62																

EGF   -   -   -   1'   1'   1'   15'   15'   15'   30'   30'   30'   60'   60'   60'

AG1478   +   +   +   +   +

tet   on   on   off   on   on   off   on   on   off   on   on   off   on   on   off

IP: ANX A1 (Sidney) - WB: Anti EGF receptor

B   HeLa wt   Wt + AG1478   K44Adyn-

175							
83							

EGF   -   1'   15'   30'   60'   -   1'   15'   30'   60'   -   1'   15'   30'   60'
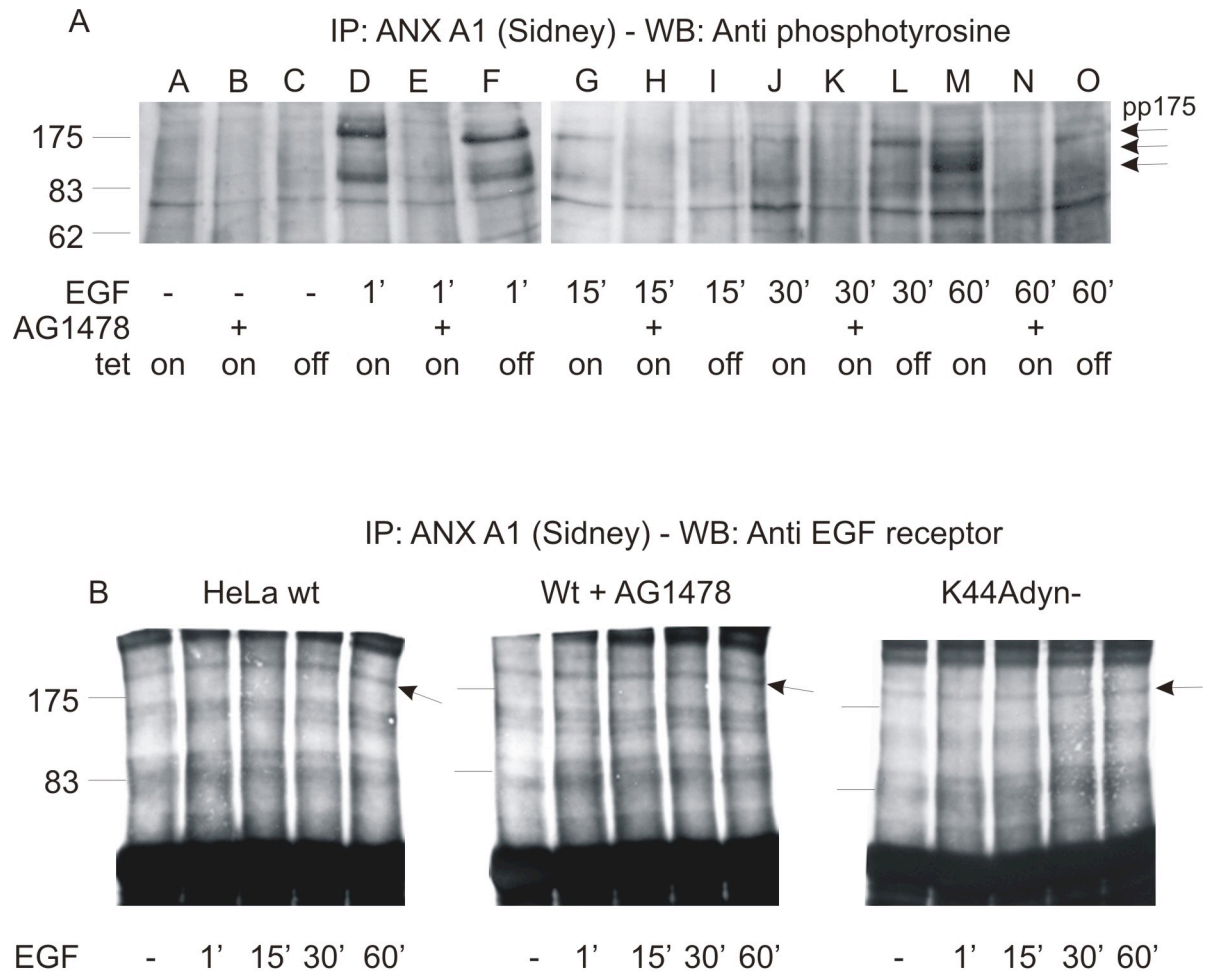
Figure 23: ANX A1 interaction with the phosphorylated EGF receptor

HeLa wt and HeLa K44Adyn- cells were serum starved over night before EGF stimulation for 1 to 60 min. In addition, HeLa wt cells were preincubated with the EGF-R kinase inhibitor AG1478 for 15 min.
Total cell lysates were prepared and immunoprecipitation using the polyclonal antibody Sidney was performed. The membranes were either decorated with phosphotyrosine (A) or with EGF-R antibody (B).

2.1.7 Interaction of endogenous ANX A1 with the EGF-R

Interactions between ANX!A1 and the EGF-R were analyzed by immunoprecipitation, applying either the polyclonal ANX!A1 antibody Sidney (Fig.23), or the polyclonal EGF-R antibody directed against the intracellular domain of the receptor (Fig.24). As for the experiments before, HeLa wt cells pre-incubated or not with the EGF-R kinase inhibitor AG1478 and HeLa cells overexpressing non-functional dynamin were also used.

Immunoprecipitation with Sidney and immunoblotting with phosphotyrosine antibodies did not identify tyrosine phosphorylated proteins in the MW range of the EGF-R when the cells were not EGF stimulated (fig.23A, lanes A-C). When HeLa wt and K44Adyn- cells were stimulated for 1!min, a phosphoprotein band at 175 kDa was detected, which was evident when AG1478 was applied (lanes D-F). After 1!min EGF, the level of p175 phosphorylation decreased so that at 15, 30 and 60!min EGF, the phosphorylation signals were hardly detectable in HeLa wt cells (lanes G, J&M). In HeLa K44Adyn- cells, the pp175 signal seemed to remain more stable and was still detectable after 30 and 60!min EGF stimulation (lanes L&O).

However, at 60!min EGF stimulation of HeLa wt cells, bands of phosphorylated proteins with a MW inferior to pp175 were found that were not detected in HeLa K44Adyn- cells (lane M&O). In the AG1478-treated Hela wt cells, a phosphorylation of p175 was not detected at any time (lanes B, E, H, K, N).

When the EGF-R antibody was applied to analyze if the EGF-R was present in the protein complex that co-precipitated with ANX A1, weak signals of a protein band at the MW of the EGF-R were found in all samples of stimulated or non-stimulated cells (fig.24B).

Next the EGF-R antibody was applied for immunoprecipitation experiments and the receptor phosphorylation status was studied in HeLa!wt and K44Adyn-!cells following EGF stimulation of 1, 5, 15, 30 and 60 min (fig.24A). After 1!min EGF stimulation, the EGF-R was highly phosphorylated in both cell types. In cells with defective clathrin-dependent endocytosis, the phosphorylation degree decreased between 1 and 5 min EGF but remained stable until the end of the kinetic. In contrast to this, the dephosphorylation of the EGF-R seemed to be slower but continuously from 5 to 60 min EGF in Hela wt cells. At 30 and 60!min stimulation time, bands of phosphorylated proteins with MW inferior to that of the EGF-R appeared that had not been detected in K44Adyn- cells.
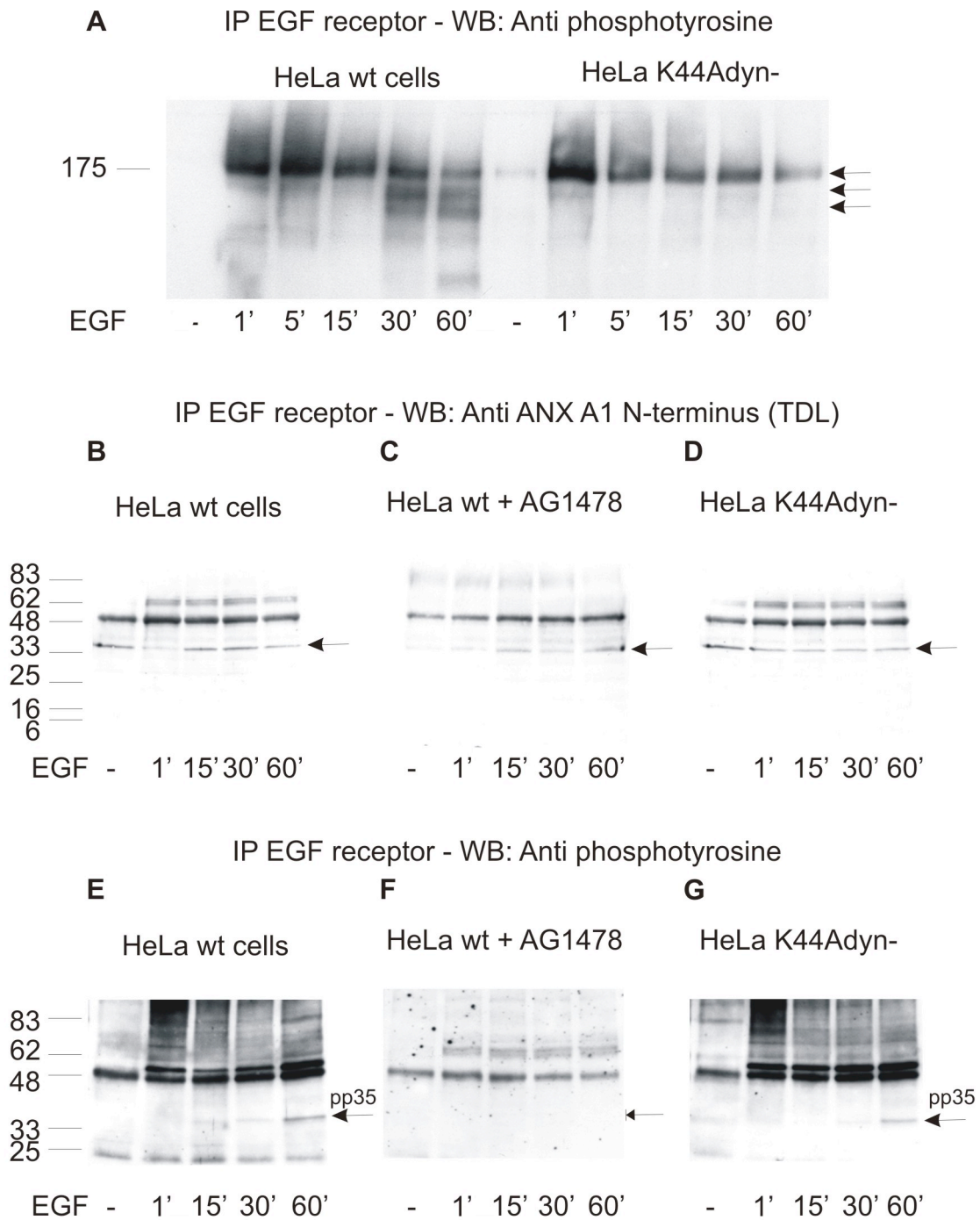
Figure 24: EGF-R phosphorylation and interaction with ANX A1 in EGF stimulated HeLa and HeLa K44Adyn- cells

HeLa wt (A, B, E) and HeLa K44Adyn- cells (A, D,G) were serum starved over night before EGF stimulated for 1 to 60 min. In addition, HeLa wt cells were preincubated with the EGF-R kinase inhibitor Ag1478 for 15 min (C and F).
Total cell lysates were prepared and immunoprecipitation using EGF-R antibody was performed. Precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose. The membranes were either decorated with phosphotyrosine antibody (A, E-G) or with the monoclonal ANX A1 N-terminal peptide antibody from Transduction Laboratories (B-D).

Applying in parallel the EGF-R kinase inhibitor AG1478, ANX A1 co-precipitated with the EGF-R was analyzed with the monoclonal ANX!A1 antibody TDL (fig.24B-D). ANX A1 had already been detected in samples of unstimulated wt cells that had not been treated with AG1478 and also in unstimulated K44Adyn- cells. After 1!min EGF stimulation, the ANX A1-signal seemed to disappear temporarily in wt cells. At 15 min stimulation, ANX A1 seemed to reappear and remained relatively stable during the next 45 min of EGF treatment (fig.24B). In AG1478 pre-incubated HeLa wt cells, protein bands at the MW of ANX A1 were hardly detected (fig.24C), whereas the signal intensity of the ANX!A1-protein band remained almost stable between 1 and 60 min of EGF stimulation in K44Adyn- cells.

The same samples were used for immunoblotting with phosphotyrosine antibodies (fig.24E-G). In wt and K44Adyn- cells, phosphoproteins superior to a MW of 50!kDa were found, especially at 1!min EGF stimulation, which had not been detected when pre-incubated with AG1478. In HeLa wt cells, a weak phosphoprotein band at 35 kDa (pp35) appeared at 15!min EGF and increased in signal intensity up to 60!min stimulation period (fig.24E). pp35 was also observed in K44Adyn- cells but with weaker signal intensity than in wt cells (fig.24G). No phosphoproteins at all were detected in AG1478-treated cells between 25 and 48!kDa (fig.24F).

So as to analyze if the EGF-R interacting phosphoprotein pp35 represented phosphorylated ANX!A1, RNA interference was applied using specific double-strand RNA oligos (siRNA), corresponding to nt642 to nt662 (counting the translational start site as nt1) of the ANX!A1-RNA. Resulting from this, the ANX A1-synthesis should be specifically down-regulated by this technique. HeLa cells transfected with siRNA or the control RNA-oligomer (invsiRNA) were used for the EGF-R immuno-precipitation experiments as described above. Total cell lysate of serum-starved and 30 and 60!min EGF stimulated cells were incubated with the EGF-R antibody and immunoblotted with phosphotyrosine antibody (fig.25). The down-regulation of the ANX!A1-expression in siRNA transfected cells was controlled by immunoblotting using the monoclonal ANX!A1 antibody Dac5 (fig.25A). The downregulation amounted to more than 50!% in siRNA *versus* invsiRNA transfected cells.

The immunoblot of phosphorylated proteins precipitated with the EGF-R antibody revealed highly tyrosine phosphorylated proteins at 175!kDa and less intense phophoprotein bands at 35!kDa in both cell types (fig.25B). pp35 was not detected in the samples of unstimulated cells but in those of stimulated cells, regardless or not if siRNA- or invsiRNA-transfected.
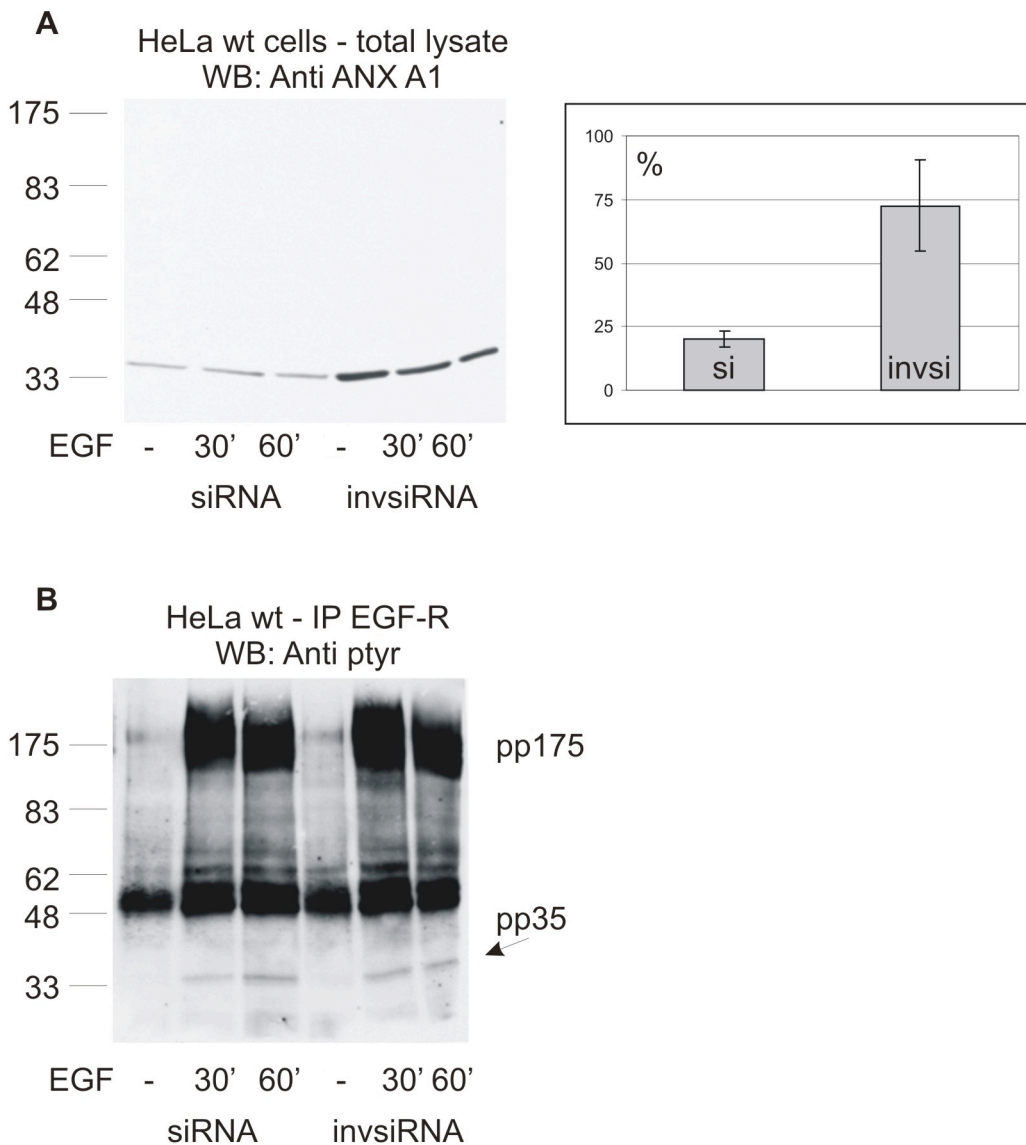
Figure 25: Effect of ANX A1-downregulation on the interaction of the EGF-R with phosphorylated proteins

HeLa wt cells were transfected with a ANX A1 silencing RNA oligomer (siRNA) or with a RNA oligomer of the inverse siRNA sequence (invsiRNA) as control.
The presence of ANX A1 in total cell lysate was analyzed 48 hrs after transfection by immunoblotting with the monoclonal ANX A1 antibody Dac5 (A). The same lysates were used for immunoprecipitation of the EGF-R (B). The precipitated proteins were separated by SDS-PAGE and immunoblotting using phosphotyrosine antibodies was performed.

2.1.8 Interaction of the EGF-R with wt ANX A1, ANX A1 core and ANX A1 mutants

The relevance of the ANX A1 N-terminal domain and the putative EGF-R phosphorylation site tyrosine 21 for the ANX A1/EGF-R-interaction was analyzed next, using the four ANX A1-GFP constructs applied previously for immunofluorescence studies: ANX!A1!full-length-GFP, core-GFP, ANX A1(Y21A)-GFP, ANX A1(Y21E)-GFP. HeLa wt cells were transfected with the ANX A1-GFP constructs and in parallel with the empty vector pEGFP.

The expression of the transfected plasmids was controlled by immunoblotting using a GFP antibody and the polyclonal ANX A1 antibody r656 (fig.R26A&B). Both antibodies detected in the lysates of cells transfected with plasmids encoding full-length ANX A1-GFP, a protein band at around 65 kDa and in cells transfected with the plasmid encoding ANX A1 core-GFP, a smaller protein of approximately 62!kDa. No protein was detected at these molecular weights in cells that were transfected with the empty vector. The molecular masses of 65 and 62 kDa correspond to the calculated masses of the fusion proteins ($MW_{ANX\ A1}$= 37!kDa, $MW_{N\text{-terminal peptide}}$= 2.5!kDa and MW_{GFP}= 25!kDa). The fusion proteins were synthesized at equal levels.

The total cell lysates of the transfected cells were used for EGF-R-immunoprecipitation. The precipitated proteins were separated and transferred to nitrocellulose membrane. The upper part of the membrane was incubated with the same EGF-R antibody that had been used for the immunoprecipitation. The result showed that equal amounts of the EGF-R were precipitated by the technique applied (fig.26C). The lower part of the membrane was decorated with the ANX A1 antibody r656 (fig.26D) revealing that all GFP fusion proteins, truncated or full-length, wt or with mutated tyrosine 21, were co-precipitated with the EGF-R.
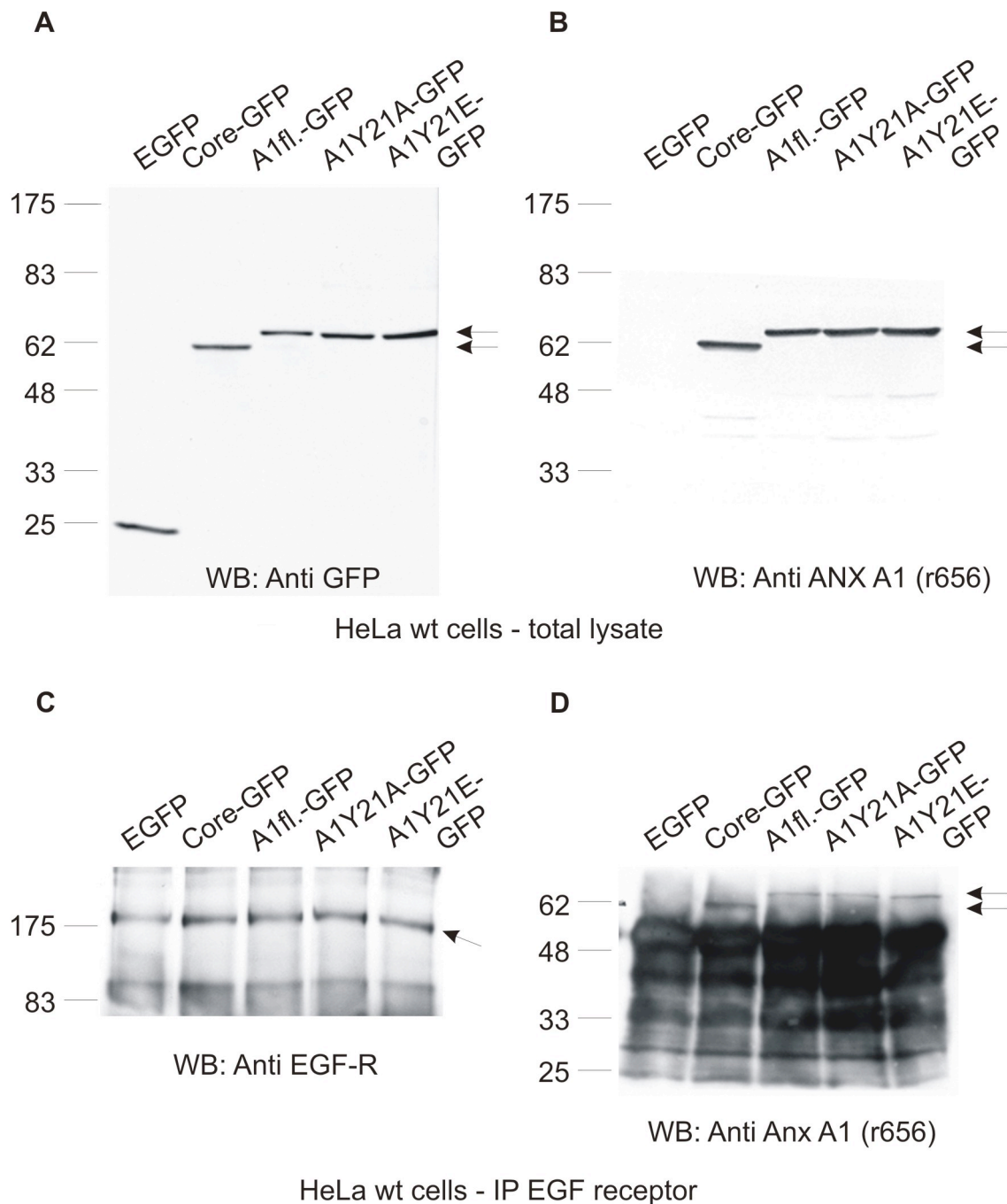
Figure 26: Interaction of the EGF-R with ANX A1-GFP fusion proteins

HeLa wt cells were transfected with four different ANX A1-GFP constructs,
panx a1 full-length-GFP, pcore-GFP, panx a1 full-lengthY21A-GFP, panx a1 full-
lengthY21E-GFP and with the empty vector as control. Total cell lysates were
prepared and analyzed with a GFP antibody (A) or the ANX A1 antibody r656 (B).
The same lysates were used for immunoprecipitation of the EGF-R.
The precipitated proteins were applied to immunoblotting was performed. The
membranes were decorated with EGF-R (C) or ANX A1 (D) antibodies.

2.2 Internalization, transport and deactivation of the EGF receptor in the ANX A1 knock out cell line JaCro!ANX!A1-/-

2.2.1 Expression of ANX A1, A2, A6 and the EGF receptor in the JaCro cell line

In order to study the synthesis of ANX A1, that of two other members of the annexin family, ANX!A2 and A6, and of the EGF receptor (EGF-R) in JaCro cells, total cell lysate of wt and ANX!A1-/- cells were prepared. The same amounts of total protein were applied to SDS-PAGE and immunoblotting. The polyclonal ANX A1 antibodies DE99603 and r656 were used, which were also characterized for HeLa cells (chapter 2.1.1).

No protein was detected in the JaCro ANX!A1-/- cell lysate at the molecular weight (MW) of ANX A1, by either antibody. In JaCro wt cell lysate, both antibodies revealed a protein band at around 37!kDa (fig.27A&B). The monoclonal ANX!A2 antibody detected a protein band inferior to that of the MW of ANX!A1 in both cell types. The intensity of the signal was lower in the ANX A1-/- lane than in the wt lane (fig.27C). The protein bands detected by the ANX!A6 antibody were equal in signal intensity in both cell types. The principal protein band was found at a MW of around 65!kDa (fig.27D). The polyclonal EGF-R antibody revealed a protein band at 175!kDa in wt and ANX!A1-/- cell lysate with lower signal intensity in the ANX!A1-/- lane (fig.27E).

Purified ANX!A1 antibodies were applied for immunofluorescence studies in JaCro cells. In JaCro!ANX!A1-/- cells, the staining of both antibodies revealing the general background was diffuse cytoplasmic and nuclear (Fig.28A&B). In wt cells, the ANX A1 full-length antibody revealed punctuate structures in the cytoplasm (fig.28C). In contrast to this diffuse staining, the labeling of the ANX!A1 N-terminal peptide antibody was very distinct at granular structures more concentrated in the perinuclear region. No nuclear or plasma membrane staining was visible (fig.28D).

A Anti ANX A1 N-terminal domain (DE99603)

B Anti ANX A1 full-length (r656)

C Anti ANX A2

D Anti ANX A6

E Anti EGF-R
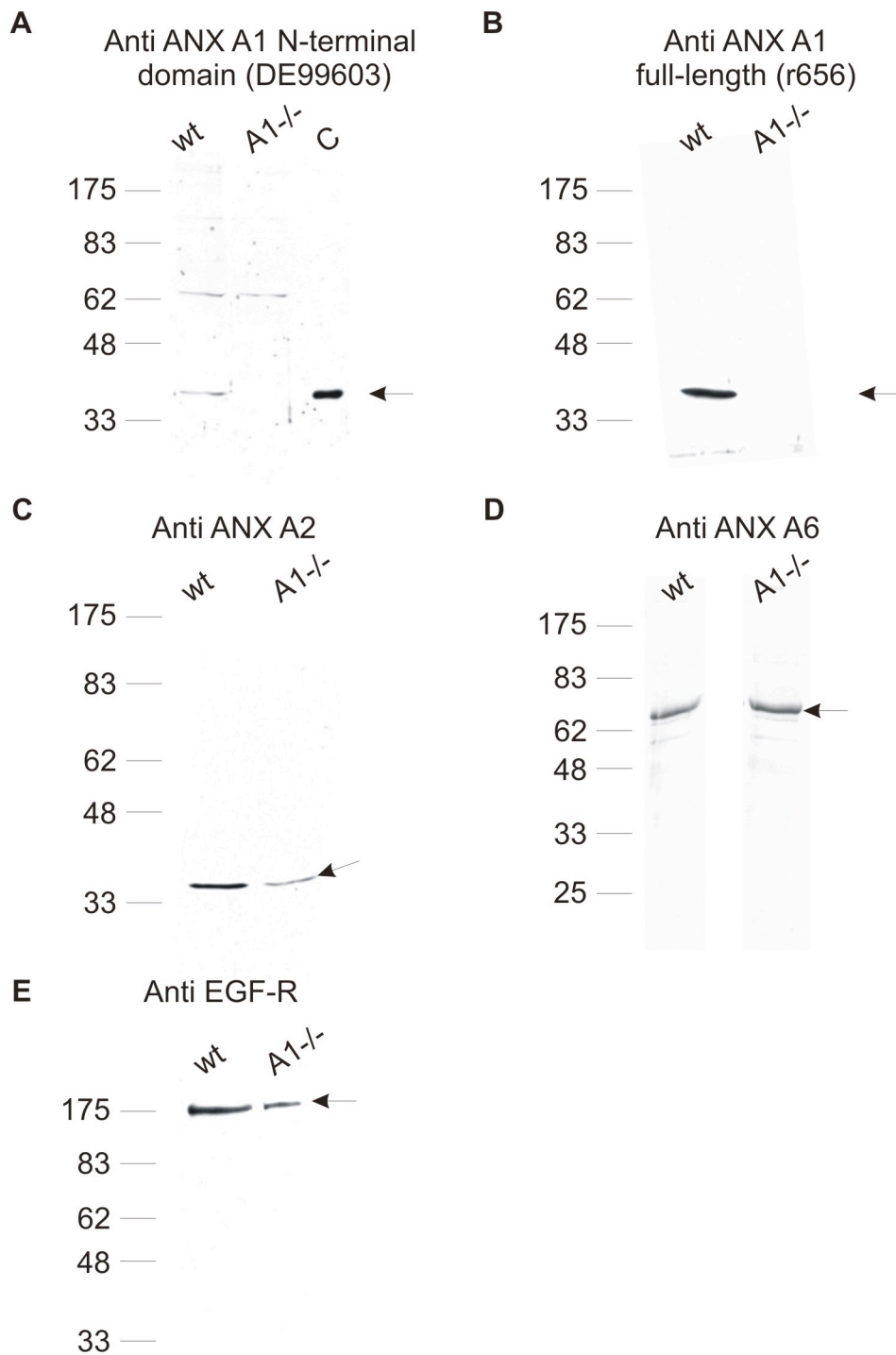
Figure 27: ANX A1, ANX A2, ANX A6 and EGF-R expression in JaCro cells

Total cell lysates were prepared from JaCro wild type and ANX A1-/- cells. The same amounts of protein were separated by SDS-PAGE and transfered to nitrocellulose membrane. Two different ANX A1 antibodies directed against the N-terminal peptide, DE99603 (A) or against the full-length protein, r656 (B), antibodies directed against ANX A2 (C), ANX A6 (D) and the EGF receptor (E) were used to compare the presence of these proteins in both cell lines. The ANX A2 antibody was monoclonal. For the immunoblots presented here, unpurified polyclonal serum were used to detect ANX A1 and ANX A6.

Anti ANX A1 full-length
(r656)

Anti ANX A1 N-terminal domain
(DE99603)
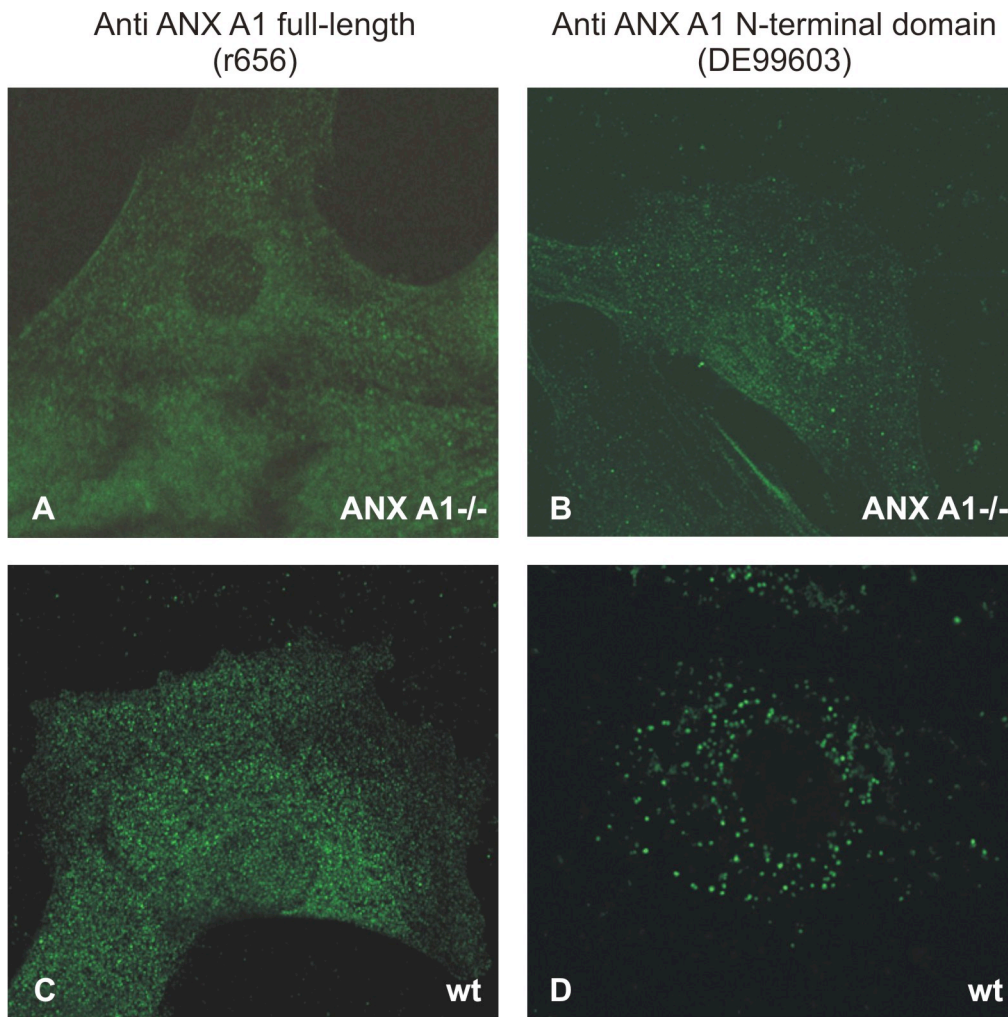
Figure 28: ANX A1 localization in JaCro wt cells

JaCro ANX A1-/- cells (A, B) and JaCro wild type cells (C, D) were fixed
using PFA, permeabilized with saponin and incubated with two different
ANX A1 antibodies, the ANX A1 full-length antibody, r656 (A, C) and the
ANX A1 N-terminal peptide antibody, DE99603 (B, D).
The serum obtained after immunization with the N-terminal peptide
or full-length ANX A1 protein were purified by specific antigen affinity
chromatography or protein A affinity chromatography respectively.

2.2.2 Co-localization of ANX A1 and late endosomal structures in JaCro wt cells

The ANX!A1 N-terminal peptide antibody (DE99603) and anti lysobisphosphatidic acid
(LBPA) antibody were used to analyze a potential co-localization in JaCro wt cells (fig.29).
The same method was applied to JaCro ANX!A1-/- cells as a control. The use of DE99603
resulted in diffuse staining in Jacro ANX!A1-/- cells and in a distinct labeling of cytoplasmic

vesicles in JaCro wt cells as described in the previous chapter (fig.29A&D). The LBPA antibody labeled in both cell lines endosomal structures that were concentrated in the perinuclear region but distributed throughout the cytoplasm (fig.29B&E). In JaCro wt cells, some of the LBPA-positive endosomes co-localized with ANX A1-positive vesicles (fig.29C). This co-localization was not observed in JaCro ANX!A1-/- cells (fig.29F).

JaCro wt

| Anti ANX A1 N-terminal peptide (DE99603) | Anti LBPA | Merge |

JaCro ANX A1-/-

| Anti ANX A1 N-terminal peptide (DE99603) | Anti LBPA | Merge |
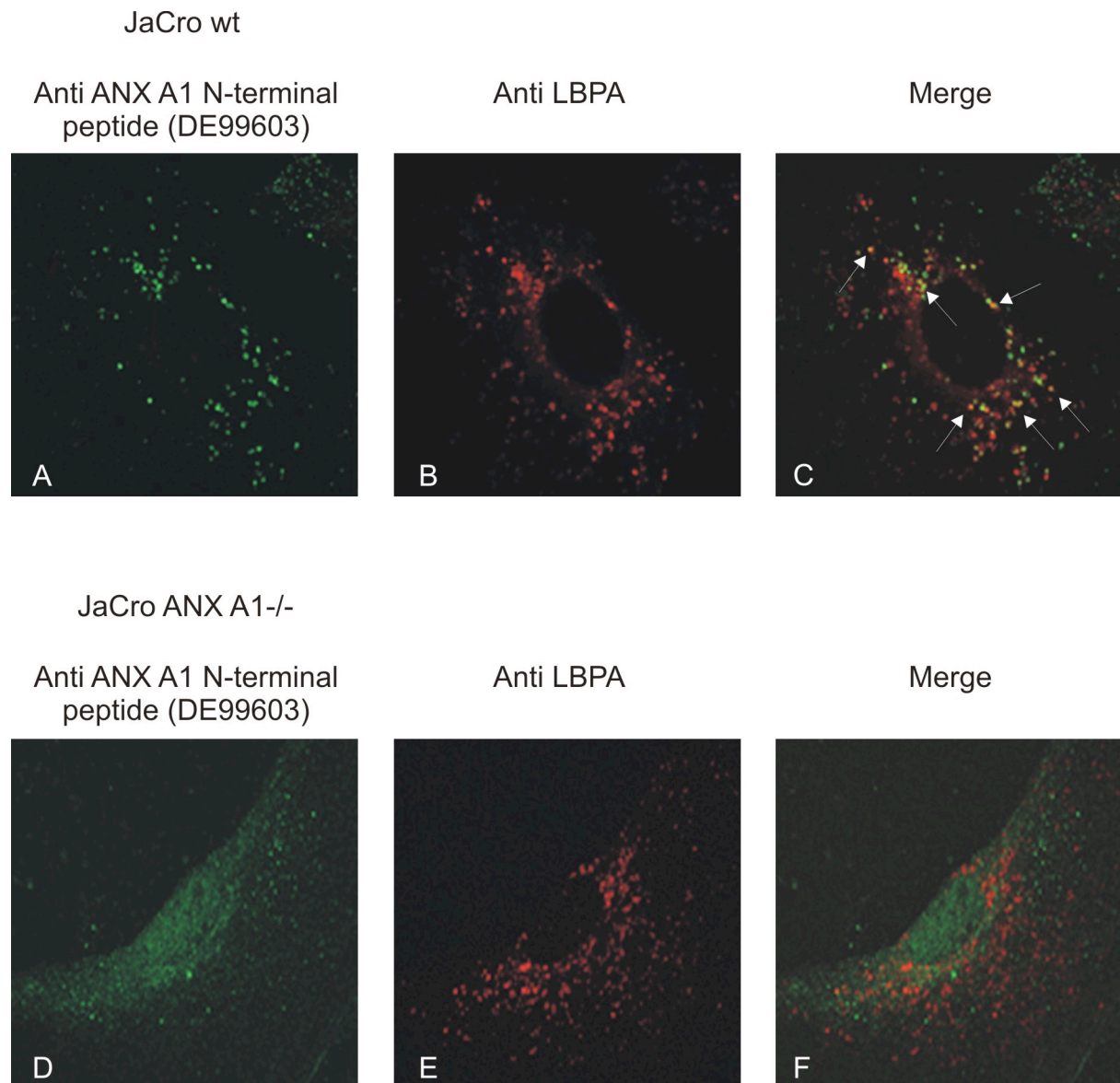
Figure 29: Co-labeling of ANX A1 and late endosomes in JaCro wt cells

PFA fixed wild type cells (A) and JaCro ANX A1-/- (B) were used for immuno-fluorescence studies applying the antibodies anti ANX A1 N-terminal peptide (DE99603) and the anti lysobisphosphatidic acid antibody (LBPA) as marker for late endosomes.

2.2.3 EGF receptor degradation, dephosphorylation and deubiquitination in JaCro cells

The aim of the following experiment was to compare the degradation, dephosphorylation and deubiquitination process of the EGF-R in the presence or absence of ANX!A1 in EGF stimulated JaCro cells.

The cells were EGF stimulated for 1 to 120 min, total cell lysates were prepared and the same amounts of protein were applied to SDS-PAGE and immunoblotting. Anti EGF-R, phosphotyrosine and ubiquitin antibodies were used to analyze the presence of the EGF-R and its phosphorylation and ubiquitination status (fig.30).

As observed previously, the p175-band stained with the EGF-R antibody was less intense in the lane corresponding to the cell lysate of unstimulated Jacro ANX!A1-/- cells compared to unstimulated JaCro wt cells (fig.29A). Within the time period of the EGF stimulation, the signal intensity of the protein band at 175!kDa corresponding to the EGF-R (p175) started to decrease between 1 and 30!min. At 120!min EGF stimulation, p175 was hardly detected in both cell types. The signal of one or several tyrosine phosphorylated proteins at the molecular weight (MW) of the EGF-R (pp175) appeared at 1!min EGF stimulation in JaCro!wt and ANX!A1-/- cells (fig.30B). The phosphorylation intensity decreased after 15!min EGF and continued decreasing up to 2!hrs EGF stimulation but still, a slight pp175-signal was detectable in Jacro wt and ANX A1-/- cells.

The ubiquitination status of proteins at 175!kDa (p175ubi) during EGF-R activation was analyzed next (fig.30C). The highest intensity of the p175ubi-signal was observed at 1!min EGF. Afterwards, the signal decreased within 120 min, ending in a hardly detectable protein band in both cell types. Signal intensity and decrease were not affected by ANX!A1 gene ablation.

The results obtained in this study were quantified by a luminescence imager (Lumi-Imager F1™, Boehringer Mannheim). The ubiquitination study was repeated three times; EGF-R and the phosphotyrosine immunoblotting were performed five times. The mean values and the standard deviations were calculated and plotted in diagrams (Fig.31). In all three cases – revealing the degradation (fig.31A), dephosphorylation (fig.31B) and deubiquitination of p175 (fig.31C) – the curves behaved in an exponential manner. The curves relating to the ANX A1 non-expressing cells (red crosses) were almost identical to the results of the wild type cells (blue squares).
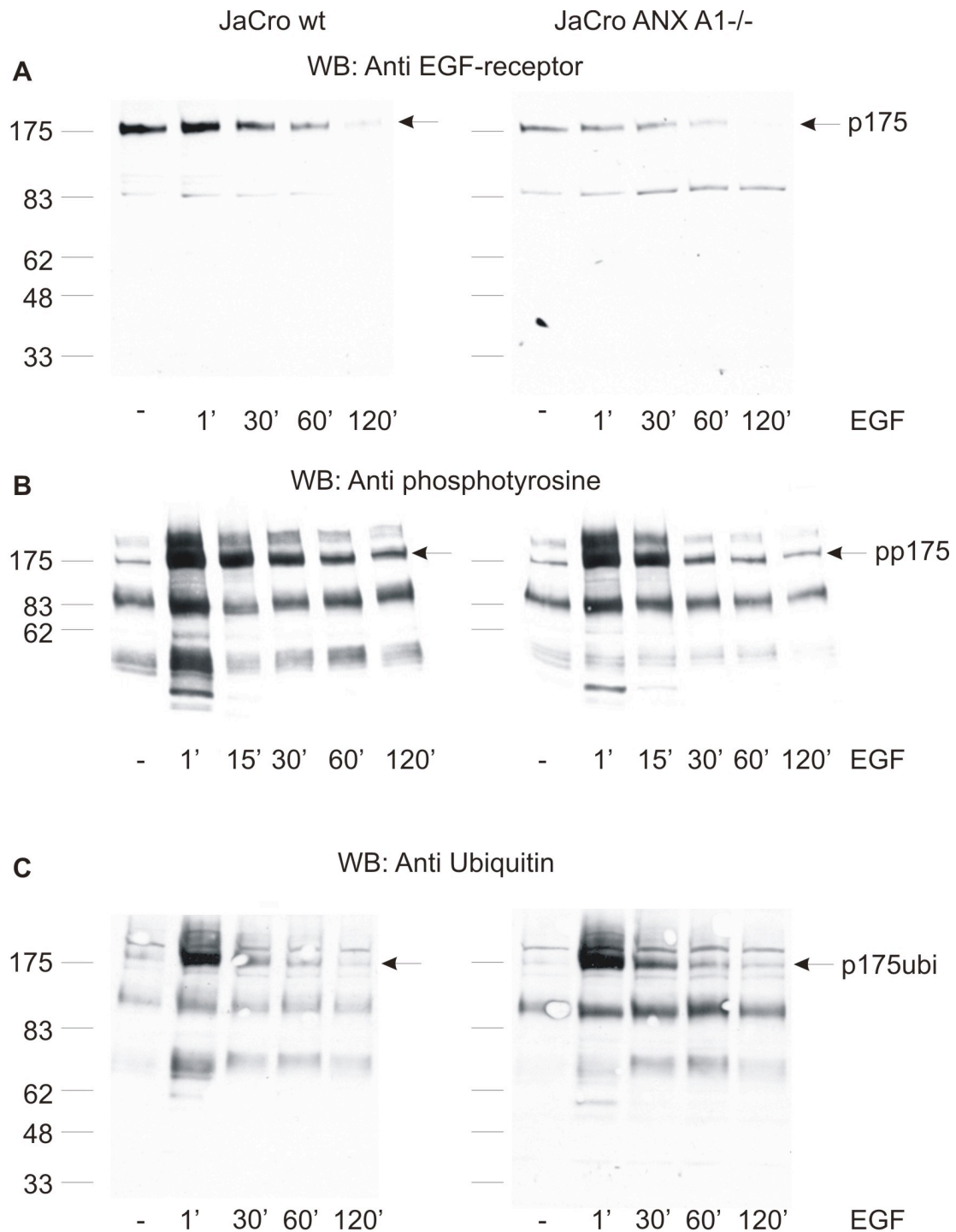
Figure 30: Degradation, dephosphorylation and deubiquitination of p175 in JaCro ANX A1-/- vs wt cells

JaCro wild type cells and JaCro ANX A1-/- cells were EGF stimulated for the indicated times and total protein lysates were prepared. The same amounts of protein were separated by SDS-PAGE and transfered to nitro-cellulose membrane. To analyze the degradation, dephosphorylation and deubiquitination of proteins with the molecular weight of the EGF-R during EGF-R activation, EGF-R (A), phosphotyrosine (B) and ubiquitin (C) antibodies were used for immunoblotting.

A

B

C
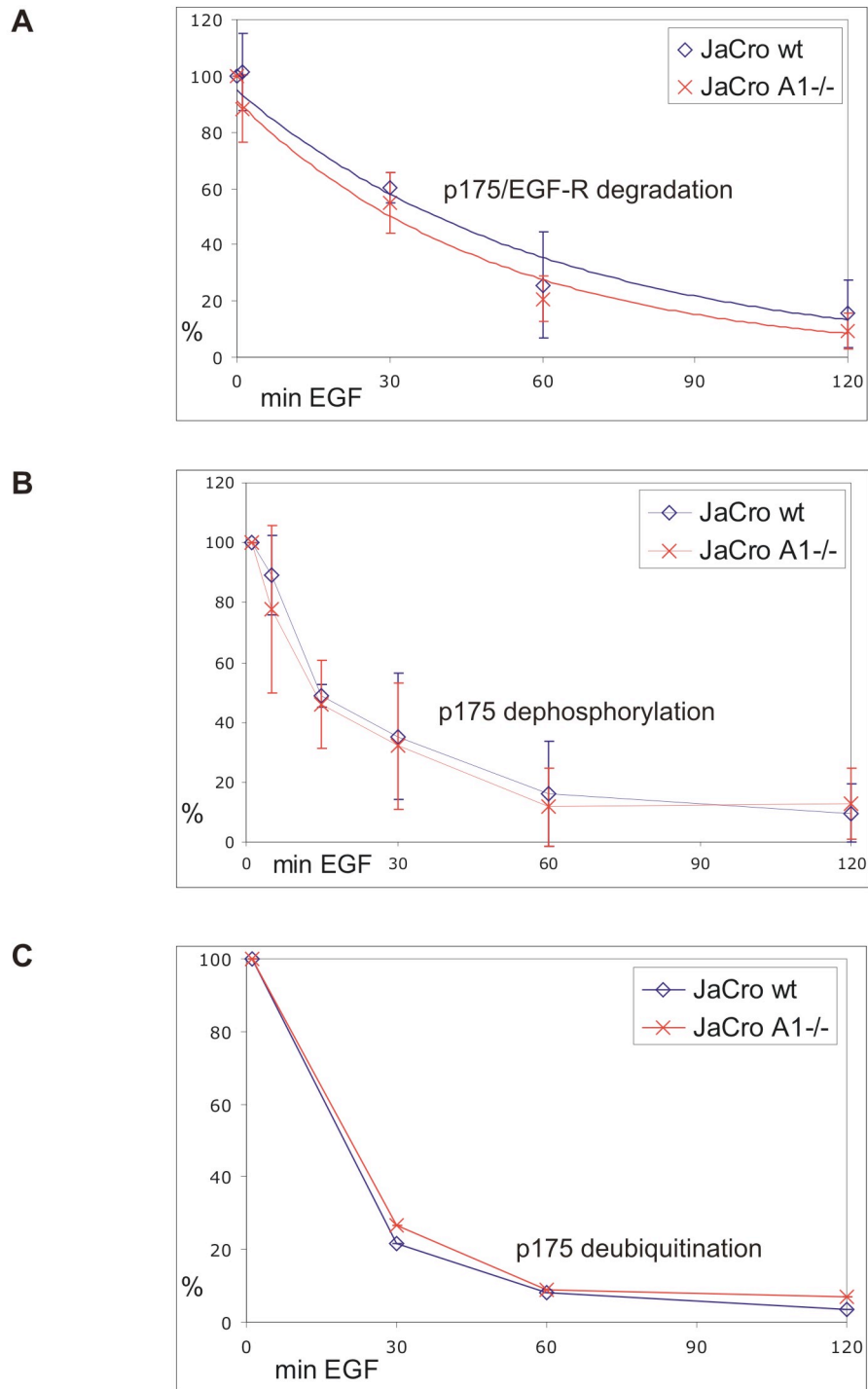
Figure 31: Quantification of the degradation, dephosphorylation and debuiquitination of p175 (figure 30)

Signals corresponding to the EGF-R protein, the tyrosine phosphorylated or the ubiquitinated p175 from the immunoblots in figures 30A-C were quantified by the Lumi-Imager Analysis System F1 (Boehringer Mannheim) and plotted in curve diagramms.
Figures A and B represent the mean values of five successive experiments and in figure C, one representative result of 3 experiments is shown.

A

IP EGF receptor
WB Anti phosphotyrosine

175 —

83 —

62 —

48 —

33 —

p(EGF-R)

EGF   ⁻   5'   15'   30'   60'   ⁻   5'   15'   30'   60'

JaCro wt   JaCro ANX A1-/-

B
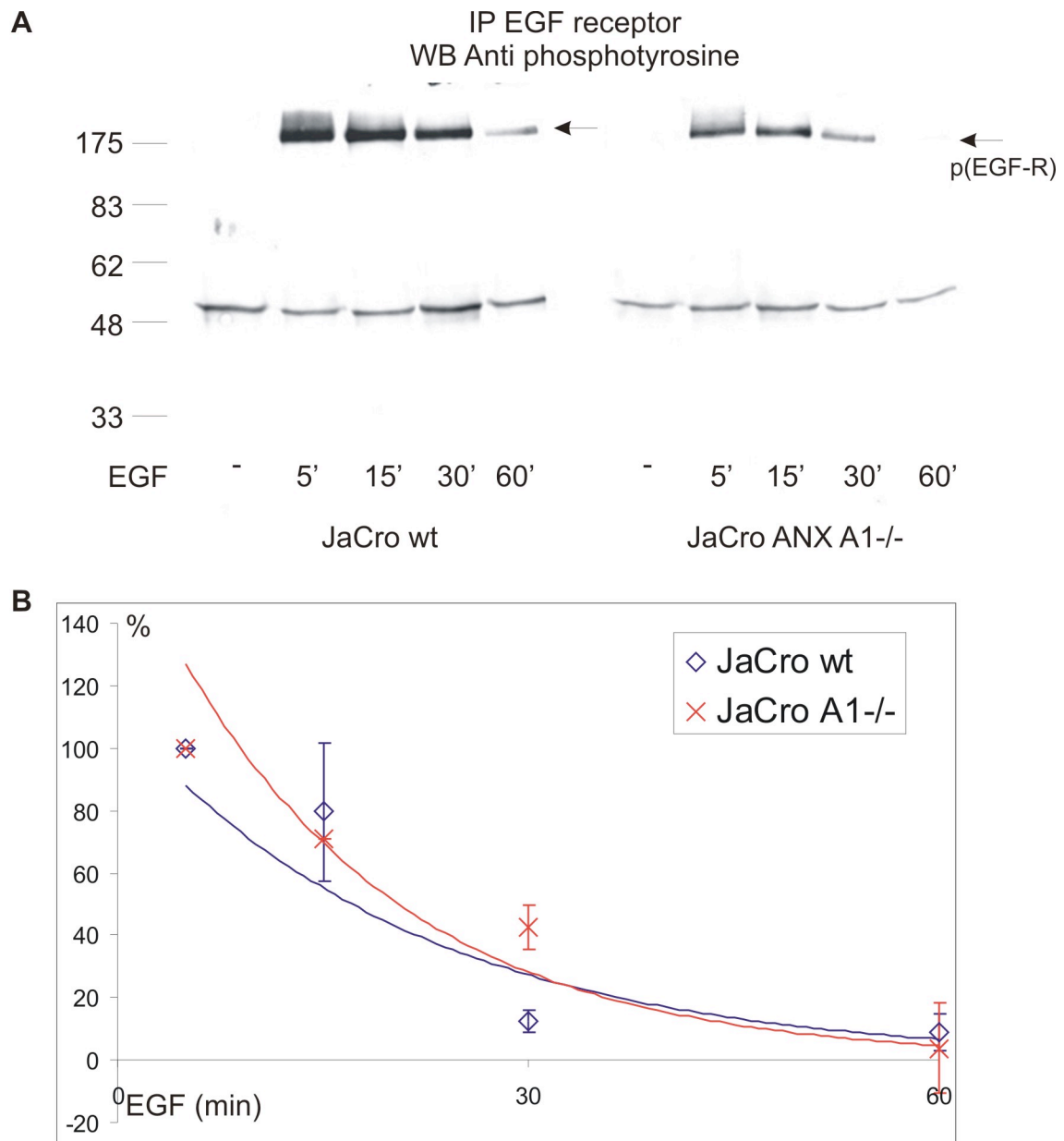
◇ JaCro wt
× JaCro A1-/-

Figure 32: Dephosphorylation of the EGF-R in JaCro ANX A1-/- vs wt cells

JaCro wild type cells and JaCro ANX A1-/- cells were EGF stimulated for the indicated times. Total cell lysates were prepared and immunoprecipitation using EGF-R antibody was performed. The precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulosis membrane.
The phosphorylation status of the EGF-R was analyzed by immunoblotting with a phosphotyrosine antibody (A). The mean values and the standard deviations of 4 experiments were quantified and presented in a curve diagramm (B).

In order to study the dephosphorylation of the EGF-R in JaCro wt and ANX A1-/- cells in more detail, immunoprecipitation using EGF-R antibodies, was performed. Tyrosine phosphorylation of the precipitated proteins, especially at 175!kDa, was analyzed by immunoblotting (fig.32). In all samples of the kinetic from 0 to 60 min EGF stimulation, equal quantities of EGF-R protein were precipitated (not shown). However, the phosphorylation status of the EGF-R changed during the EGF stimulation (fig.32A). In neither cell line was any EGF-R phosphorylation detected in non-stimulated, serum starved cells. Although the phosphorylation of the EGF-R was always stronger in JaCro wt cells than in ANX A1-/- cells, presumably because of higher receptor level, the kinetic of the EGF-R dephosphorylation was similar in both cell types. At 5 min EGF treatment, the phosphorylation signal was most intense, between 5 and 15 the signal intensity started to decrease and after 60 min EGF stimulation, a phosphorylation of the EGF-R was hardly detectable especially in ANX A1-/- cells. The calculated mean values and standard deviations were plotted in a diagram (fig.32B) revealing almost identical exponential decays.

2.2.4 EGF uptake by JaCro wt and ANX A1-/- cells

After having studied the phosphorylation and dephosphorylation of the EGF-R stimulated by its ligand EGF, the EGF uptake by JaCro cells should be analyzed by cell biological methods. The starved cells were stimulated with TexasRed®-conjugated EGF for 5, 30 and 60!min and then PFA fixed. The EGF uptake was observed by confocal microscopy (fig.33).

After 5 min EGF-TexasRed® treatment, punctuate, red fluorescent structures were visible evenly distributed in the cytosol of JaCro!wt and ANX!A1-/- cells (fig.33A&B). These signals were not detected in non-stimulated cells (not shown). At 30!min, the fluorescent structures became more intense and concentrated closer to the nucleus in JaCro!wt cells but not in JaCro ANX!A1-/- cells (fig.33C&D). JaCro!wt cells stimulated with EGF-TexasRed® for 60!min still showed the fluorescent punctual structures in the cytosol (fig.33E). The EGF-positive structures also became more intense in JaCro!ANX!A1-/- cells after 60!min EGF but they remained distributed throughout the cell and were not as concentrated in the perinuclear region as had been observed in wt cells (fig.33F).

JaCro wt   JaCro ANX A1-/-
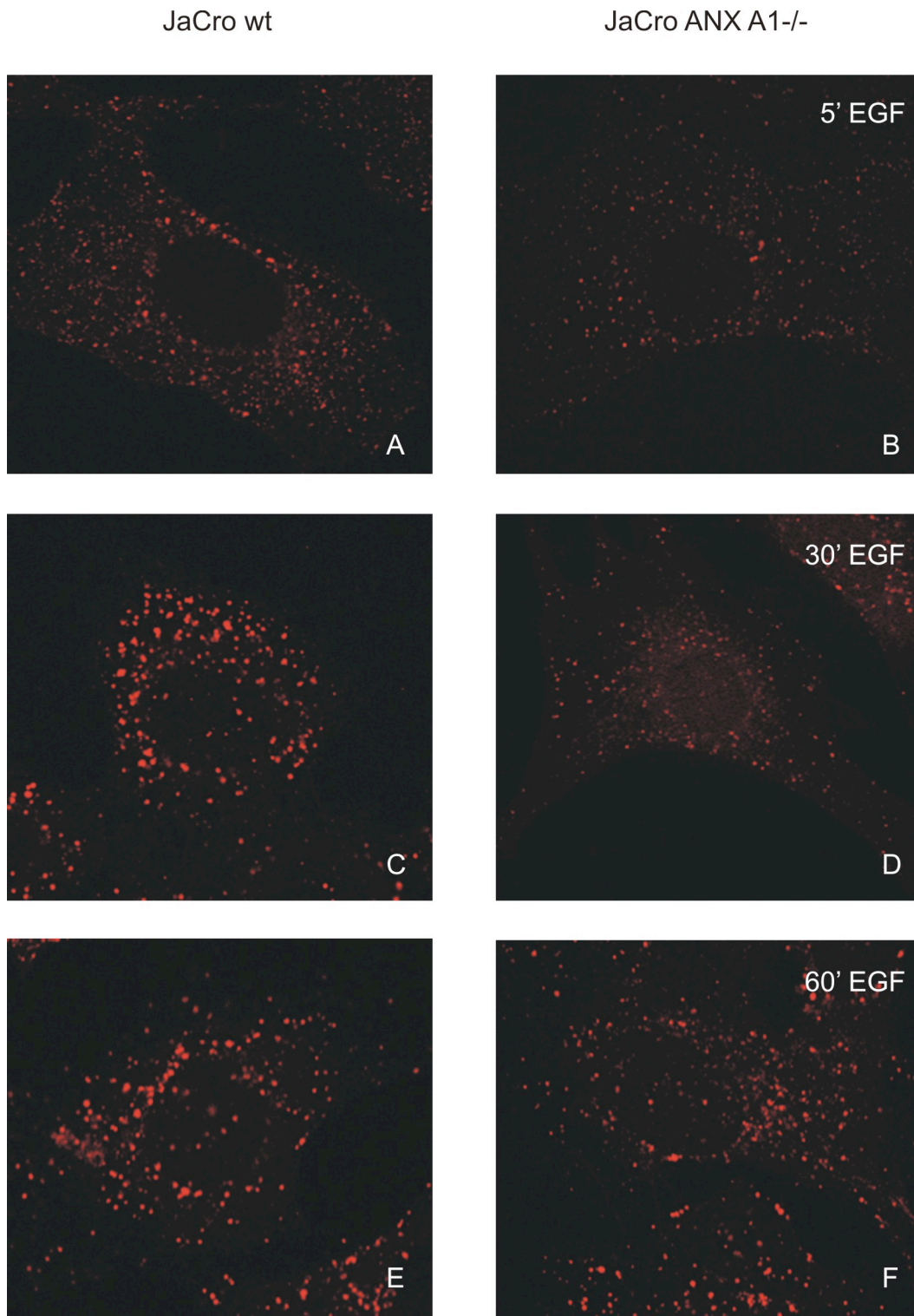
Figure 33: EGF uptake in JaCro wild type versus Jacro ANX A1-/- cells

For the analysis of the EGF-uptake ratio in the presence and in the absence
of ANX A1, JaCro wild type (A, C, E) and ANX A1-/- (B, D, F) cells were serum
starved over night and then stimulated with EGF-Texas Red for 5 min (A, B),
for 30 min (C, D) and 60 min (E, F).

2.2.5 Transferrin and dextran uptake by JaCro wt and ANX A1-/- cells

The uptake of another membrane receptor ligand, transferrin (fig.34), and pinocytosis kinetics in the JaCro cell lines was studied next (fig.35).

Incubation of the cells with TexasRed®-conjugated transferrin for 5!min, provoked the uptake of transferrin in early endosomal structures. After 30!min transferrin uptake, the whole transferrin receptor pathway consisting of sorting endosomes and recycling endosomes, was visualized. The pulse/chase-experiment, 5!min transferrin treatment and 25!min transferrin-free incubation, mainly showed the recycling endosomes.

In JaCro wt as in ANX!A1-/- cells, 5!min of transferrin treatment revealed fine vesicular structures throughout the cytosol (fig.34A&B). After 30!min stimulation, the transferrin TexasRed® positive structures became bigger and more visible in the cytoplasm of both cell types (fig.34C&D). Last but not least, the pulse/chase-experiment resulted in a smaller number of transferrin-positive vesicles than observed after 30!min transferrin uptake in wt and ANX A1-/- cells. In addition, the vesicles were less distributed in the cytoplasm (fig.34E&F). No apparent difference was visible between wt and ANX A1-/- cells.

Pinocytosis analyzed by liquid phase uptake, was also similar in wt or ANX A1-/- JaCro cells. Here, cultured cells were incubated in the presence of dextran conjugated with a red fluorescent dye for 5, 30 and 60 min. The longer the cells were incubated with the dextran solution the more visible and numerous the intracellular vesicles became. At 5!min, the dextran positive structures were fine and diffuse throughout the cytoplasm of JaCro!wt and ANX!A1-/- cells (fig.35A&B). The fluorescence intensity became stronger up to 30!min dextran uptake (fig.35C&D) and continued increasing in the following 30!min (fig.35E&F).

JaCro wt　　　　　　　　　　JaCro ANX A1-/-

5' transferrin

30' transferrin

5' transferrin-
pulse
25' chase
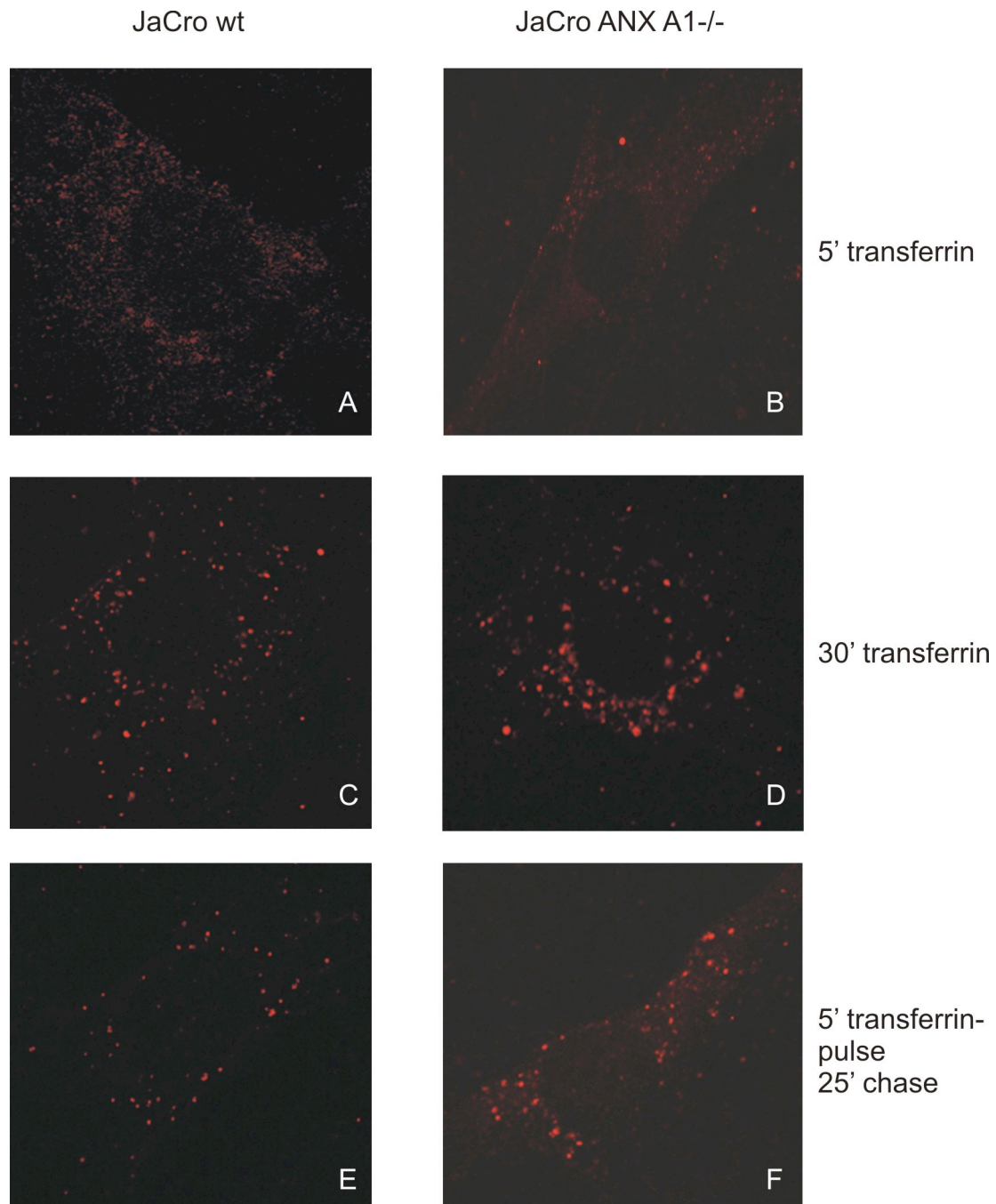
Figure 34: Transferrin uptake in JaCro wild type and ANX A1-/- cells

JaCro wild type (A, C, E) and ANX A1-/- (B, D, F) cells were serum starved for at least one hour and then incubated with transferrin-TexasRed® for either 5 min to label the early endosomal compartment (A, B) or for 30 min to label the complete early endosomal and the recycling pathway of the transferrin receptor (C, D). The pulse/chase experiment (5 min transferrin incubation followed by 25 min transferrin free medium) was performed to label mainly the recycling compartment (E, F).

JaCro wt   JaCro ANX A1-/-
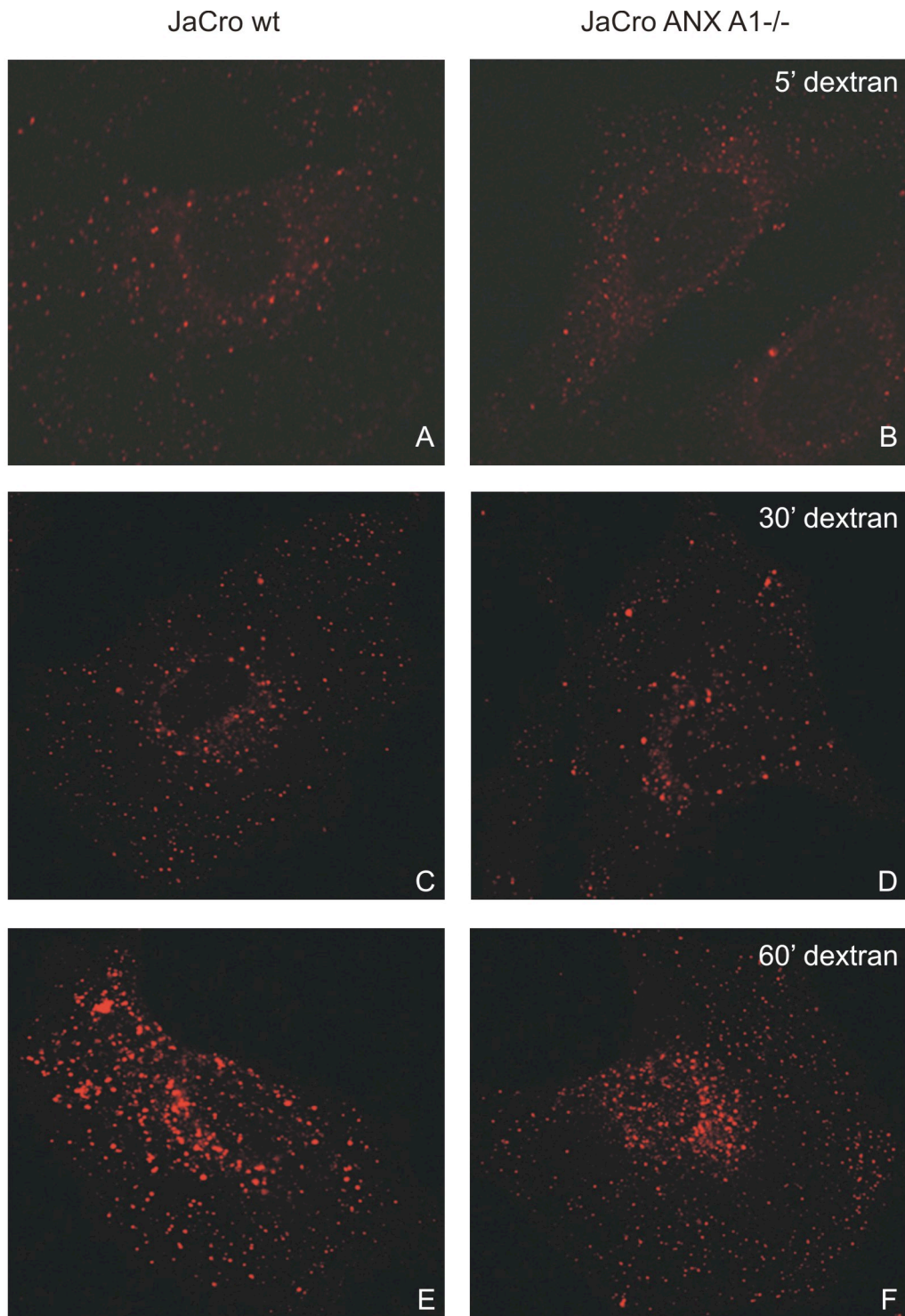
Figure 35: Pinocytosis study in JaCro wild type and Jacro ANX A1-/- cells

JaCro wild type (A, C, E) and ANX A1-/- (B, D, F) cells were incubated with dextran-Alexa 568 for 5 min to label early pinosomes (A, B) and for 30 (C, D) and 60 min (E, F) to visualize the later pinosomal pathway inside the cells by the uptake of the fluid phase marker dextran.
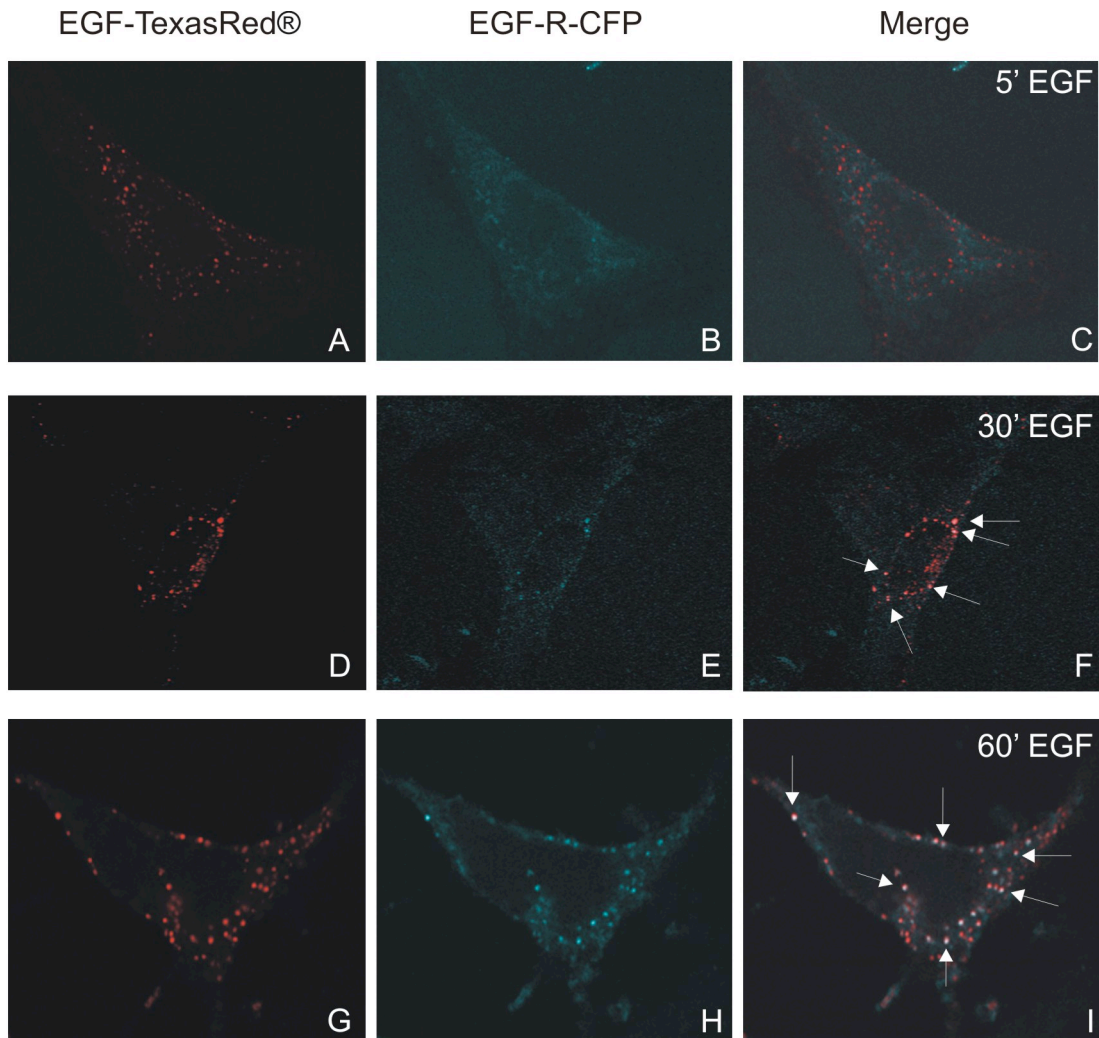
Figure 36: EGF uptake in EGF-R-CFP transfected JaCro ANX A1-/- cells*.*

JaCro ANX A1-/- cells were transfected with the construct pEGF-R-CFP
by electroporation. 24 hrs later, the cells were serum starved over night and
stimulated for 5, 30 and 60 min with Texas Red® conjugated EGF.

2.2.6 EGF uptake by JaCro ANX A1-/- cells overexpressing the EGF receptor

The following study was performed in order to ascertain if the different EGF uptake rates in ANX!A1-/- cells compared to JaCro wt cells (chapter 2.2.4) were due to the fact that JaCro ANX A1-/- cells showed less EGF receptor.

Therefore, the EGF uptake experiment was repeated using cells that had previously been transfected with a vector encoding the EGF-R fused to the cyano fluorescent protein (EGF-R-CFP). The transfected cells were serum starved over night before being stimulated with EGF-TexasRed® for 5, 30 and 60 min (fig.36).

After 5 min stimulation, numerous fine EGF- and EGF-R-positive vesicles appeared in cytoplasm (fig.36A-C). Co-localization of EGF-TexasRed® and EGF-R-CFP was hardly detected at this short stimulation time. After 30 min of EGF treatment, the vesicles became bigger in size and concentrated in the perinuclear region (fig.36D-F) as observed in untransfected JaCro!wt!cells (fig.33). At this time, co-localization could be observed.

After 60 min of EGF stimulation, numerous EGF-positive vesicles of bigger size than those observed at 5 and 30!min formed, which co-localized closely to the nucleus with the EGF-R as in untransfected wt cells (fig.36G-I).

2.2.7 Expression and localization of ANX A1-GFP fusion proteins in JaCro A1-/- cells

JaCro ANX!A1-/- cells were transfected by electroporation with the four different ANX!A1-GFP expression vectors described previously (chapter 2.1.5). The vectors encoded ANX!A1 full-length protein, ANX!A1 core without the N-terminal domain, two mutants of full-length ANX!A1 with an exchange of the potential EGF-R phosphorylation site tyrosine-21 to alanine or to glutamate.

The expression of the ANX!A1-GFP fusion proteins was analyzed by immunoblotting (fig.37A). The expression level of the four fusion proteins was equal.

Confocal microscopy analysis of cells transfected with the empty vector revealed diffuse cytosolic and nuclear staining (fig.37B). In cells transfected with the vectors containing an ANX A1 insert, vesicular structures in the cytosol were detected (fig.37C-F). The vesicles seemed to be concentrated on one side of the nucleus, creating a cap-looking formation in the perinuclear region, especially in the case of ANX!A1 core- and ANX!A1 full-length-GFP (fig.37C&D). Fewer ANX A1-GFP positive vesicles seemed to be formed in cells expressing one of the ANX A1 point mutants (fig.37E&F).
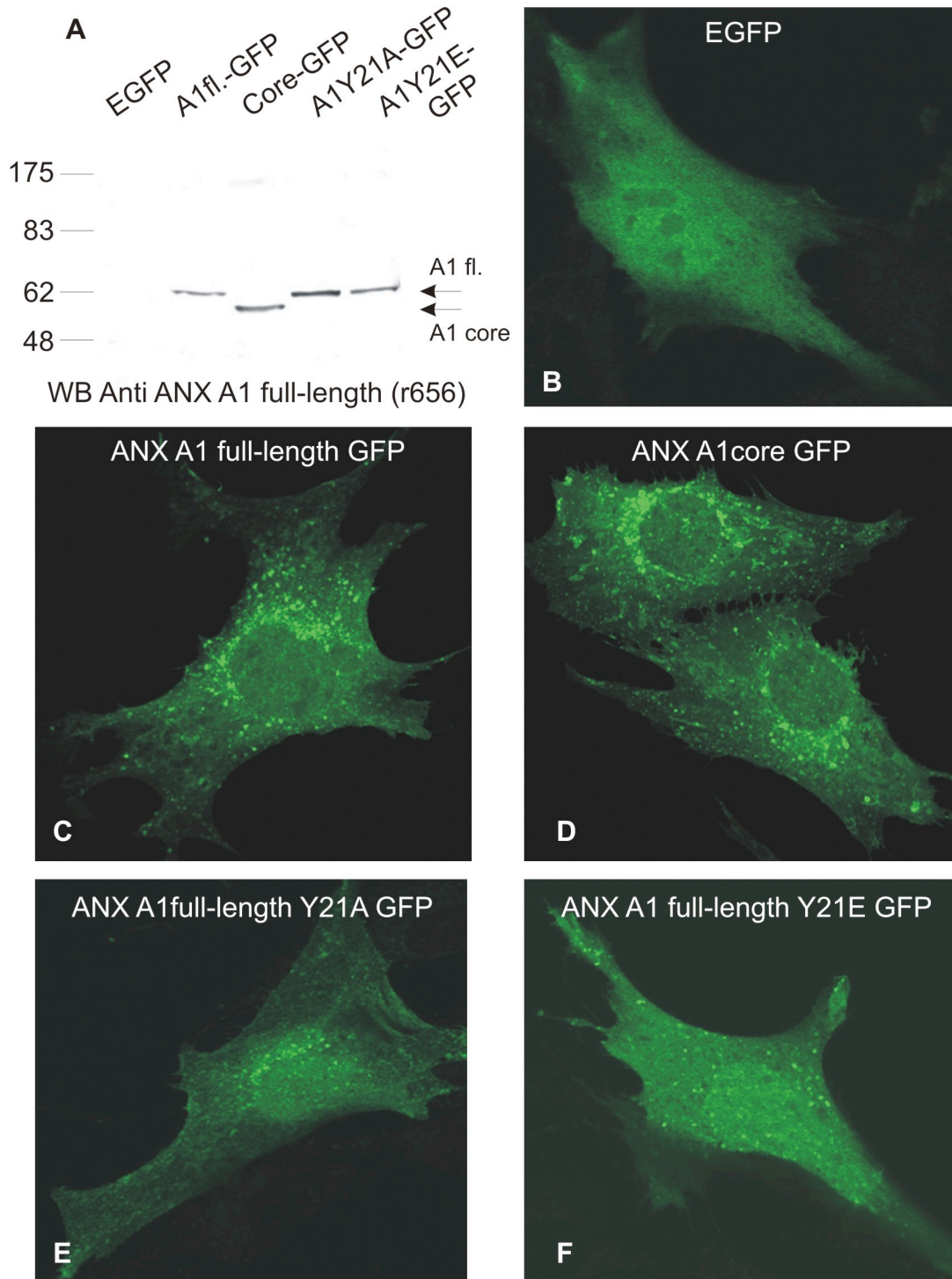
Figure 37: Expression of ANX A1-GFP fusion proteins in JaCro cells

JaCro ANX A1-/- cells were transfected with the vector without insert (pEGFP), panx a1 full-length-GFP, pcore-GFP, panx a1 full-length(Y21A)-GFP and panx a1 full-length(Y21E)-GFP by electroporation. Expression of the fusion proteins was analyzed by immunoblotting (A) and confocal microscopy (B-F).

Transfected JaCro cells were serum starved and stimulated for 5 and 30!min with EGF-Texas-Red® (EGF-TxRd) to study the co-localization of ANX A1- and EGF-positive vesicles (Fig.R38). In cells expressing wild type ANX A1 fused to GFP, almost no co-localization of ANX A1-GFP and EGF-TxRd could be identified (fig.38A-C). A 25 min longer stimulation with EGF did not provoke visible changes. Co-localization events were hardly detected (fig.38D-F) although some EGF-positive and ANX A1 containing vesicles seemed to reside close to each other in the cytoplasm at both times. As observed with full-length ANX A1-GFP, co-localization of the ANX A1 core and EGF-TxRd were hardly detected at 5 min EGF (fig.38G-I). At 30 min EGF, co-localizations could be detected but it also appeared that most of the EGF- and ANX A1 core-positive vesicles were very close to each other but not co-localizing. In JaCro cells expressing the ANX A1(Y21A) mutant, more co-localization events were found at 5 min than at 30 min EGF stimulation (fig.38M-R). Indeed, after 30 min EGF fewer vesicles were observed but still co-localizing vesicles were detected. The results obtained with cells transfected with the second mutant ANX A1(Y21E)-GFP were comparable with the first tyrosine-21 mutant. Co-localization events were better detected at short than at longer EGF stimulation (fig.38S-X).
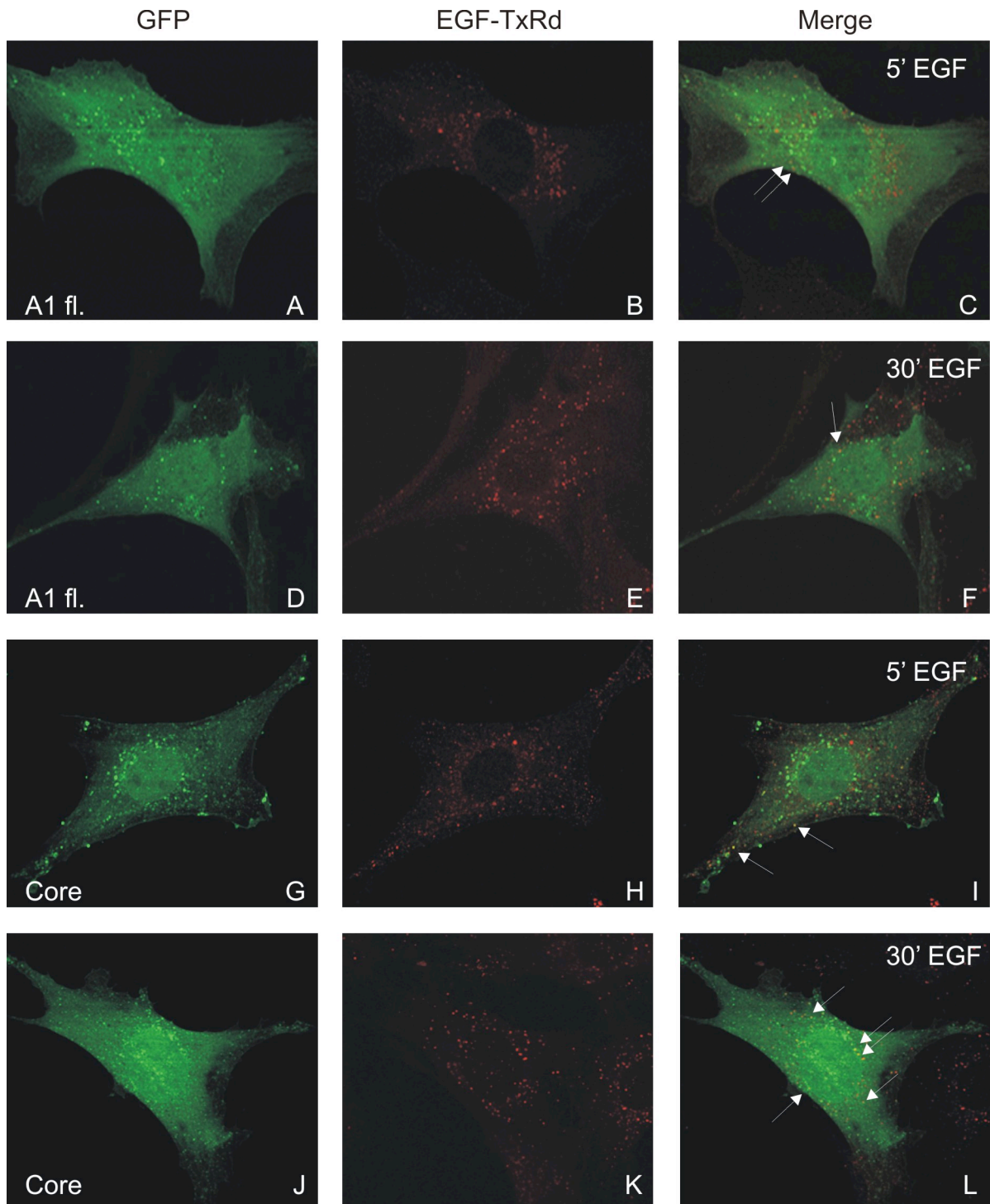
Figure 38 A-L: EGF uptake in JaCro ANX A1-/- cells transfected with ANX A1 fl.- and ANX A1 core-GFP

JaCro ANX A1-/- cells were transfected with panx a1fl.-GFP(A-F), pcore-GFP (G-L), panx a1fl. Y21A-GFP (M-R) or panx a1fl. Y21E-GFP (S-X). 24 hrs later, the cells were serum starved over night and finally stimulated for 5 and 30 with Texas-Red® conjugated EGF (EGF-TxRd).
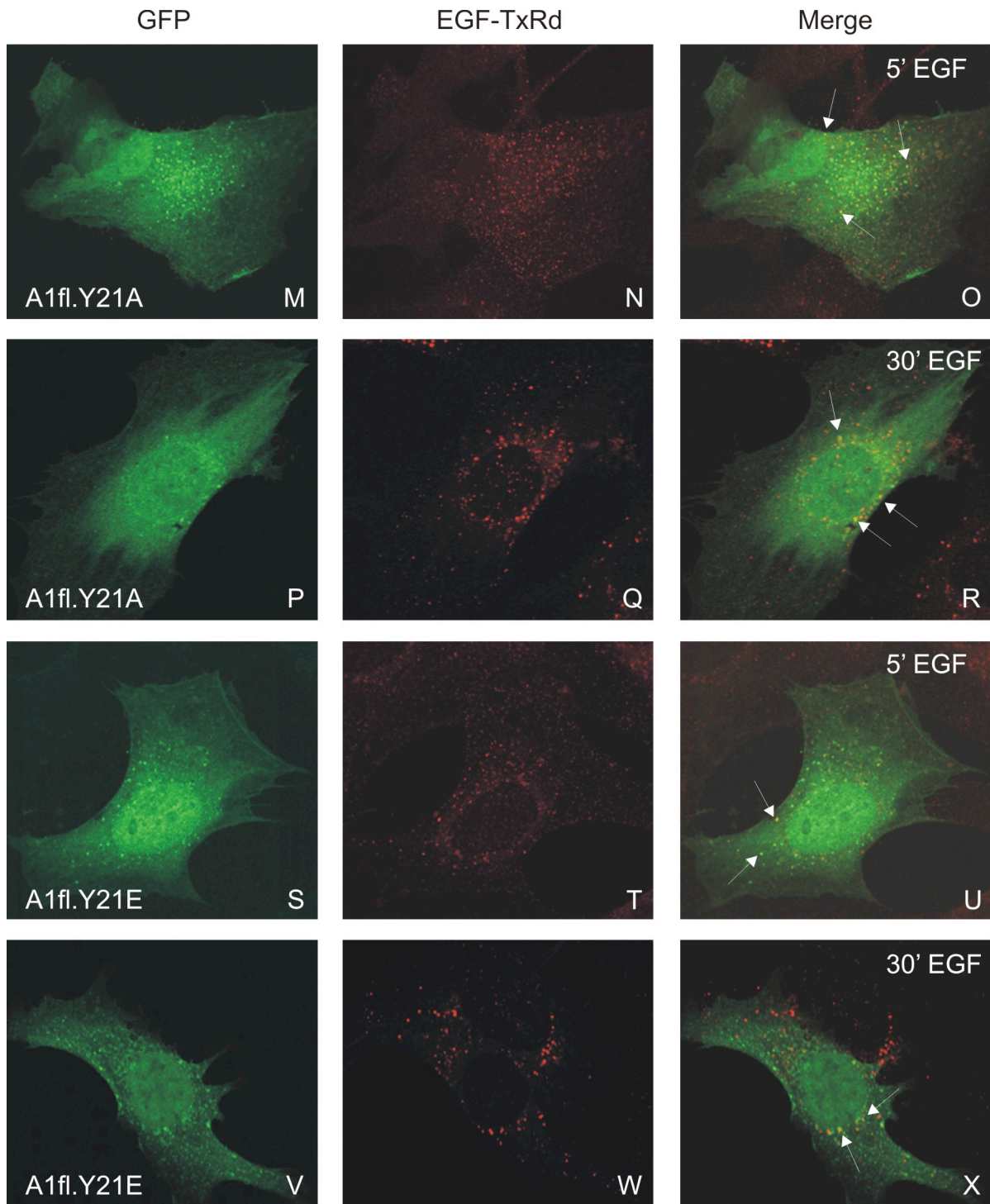
Figure 38 M-X: EGF uptake in JaCro ANX A1-/- cells transfected with ANX A1(Y21A)- and ANX A1(Y21E)-GFP

3. DISCUSSION

ANX A1 has the capability to mediate membrane aggregation, vesicle formation and fusion (Raynal and Pollard 1994, Gerke and Moss 1997 and 2002). The physiological functions of this as well as others annexins are still not completely understood. However, putative roles of ANX A1!in inflammation processes, signal transduction, cell proliferation and differentiation and also in intracellular trafficking have been described.

It has been shown – mostly by *in vitro* studies – that ANX A1 is a substrate for the EGF-R (Pepinsky 1991). Futter *et al.* (1993) discovered ANX A1 to be present together with the still active EGF-R in multivesicular bodies. Tyrosine phosphorylated ANX!A1 is more sensitive toward proteolysis and has altered requirements for Ca^{2+} when interacting with phospholipids including those of endosomal membranes (Ando *et al.* 1989, Futter *et al.* 1993). Therefore, it has been suggested that tyrosine phosphorylated ANX!A1 may be implicated in the fusion of endosomal membranes, inward vesiculation and maturation of late endosomes during their transport toward lysosomes.

In the present work protein interactions of ANX A1, its phosphorylation and other modifications such as proteolytic cleavage following activation and endocytic transport of the EGF-R were analyzed to define a possible role of ANX!A1 in EGF-R internalization, trafficking and degradation.

3.1 Detection and localization of endogenous ANX A1 in HeLa and JaCro cells

Five different antibodies have been used to detect ANX A1 and to analyze its localization in the modified HeLa cell line HeLa wt/ HeLa K44Adyn- (S.!Schmid, La!Jolla) and in the fibroblast cell line JaCro (Dr J. Croxtall, London).

One of the polyclonal antibodies named Sidney was generated in this work (fig.7). It could be used for immunoprecipitation and immunoblotting on HeLa total cell lysate (fig.8) and also for immunofluorescence studies after affinity chromatography (fig.9). Two different protocols were applied to purify the antibody. The first method was a protein-A chromatography to purify IgGs and the second was more specific using immobilized antigen. The specific purification selected an antibody fraction that specifically recognized the plasma membrane bound form of ANX A1. The protein-A purified fraction detected in addition ANX A1 that interacted with vesicular structures. Moreover, this antibody revealed clustered structures,

which localized closely to the nucleus. Antigen competition identified these structures as unspecific.

Sidney seems to contain antibodies, which recognize in addition to different epitopes different intracellular ANX A1 fractions as well. It is common knowledge that more than one annexin-pool can be present inside the cell *i.e.* one pool at the plasma membrane and another in the cytosol (Gerke and Moss 2002). It is probable that there is a dynamic exchange between theses fractions. The analysis of the ANX A1 structure has shown that Ca^{2+} might be able to trigger a conformational change in the ANX!A1 core domain (Rosengarth *et al.* 2003). Thus, it is possible that a specific ANX A1 antibody, which labels ANX A1 at vesicular structures, is not able to detect it at the plasma membrane when its epitope is not accessible due to a conformational change in a different local Ca^{2+}-environment.

The serum DE99603 directed against the N-terminal domain of ANX A1 was purified by specific antigen chromatography and then also used for immunofluorescence studies. The staining of this antibody revealed a punctuate structure in the cytosol but not at the plasma membrane (fig.9). The cytosolic labeling was specific as tested by peptide competition analysis (not shown).

Moreover, the commercial monoclonal antibody TDL was used for immunofluorescence studies in HeLa cells. It labeled the plasma membrane and intracellular vesicles that were comparable with the structures stained by Sidney and DE99603. The epitope of this antibody is unknown. But considering the results described here and the fact that this antibody is not able to recognize an ANX A1 core-GFP constructs (not shown) it is probable that the epitope is localized on the N-terminal region. This differs from the epitopes of DE99603 because TDL is able to detect the plasma membrane bound form of ANX A1, whereas DE99603 only recognizes vesicular ANX A1.

Two further antibodies have been applied for immunoblotting, immunoprecipitation and immunofluorescence studies: r656 and Dac5. The best results with the lowest background were obtained when immunoprecipitation was performed with Sidney or Dac5 and immunoblotting with Dac5 or TDL (fig.8). R656 could not be used for interaction studies between the EGF-R and ANX A1 because this serum interacted with a protein having the same molecular mass as the EGF-R (175!kDa). The results obtained with Sidney had much less background compared to r656 especially at high molecular masses.

3.1.1 ANX A1 localization at the cytoskeleton in HeLa cells

ANX A1 positive vesicles were partially orientated along microtubules, intermediate filaments and F-actin. Partial co-localizations of ANX A1 stained with Sidney or DE99603 were found with α-tubulin, α-cytokeratin and α-vimentin. This could indicate that ANX!A1 interacts with the tested cytoskeletal proteins *via* its N-terminal domain. Indeed, co-localizations of ANX A1 with the cytoskeletal proteins actin (Glenney *et al.* 1987, Schlaepfer and Haigler 1987), tubulin (Traverso *et al.* 1998) and cytokeratins (Traverso *et al.* 1998, Croxtall *et al.* 1998) have been observed previously.

3.1.2 ANX A1 localization at vesicular structures in HeLa and JaCro cells

DE99603 was used for localizing ANX!A1 to different endosomal structures (fig.10). Clathrin-coated endosomes, early endosomes, late endosomes and lysosomes were tested. The data showed that ANX A1 was present to some extend on all endosomal structures tested except lysosomes stained with Lamp1 antibody. DE99603 is directed against the N-terminal peptide amino acid aa15-26. The protein detected could therefore represent the full-length protein or the N-terminal domain separated from the core domain. Indeed, there is a potential calpain cleavage-site at lysine-26 (Ando *et al.* 1989).

Co-localization studies were also performed in JaCro cells where the specifically purified DE99603 revealed a specific punctuate staining without background in wild type cells (fig.29) but not in ANX!A1-/- cells. As before in HeLa cells late endosomal structures were labeled with an antibody directed against the phospholipid lysobisphosphatidic acid that is enriched in internal membranes of late endosomes or so called multivesicular bodies (MVB) (Kobayashi *et al.* 1998) (fig.29). The double-labeling experiment revealed that parts of the ANX A1 positive structures represented this special kind of endosomal vesicles, thus confirming the results obtained in HeLa cells.

Endogenous ANX A1 localizes in HeLa wt cells at the plasma membrane and in addition at early and late endosomes. The N-terminal domain of the protein is at least partially present in these structures. Some ANX A1 positive vesicles are oriented along the cytoskeleton.

3.2 Localization of ANX A1-GFP in HeLa cells

ANX A1-GFP was detected at vesicular structures in the cytoplasm of HeLa cells (fig.13) and also at the plasma membrane (fig.14D&M, 15D). Co-localization studies showed that full-length ANX!A1-GFP was neither located in mitochondria nor at the trans Golgi network (fig.14). Clathrin-coated endosomes and few lysosomes labeled by DQ-BSA appeared ANX!A1-GFP positive. Surprisingly, no localization of ANX A1-GFP appeared in late endosomes, whereas DE99603 did detect ANX!A1 in late endosomes or MVBs. It is possible that DE99603 detected the N-terminal domain already cleaved from its protein core in MVBs. This suggestion would be supported by the findings of Rescher *et al.* (2000). Using the same GFP constructs as used in this work they localized the full-length protein mainly at early endosomes, whereas the protein core without N-terminal region was localized at late endosomes. Thus, the N-terminal region directs ANX!A1 to early endosomes where it remains or is exchanged with ANX A1 from the plasma membrane by means of a dynamic switch between these two cellular ANX A1 pools.

However, when ANX A1 full-length- or core-GFP transfected HeLa cells were incubated with transferrin-TexasRed® for 5!min (early endosomes) or 30!min (early endosomes and recycling compartment), both constructs seemed to be localized at early endosomes and in the recycling compartment. This core localization contradicts with the results of Rescher *et al.* (2000) (fig.15). Thus, it would appear that in the cell system used here no differential localization between full-length ANX A1 and the core is visible. This might be due to the fact that different cell lines are used in the two studies. As it is known that HeLa cell lines have the property to change during cell culture it is possible that HeLa pools can be very different genetically. Hence, specific studies are not feasible with all HeLa pools. Indeed, several cell lines have been tested for the transferrin study including HepG2 cells (human hepatocyte cell line) with the result that the different localization of full-length ANX!A1 and the core was not visible (according to Dipl. Biol. Judith Austermann).

3.3 Interaction of ANX A1-GFP and the EGF-R in HeLa cells

Transfected cells were EGF stimulated to study the transport of the EGF-R in comparison to the localization of ANX!A1 full-length and core-GFP so as to study if ANX!A1 and the EGF-R co-localize during receptor trafficking.

The kinetics of the EGF-R activation, its trafficking and degradation have been described by Burke *et al*. (2001) in different human endothelial and epithelial cell lines. The EGF activated EGF-R was internalized in less than 5!min, remained in the early endosomal compartment for approximately 20!min and was then detected in the late endosomal compartment under 60!min of total EGF stimulation. Afterwards, the EGF-R was sorted toward lysosomes for degradation.

Co-localizations of the EGF-R and both ANX A1 fusion proteins were detected at 5 min of EGF treatment in early endosomes (fig.16). At 30 min the results showed a difference between the core and ANX A1 full-length. The EGF-R and ANX A1 full-length were both closely localized to the nucleus in a compact late endosomal compartment. In contrast there were only few co-localizations of the core and the EGF-R detected in the cytoplasm.

At early time points following activation both fusion proteins might accompany the EGF-R, *i.e*. during its transport towards clathrin-coated pits, the receptor internalization into early endosome and its transport in the early endosome, or might shuttle between cytosol and the membranes where the EGF-R is localized. Therefore, no differences in EGF-R co-localization with the core-GFP or ANX A1 full-length GFP were visible. However, only in the cells transfected with full-length ANX A1-GFP intense co-localization with the receptor are visible at later stages of endocytosis.

The co-localization study of the EGF-R and ANX A1 has not shown if these proteins interacted already at the plasma membrane although full-length ANX A1-GFP and core-GFP have been detected at the plasma membrane. This might be due to the fact that the EGF-R was not concentrated enough at the plasma membrane for visualization by confocal microscopy. To clearly visualize plasma membrane-associated EGF-R artificial clustering was performed with an EGF-R antibody directed against an external domain and a secondary anti-mouse IgG antibody. Several monoclonal antibodies directed against the exterminal domain of the EGF-R have been developed that can compete with EGF for binding sites (Mendelsohn 1988). Some of these antibodies, among them the antibody 528 used here, were able to block cell

proliferation of the A431 squamous carcinoma cell line (Kawamoto *et al.* 1983). In addition 528 was also able to block self-phosphorylation of the EGF-R *in vivo* (Gill *et al.* 1984). Although these antibodies induced receptor dimerization accompanied by receptor down-regulation (Fan *et al.* 1994), compared to EGF a rapid down-regulation was not observed with 528 (Kawamoto *et al.* 1983).

Transfected HeLa wt cells were used for the cluster study (fig.17). Parallel to wt full-length ANX A1-GFP and core-GFP, two ANX A1 mutants with an exchange of tyrosine-21 to alanine or glutamate were applied. The alanine-mutant was also named the inactive mutant and the glutamate-mutant the constitutively active mutant as in the first case the residue-21 cannot be phosphorylated anymore and in the second case, the tyrosine phosphorylation is mimicked by the negative charge of the glutamate-residue.

In cells that were transfected with ANX A1 wt and the constitutive active ANX A1 mutant the formation of relatively large clusters was triggered where the EGF-R and ANX!A1 co-localized. Both proteins were at least partially internalized. Co-localizations were also detected inside the cell in compartments close to the nucleus. The same results were obtained when the experiment was repeated with the monoclonal ANX!A1 antibody TDL directed against an epitope on the N-terminal peptide.

Core-GFP and the inactive mutant also co-localized with the antibody-induced EGF-R clusters. However, the clusters were smaller and less numerous. Deep invaginated pits were rarely found. However, small vesicles containing EGF-R and either one of the fusion proteins were observed inside the cell.

The interaction with the EGF-R cluster was specific for ANX!A1 as other annexin family members such as ANX A3- and ANX A6 did not show the same effect.

Interactions between the ANX A1-GFP fusion proteins and the EGF-R were confirmed by immunoprecipitation applying EGF-R antibody on cell lysate of transfected cells. All fusion proteins, including core-GFP, wt ANX A1-GFP, A1Y21A- and A1Y21E-GFP interacted with the EGF-R. Hence, the direct or indirect interaction site for the EGF-R was not localized in the N-terminal domain. Phosphorylation of tyrosine-21 was also not required for EGF-R/ANX A1 interactions.

Wild type ANX A1 conjugated with the green fluorescent protein is localized at the plasma membrane and on early endosomes. In EGF stimulated HeLa cells, ANX A1-GFP appears together with the EGF-R in early and also in late endosomes and seems to accompany the EGF-R during its trafficking. However, without its N-terminal domain ANX A1 does not seem to be transported with the EGF-R to late endosomes or is not bound to late endosomes. Interaction studies show that full-length ANX!A1 or truncated, non-active or constitutively active mutants are able to interact with the EGF-R. However, for continued or prolonged interactions of ANX A1 with the EGF-R during its complete trafficking through the cell toward lysosomes, the N-terminal domain would appear to be necessary.

3.4 Activation of the EGF-R and interaction with endogenous ANX A1 in HeLa cells

The EGF-R was rapidly tyrosine phosphorylated within 1!min of EGF stimulation (fig.24A). The phosphorylation status remained almost stable in K44Adyn- cells, which expressed a dominant-negative dynamin mutant under tetracyclin control so that membrane proteins could not be internalized in a dynamin- and clathrin-dependent manner (fig.6). A certain rate of receptor dephosphorylation took place nevertheless. This was probably due to the fact that the non-functional dynamin was not overexpressed in all cells, but only in 80-90!% of them.

In HeLa wt cells the EGF-R was dephosphorylated progressively and also degraded, as visualized by the tyrosine phosphorylated protein bands of a molecular mass inferior to that of the EGF-R especially at 30 and 60!min of EGF stimulation. These phosphoproteins interacted with the EGF-R antibody and were hardly detectable in K44Adyn- cells.

Vieira *et al*. (1996) found that the EGF-R was phosphorylated to a lesser extent in K44Adyn- cells compared to wt cells. This finding could be confirmed here (fig.24A). The authors proposed that the EGF-R phosphorylation continued in endosomes and that only in cells with functional endocytotic trafficking full EGF-R tyrosine phosphorylation and thus activation could be achieved.

The analysis of an ANX A1 interaction with the EGF-R was done by immunoprecipitation with EGF-R antibody and immunoblotting with the monoclonal antibody TDL directed against the N-terminal domain of ANX!A1 (fig.24B-D).

In K44Adyn- cells the protein band corresponding to ANX!A1 showed almost the same intensity over the kinetic from 1 to 60!min of EGF treatment and was visible even in the unstimulated sample. Therefore, ANX A1 and the EGF-R interacted directly or indirectly even in serum starved cells.

As the interaction was maintained in the cell line without dynamin-dependent internalization it could be suggested that the EGF-R interacted with ANX A1 already at the plasma membrane. The same protein band was hardly detected in cells that were treated with EGF-R kinase inhibitor, which could mean that the interaction was interrupted when the EGF-R kinase activity was inhibited and also that the EGF-R might always show a certain activity even in non-stimulated and serum-depleted cells. Indeed, the assumption has been made that the EGF-R might be less tightly autoinhibited in comparison to other receptor tyrosine kinases as a highly conserved tyrosine residue in the activation loop was not required for EGF-R kinase activation but for other receptor tyrosine kinases (Gotoh *et al.* 1992).

In contrast to HeLa K44Adyn- cells ANX A1 presence in EGF-R protein complexes of HeLa wt cell samples did show alterations in intensity during the EGF stimulation course (fig.25B). Between 1 and 15!min EGF stimulation the protein band disappeared, reappeared at 15!min, remained until 30!min and weakened between 30 and 60!min. In unstimulated cells ANX A1 was again present in the EGF-R protein complex. The interaction between ANX!A1 and the EGF-R could be interrupted at 1!min EGF, or ANX!A1 was cleaved at its N-terminal domain during the EGF-R internalization process. It is therefore possible that ANX A1 participates in the internalization mechanism. The reappearance of ANX A1 in the EGF-R complex could be explained by a new interaction between both proteins established following internalization.

As it is known that the EGF-R partially localizes in specific glycosphingolipid- and cholesterol-enriched microdomains of the plasma membrane in unstimulated cells *e.g.* caveolae (Carpenter 2000), and as it is shown here that the EGF-R and ANX A1 interact in the absence of EGF, it might be possible that ANX A1 is also present in these complexes of distinct protein and lipid components. However, it is unlikely that ANX A1 was a direct part of the detergent resistant microdomain since in contrast to ANX!A2 and ANX!A6 it is not prevented from extraction by detergents from lipid microdomains of smooth muscles sarcolemma when the Ca^{2+}-concentration is elevated (Babiychuk and Draeger 2000). Moreover, Babiychuk *et al.* (2002) showed by proteolytic cleavage that ANX!A1 interacted with non-raft glycerolipid regions, whereas ANX!A2 interacted preferentially with membrane

rafts. In this study ANX A1 was proteolytically degraded by calpain, whereas ANX!A2 degradation was only limited probably due to its localization in protective microdomains.

As far as a possible phosphorylation of ANX A1 in the EGF-R complex of stimulated cells was concerned tyrosine phosphorylated proteins (pp35) at a molecular mass of ANX A1 were detected only at 30 and 60!min EGF (fig.24E-G) in wt cells and also in K44Adyn- cells, but to a lesser extent.

Application of siRNA in order to downregulate ANX!A1-expression in HeLa wt cells did not show if pp35 represented ANX A1 or not as the phosphorylation intensity did not alter (fig.25). However, as the downregulation by siRNA was incomplete and as it can be suggested that only a very small ANX!A1-portion of the total cellular ANX A1 pool interacted with the EGF-R and was tyrosine phosphorylated, it was possible that the remaining ANX A1 was sufficient for its function in EGF-R signaling.

When the interaction study was carried out with the ANX!A1 antibody Sidney to immunoprecipitate proteins and with anti-phosphotyrosine or EGF-R antibodies to detect them, the ANX A1/EGF-R interaction described previously was confirmed (fig.23). The blots decorated with EGF-R antibodies showed that the EGF-R seemed to interact with ANX!A1 during the whole time course. The phosphotyrosine blot revealed a phosphoprotein at the molecular mass of the EGF-R that was not present in the precipitated samples of EGF-R kinase inhibitor pretreated cells. Hence, it can be concluded that the phosphorylated 175!kDa protein represented the activated EGF-R. In HeLa wt cells the presence of the phosphorylated EGF-R decreased progressively in the ANX A1 protein complex. At 60 min the tyrosine phosphorylated products of the EGF-R degradation also precipitated with the ANX A1 antibody. The degradation was not visible in K44Adyn- cells as observed previously (fig.24A).

The ANX!A1 presence in the EGF-R protein complex might reflect its hypothesized capability to aggregate membranes. Thus, it could play a role in EGF-R internalization into early endosomes and also in inward vesiculation of late endosomes as proposed by Futter *et al.* (1993).

Based on results of *in vitro* studies and the data obtained by cristallography several models concerning mechanisms of ANX!A1-mediated membrane aggregation had been proposed. First of all, aggregation activity requires the approach and the link of two membrane surfaces. This can be achieved by protein-protein interactions of annexins with other proteins *i.e.* of the S100 family or by protein-lipid interaction.

It has been postulated that two phospholipid-binding sites are present in the ANX A1 protein structure. The primary membrane-binding site would correspond to the Ca^{2+}-binding site on the convex surface of the protein core and the secondary interaction site could be on the concave site of ANX A1 where the N-terminal domain is located (Bitto *et al.* 2000). The amphipathic α-helix in the N-terminal region of ANX A1 (residue 2 to 12) interacts with the S100A11 protein (Mailliard *et al.* 1996) and might be able to interact with membranes (Rosengarth *et al.* 2001). However, the second membrane-binding site identified by Bitto *et al.* differs from the S100 site.

It might be possible that ANX A1 forms heterotetramers with its ligand S100A11 (ANX $A1_2$:$S100A11_2$) to connect membranes or that only one ANX A1 molecule is able to provide a link between two membrane surfaces (Rosengarth *et al.* 2001, Gerke and Moss 2002).

The EGF-R and ANX A1 seem to interact directly or indirectly at the plasma membrane and also at endosomal structures. The intrinsic kinase activity of the EGF-R seems to be required for ANX A1/EGF-R interaction. There are indications that the interaction is maintained even with partially degraded EGF-R.

It remains to be elucidated whether or not the interaction between both proteins is maintained during the entire transport from the plasma membrane to the lysosomes, if both proteins are not internalized together but come together in early endosomes by membrane fusion or if ANX!A1 is dynamically changing its localization between the cytoplasm and the EGF-R containing membrane structures. Moreover, it is not yet clear if the tyrosine phosphorylation of ANX!A1 takes place during endocytosis and if the N-terminal domain is affected by proteolytic cleavage.

3.5 EGF dependent ANX A1 phosphorylation in HeLa cells

ANX A1 is abundantly expressed in HeLa wt and K44Adyn- cells (fig.18A). Since ANX A1 represents a good *in vitro* substrate for the EGF-R (Pepinski 1986, Futter *et al.* 1993), EGF-dependent ANX!A1-phosphorylation should be able to be detected with HeLa cells. Using the cells defective in dynamin-dependent vesicle budding the aim was to analyze whether ANX A1-phosphorylation depended on EGF-R localization in the endosomal compartment.

In HeLa total cell lysate several proteins could be distinguished that were EGF-dependently tyrosine phosphorylated (fig.18B).
Some proteins showed stronger phosphorylation in wt cells than in K44A dyn- cells and vice versa (fig.18B). The here presented results matched with the findings of Vieira *et al.* (1996). One phosphoprotein that has also been observed but was not identified by Vieira *et al.* (1996) appeared at the molecular weight of full-length ANX A1 (37!kDa). In addition a phosphoprotein band of smaller molecular weight of 35!kDa was detected. pp35 was not as strongly phosphorylated in K44Adyn- cells as in wt cells. Thus, tyrosine phosphorylated ANX!A1 could be a part of either one or both of these phosphoprotein bands. In the latter case it would be tyrosine phosphorylated at early stimulation times (1!min) and might be proteolytically cleaved later (between 1 and 5!min) due to the increased accessibility of the N-terminal region after Ca^{2+}-release and its phosphorylation. If ANX A1 was a part of both phosphoprotein bands the site for proteolytic cleavage must be located N-terminal of tyrosine-21. The proteolytic cleavage might not take place at the plasma membrane after 15!min EGF stimulation, but after internalization in endosomal structures as has been suggested by Futter *et al.* (1993) who identified ANX A1 as a main *in vitro* substrate for EGF-R in multivesicular bodies.

In order to separate the pool of phosphoproteins of the pp37- and pp35-band more efficiently, two-dimensional gel electrophoresis was performed (fig.19&20). Due to the negative charge of the phosphate group an additional phosphorylation can provoke a shift towards a more acidic pH. Therefore, changes in the isoelectric point of ANX A1 due to phosphorylation or, as well to proteolytic cleavage could be observed by using this technique.
However, the phosphotyrosine and anti ANX A1 decorated blots of the 2D-gels did not reveal unambiguous results by the tyrosine phosphorylation of ANX A1 or to a change of the molecular mass of ANX A1 as expected after proteolytic cleavage. However, a shift to more acidic pH of ANX!A1 signals was visible after 15!min EGF treatment in HeLa wt, but not in

K44Adyn- cells. These signals were not recognized by the anti phosphotyrosine antibodies and they could represent ANX!A1 phosphorylated on serine, or threonine by *e.g.* protein kinase C. As the variety of ANX A1 signals at different pH was only visible in wt cells endocytosis might be necessary for the mechanism of ANX!A1 modification. It is possible that this did not occur at the plasma membrane, but in endosomal structures.

When cells were pretreated with a m-calpain inhibitor prior to the EGF-R stimulation the 2D-blots revealed different results. A smaller number of phosphoproteins seemed to be present in the lysates of wt and dyn- cells. Moreover, the switch to an acidic pH of ANX A1 signals did not take place in the presence of the inhibitor. Hence, m-calpain had an effect on the EGF-dependent modification of ANX A1.

Proteolytic cleavage of ANX A1 in the N-terminal domain has been observed in neutrophils (Movitz and Dahlgren 2000) and also in epithelial cells (Liu *et al*. 1995, Tsao *et al*. 1998) and might be implicated in endocytic vesicle maturation (Sjolin and Dahlgren 1996, Movitz *et al*. 1999). Proteases implicated so far in this process were metalloprotease (Movitz *et al*. 1999) and calpain (Ando *et al*. 1989, Liu *et al*. 1995).

Calpains represent a family of intracellular cysteine proteases, which are Ca^{2+}-dependently activated. In this work an inhibitor against m-calpain has been implemented. M-calpain and other calpain isoforms have been localized in nuclear and cytosolic compartments as well as at the inside of plasma membranes, coated pits and coated vesicles of a variety of cell types (Glading *et al*. 2001, Nakamura *et al*. 1992, Sato *et al*. 1995). Moreover, certain calpains seem to play a role in membrane fusion and have been implicated in endoytosis and/or exocytosis processes stimulated by Ca^{2+} (Nedrelow *et al*. 2003, Hayashi *et al*. 1992, Sato *et al*. 1995). Moreover, calpain activity, including m-calpain can be triggered by the EGF-R (Glading *et al*. 2001).

Taking all these findings into account m-calpain could be activated in EGF stimulated cells when Ca^{2+}-concentration was increased and provoke ANX A1 cleavage at its N-terminal domain.

As ANX A1 tyrosine phosphorylation could not be identified unambiguously in total cell lysate ANX A1 was concentrated by immunoprecipitation and then immunoblotted with anti tyrosine antibodies (fig.21). Total cell lysate of HeLa wt preincubated or not with AG1478 and HeLa K44Adyn- cells were subjected to this immunoprecipitation. Except for differences in the intensities of the phosphoprotein bands at the molecular mass of ANX A1 (pp37) in kinase inhibitor treated cells a difference concerning the tyrosine phosphorylation during the time course could not be detected following EGF stimulation. Even in non-stimulated cells pp37 had the same intensity in phosphotyrosine blots as in stimulated samples.

Hence, a part of the intracellular ANX!A1 pool might always be present in its tyrosine phosphorylated form even in resting cells. It is possible that a residual EGF-R activity is sufficient to maintain a small part of the cellular ANX A1 pool in its tyrosine phosphorylated state.

Additional tyrosine phosphorylated proteins have been found, which co-precipitated with ANX A1. Some of them seemed to be EGF-dependently posphorylated and one of these had the molecular mass of the EGF-R (pp175). Thus, ANX A1 could be a part of a large complex of tyrosine phosphorylated proteins, which might include the EGF-R. Proteins co-precipitating with ANX A1 could be SH2- or PTB-domain presenting proteins such as Grb2 (Allridge *et al*. 1997).

When tyrosine phosphorylated proteins were precipitated with anti phosphotyrosine antibodies and ANX A1 phosphorylation could be detected, especially at 15!min, in HeLa wt cells. The effect was less obvious in K44Adyn- cells and almost absent in cells pretreated with AG1478 (fig.22A-C).

The decrease of the signal after 15!min might be due to ANX A1 dephosphorylation and/or proteolytic cleavage of the N-terminal peptide.

This experiment did not show if ANX A1 was directly precipitated with the anti phosphotyrosine antibodies or indirectly in a protein complex. Therefore, the same experiment was repeated with the difference that the immunoblot was decorated with the same anti phosphotyrosine antibody used for the immunoprecipitations (fig.22D-F). The results showed that again at 15!min an intense protein band of a tyrosine phosphorylated protein (pp35) appeared that decreased afterwards in HeLa wt cells not in K44Adyn- cells. In AG1478 pretreated cells this phosphoprotein band was not detected at all.

These contradictive results concerning ANX A1 phosphorylation are probably due to unspecific interactions of the ANX A1 antibody with other tyrosine phosphorylated proteins of the molecular mass of ANX A1 so that by using this method an elevated ANX A1 phosphorylation could not be detected.

ANX A1 appears tyrosine phosphorylated in stimulated cells after 15!min EGF treatment. After prolonged EGF stimulation, ANX A1 seems to be dephosphorylated or proteolytically cleaved at a site located C-terminally of tyrosine-21. These processes are influenced by dynamin-dependent internalization processes as the effects are not as visible in cells expressing a non-functional dynamin mutant. It remains to be seen whether or not this phosphorylation is directed or whether ANX A1 is phosphorylated by another kinases which is phosphorylated by the EGF-R.

3.6 Influence of ANX A1 on internalization and trafficking of the EGF-R in JaCro cells

In this study, the fibroblast cell line JaCro ANX A1-/- which was derived from the ANX A1 knock out mouse was used. The experiments were performed in parallel with a similarly prepared cell line from a wild type (wt) mouse.

Work published so far about the knock out cell line showed that ANX A2, 4 and 6 expression is upregulated in the ANX A1-/- cells compared to wt cells. It has further been suggested that these annexin family members could compensate the absence of ANX A1 (Croxtall *et al.* 2003). However, in this study ANX A2-expression was more pronounced in wt cells than in ANX A1-/- cells and almost equal expression levels in both cell lines were detected for ANX A6 (fig.27C&D). In addition a lower EGF-R concentration was observed in ANX A1-/- cell lysate than in wt cells (fig.27E). The alterations in the expression pattern of annexins can be due to different cell pools, to the different sera used in cell culture and to general differences in cell culture techniques.

Some differences between JaCro wt and ANX A1-/- have been described by Croxtall *et al.* (2003) that could be confirmed in the present study, namely that the proliferation rate of ANX!A1-/- cells was slower compared to wt cells, and that ANX!A1-/- cells showed a different spindle shaped-morphology. However, the cell size was similar.

As ANX A1 was localized partially in late endosomes (fig.29) and it might carry out a special function in these structures concerning EGF-R trafficking and sorting towards lysosomes, EGF-R deactivation processes in EGF stimulated cells were analyzed by quantification of the EGF-R protein levels, its phosphorylation and ubiquitination status (fig.30-32). The results showed no different behavior as far as the degradation, dephosphorylation or the ubiquitination/ deubiquitination of the EGF-R in JaCro wt or ANX!A1-/- cells were concerned. Thus ANX A1 is either not involved in EGF-R inactivation in mouse fibroblasts, or its activity is replaced by other members of the annexin protein family as discussed previously (Croxtall *et al.* 2003). Indeed, ANX!A2 was localized to membranes of the endosomal system in living BHK cells (Zeuschner *et al.* 2001, Jost *et al.* 1997), ANX A4 and 6 have been detected in multivesicular bodies prepared from rat liver (Ortega *et al.* 1998, Pol *et al.* 1997) and ANX A5 in late endosomes of J774 macrophages (Diakonova *et al.* 1997). These annexins could therefore compensate for the ANX!A1 absence.

EGF-transport was studied by confocal microscopy after incubating both cell lines with EGF-TexasRed® for 5, 30 and 60 min (fig.33). Differences in the uptake were visible, especially at 30 min EGF stimulation when in wt cells the EGF was concentrated in relatively big vesicles localized in a distinct cellular compartment around the nucleus. On the other hand the EGF positive vesicles were smaller and more diffuse throughout the cell of the ANX!A1-/- cell line. This suggests that in wt cells the EGF-R reached after 30!min an advanced stage of the endosomal trafficking, whereas in ANX A1-/- cells the trafficking was slower.

The transferrin-uptake and kinetics of pinocytosis were also compared in both cell lines (fig.34&35). The results of these experiments did not show any differences between wt and ANX!A1-/- cells, neither in transferrin uptake and transport, nor in pinocytosis.

Yet the effect of EGF accumulation in cells expressing ANX!A1 after 30!min of EGF treatment in contrast to cells lacking ANX A1 in which EGF containing endosomes appeared more dispersed in the cytosol could still be due to the higher EGF-R concentration in wt cells. So as to enhance the EGF-R expression JaCro ANX A1-/- cells were transfected with a vector encoding the human EGF-R fused to cyano fluorescent protein. The transport of EGF-TexasRed® appeared accelerated in EGF-R-CFP expressing cells as compared to untransfected ANX A1-/- cells and similar to the transport rate of EGF positive endosomes in

JaCro wt cells (fig.36). Hence, the difference in the EGF transport rate in both JaCro cell lines seemed to be primarily due to the lower EGF-R concentration.

Jacro cells were also transfected with the same ANX A1 constructs used before in HeLa cells encoding green fluorescent protein fused at the C-terminus with wt full-length ANX A1, ANX A1 core and the two ANX A1 mutants with an exchange of tyrosine-21 to alanine or glutamate.

The transfected cells were used for EGF-uptake studies to elucidate if a transient transfection of ANX!A1 in JaCro ANX!A1-/- cells would provoke an alteration in the EGF transport. However, no visible changes could be observed concerning the EGF localization at 30!min!EGF stimulation. With all constructs very few co-localizations of ANX A1-GFP positive and EGF-TexasRed® positive early endosomes (5!min) and late endosomes (30!min) were detected.

Although differences concerning the EGF transport in the fibroblastic ANX A1-/- cell line compared to wt cells are detectable, pronounced in more dipersed EGF containing late endosomal structures, biochemical EGF-R deactivation studies do not reveal pronounced differences in EGF-R degradation in cells expressing or lacking ANX A1. This could suggest that another annexin family member compensates for absence of ANX!A1.

3.7 Conclusion

The results presented here are summarized in a hypothetical scheme to show ANX A1/EGF-R interaction during intracellular EGF-R trafficking (fig.39).

ANX!A1 and the EGF-R can interact directly or indirectly in one protein complex at the plasma membrane of HeLa wt cells. This interaction, shown biochemically and by localization of ANX A1 in EGF-R clusters seems to be mediated by the protein core and does not depend on ANX!A1 tyrosine phosphorylation. However, kinase activity of the EGF-R seems to be required (1).

After short stimulation times the activation of the EGF-R triggers signal transduction cascades that provoke an increase of the Ca^{2+}-concentration. Due to the elevated Ca^{2+}, a conformational change of the ANX A1 structure can take place so that its N-terminus is more accessible to kinases and proteases. The activated EGF-R is transported to clathrin-coated pits where membrane invagination occurs. ANX A1 might be internalized or not together with the EGF-R into early endosomes (2A&B).

Early endosomes, containing the EGF-R can fuse with other vesicles, which are ANX!A1 positive or cytosolic ANX!A1 can bind to endosomal structures thereby providing a close spatial link between the internalized EGF-R and ANX!A1. The membrane bound ANX A1 fraction could be part of the protein complex assembled at the EGF-R at endosomal structures (3). At later stimulation times *i.e.* following internalization of the EGF-R, ANX A1 appeared highly tyrosine phosphorylated by the EGF-R (4B) or another kinase (4A). Phosphorylation of ANX A1 might trigger cleavage at its N-terminal domain and this process might be linked to MVB formation (5). Indeed an interaction between ANX!A1 and partially degraded EGF-R is also observed (6).

Despite the observation that ANX!A1 interacts with the EGF-R during its intracellular transport, cells lacking ANX!A1 do not show significant differences in EGF-R dephosphorylation, degradation or deubiquitination patterns. Thus ANX!A1 might be replaced by another member of the annexin family in the ANX!A1-/- cells. However, late endosomal structures containing fluorescent EGF appeared more dispersed in ANX!A1-/- cells than in ANX A1 positive cells, which show more clustered structures containing EGF internalized for 30!min. This effect could also be due to a lower EGF-R concentration.

Hence, based on these observations it is yet not possible to conclude that ANX A1 functions in regulating EGF-R trafficking.

Figure 39: *Hypothetical implication of ANX A1 in EGF-R trafficking*
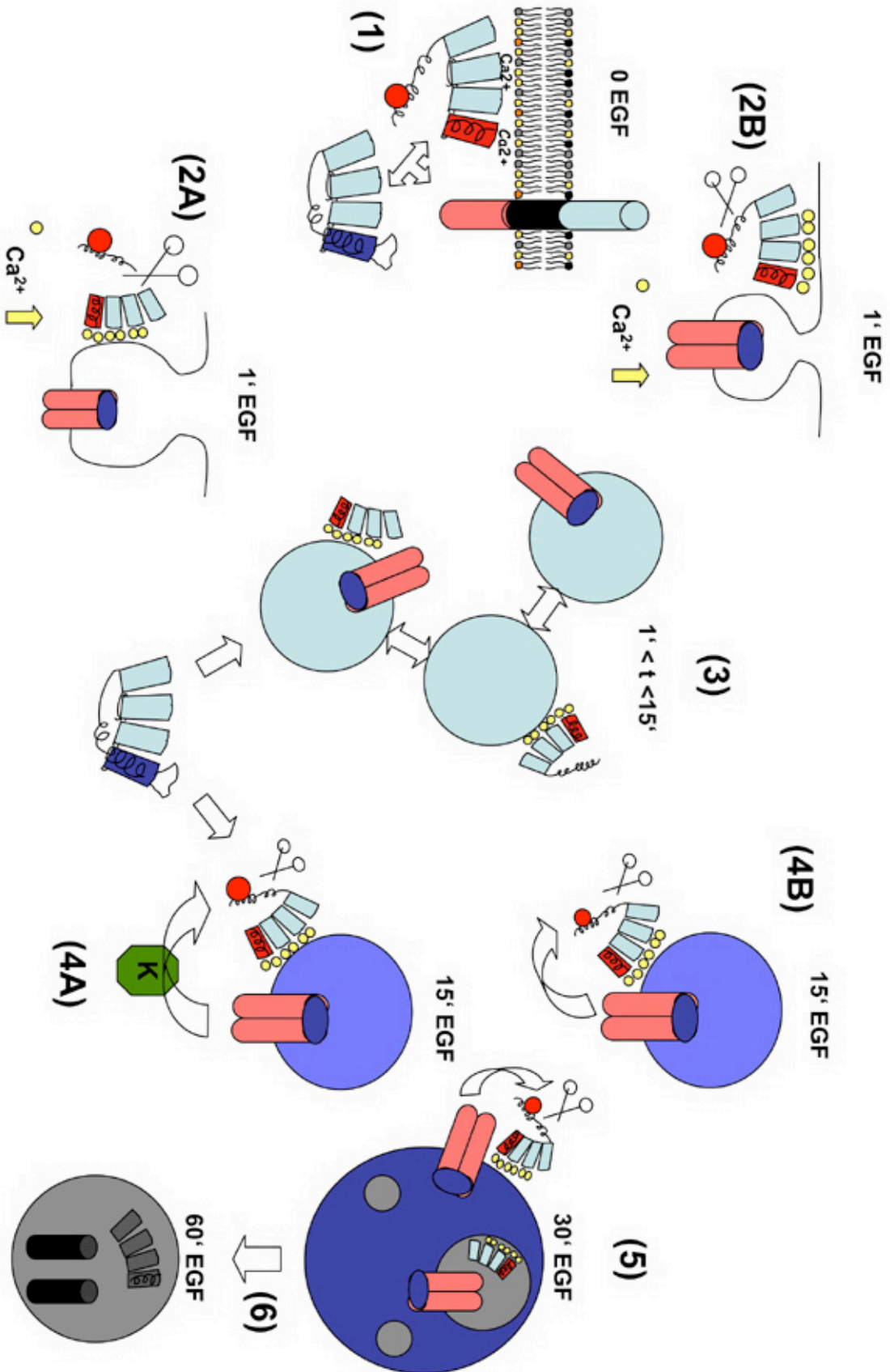
4.1 ABSTRACT

ANX A1 is a member of the annexin family of proteins that are able to interact with phospholipids in a Ca^{2+}-dependent manner. Annexins are composed of a highly conserved protein core, which can bind to membranes *via* Ca^{2+}-bridges and a domain at the N-terminus, which shows a high variability concerning sequence and length and contains specific sites for post-translational modification. ANX A1 is a substrate for several kinases including the EGF receptor (EGF-R). Phosphorylation can change biochemical properties of ANX A1 and also its sensitivity for proteolytic cleavage. The intracellular function of ANX A1 is still not understood although an involvement in cell proliferation, signal transduction, membrane aggregation and vesicle fusion during endocytic vesicular transport has been reported. In line with a role in endocytosis, full-length ANX A1 has been localized on early endosomes and only the protein core on late endosomes. Moreover, the protein seems to be present in multivesicular bodies where its phosphorylation by the EGF-R could affect the sorting of the internalized receptor to the degradative pathway.

In the present study, the role of ANX A1 in endocytic EGF-R trafficking was addressed. Using a HeLa cell line expressing an inducible dominant-negative dynamin mutant, it was shown for the first time that ANX A1 and the EGF-R are present in the same protein complex in cells stimulated with EGF and in non-stimulated cells. The ANX A1 core domain has sufficient surface to mediate this interaction. Furthermore, ANX A1 cleavage in its N-terminal domain appears to be triggered during EGF-R internalization and endocytic trafficking. The phosphorylation status of the total cellular ANX A1 pool does not seem to be affected by EGF treatment and tyrosine phosphorylated protein was even present in the absence of growth factor. However, some highly phosphorylated ANX A1 appeared to be present following 15!min of EGF treatment only in cells with functional dynamin dependent receptor endocytosis, suggesting that some ANX A1 phosphorylation might occur in endosomal structures. ANX A1 deficient cells show a slower EGF transport as compared to ANX A1 expressing cells although biochemical approaches did not reveal a retarded EGF-R degradation in ANX A1-/- cells.

In conclusion, ANX A1 seems to be present with the EGF-R during its total intracellular transport pathway ending with degradation in lysosomes and could be implicated in membrane aggregation and vesicle fusion during internalization and endosome maturation.

- 124 -

4.2 Résumé

L'annexine A1 (ANX A1) est un membre de la famille des annexines, protéines qui sont capables d'interagir avec des phospholipides en présence de Ca^{2+}. Les annexines sont composées à leur extrémité C-terminale d'un domaine conservé, le cœur, qui peut se lier aux membranes *via* des ponts calciques et à leur extrémité N-terminale d'un domaine variable en séquence et en taille. La région N-terminale contient des sites spécifiques pour des modifications post-traductionnelles. L'ANX A1 est un substrat des plusieurs kinases dont le récepteur à l'EGF (R-EGF) notamment. La phosphorylation de l'ANX A1 peut changer ses propriétés biochimiques ainsi que sa sensibilité au clivage protéolytique. La fonction intracellulaire de l'ANX A1 n'est toujours pas comprise bien qu'un rôle dans la prolifération cellulaire, la transduction du signal, l'aggrégation membranaire et la fusion vésiculaire pendant le transport endocytaire ait été rapportée. En ce qui concerne son rôle dans l'endoytose, l'ANX A1 entière a été trouvée sur des endosomes précoces alors que le cœur était trouvé sur des endosomes tardifs. De plus, l'ANX A1 semble présente dans les corps multivésiculaires où sa phosphorylation par le R-EGF pourrait avoir un effet sur le transport du récepteur vers la voie de dégradation.

Cette étude porte sur le rôle de l'ANX A1 dans le traffic endocytaire du R-EGF. Avec une lignée cellulaire HeLa exprimant un mutant dominant-negatif inductible de la dynamine, il a été démontré pour la première fois que dans les cellules stimulées à l'EGF ou non-stimulées, l'ANX A1 et le R-EGF étaient présents dans le même complexe protéique. Le cœur de l'ANX!A1 a une surface suffisante pour médier cette interaction. En outre, l'ANX!A1 apparaît clivée à son domaine N-terminal pendant l'internalisation et le traffic du R-EGF. Le ratio de l'ANXA1 phosphorylée par rapport à l'ANXA1 totale dans la cellule ne semble pas altéré par un traitement à l'EGF et même la protéine phosphorylée sur tyrosine était présente en absence du facteur de croissance. Pourtant, l'ANX A1 fortement phosphorylée apparaît après 15 minutes de traitement par l'EGF uniquement dans les cellules exprimant la dynamine fonctionnelle suggérant que la phosphorylation de l'ANX!A1 peut avoir lieu dans des structures endosomales. Dans les cellules déficentes en ANX!A1, le transport de l'EGF est plus lent que dans les cellules normales bien que des études biochimiques n'aient pas révélé une dégradation du R-EGF retardée.

En conclusion, l'ANX!A1 semble associée au R-EGF pendant tout son transport intracellulaire jusqu'à la dégradation intra-lysosomale et pourrait être impliquée dans l'aggrégation

membranaire et dans la fusion vésiculaire pendant l'internalisation et la maturation endosomale.

4.3 ZUSAMMENFASSUNG

ANX A1 ist ein Mitglied der Annexin-Proteinfamilie, die in Kalzium-abhänginger Weise mit Phospholipiden interagieren kann. Annexine bestehen aus einem hochkonservierten Proteinkern, der über Kalziumbrücken an Membranen bindet und aus einer Domäne am aminoständigen Proteinende,? die hohe Variabilität betreffend Aminosäuresequenz und Peptidlänge aufzeigt und spezifische Stellen für post-translationale Modifizierungen enthält. ANX!A1 ist ein EGF-Rezeptor-Substrat (EGF-R). Seine Phoshorylierung kann eine Veränderung biochemischer Eigenschaften und eine erhöhte Sensibilisierung für Proteolyse auslösen. Die innerzelluläre Funktion von ANX!A1 ist unbekannt, obwohl über eine Beteiligung in der Zellteilung, Signalübertragung, Bildung von Membran-Aggregaten und Vesikelfusion während Endozytose berichtet wurde. Tatsächlich ist vollständiges ANX!A1 auf frühen und der Proteinkern auf späten Endosomen lokalisiert worden. Außerdem scheint ANX!A1 in multivesikulären Körpern vorzukommen, in denen eine Phoshorylierung durch den EGF-R einen Effekt auf den Transport des Rezeptors zu Lysosomen haben kann.

In der hier präsentierten Studie wurde die Bedeutung von ANX!A1 während des EGF-R-Transports untersucht. Indem eine HeLa-Zellinie verwendet wurde, die eine induzierbare dominant-negative Dynaminmutante exprimierte, wurde gezeigt, dass ANX!A1 und der EGF-R in EGF-stimulierten oder -unstimulierten Zellen im gleichen Proteinkomplex vorkommen. Der ANX!A1-Proteinkern ist dabei eine ausreichende Interaktionsfläche. Außerdem wird ein aminoständiger Abbau von ANX!A1 während der EGF-R-Internalisierung und des endozytotischen Transports hervorgerufen. Der Phosphorylierungsstatus des gesamten zellulären ANX!A1-Vorkommens wird nicht durch die EGF-Behandlung beeinflusst. Eine Tyrosinphoshorylierung war sogar in Wachstumsfaktorabwesenheit detektierbar. Stark phosphoriliertes ANX!A1 konnte nach 15-minütiger EGF-Behandlung detektiert werden, allerdings nur in Zellen mit funktionierender dynaminabhängiger Rezeptorendozytose. Deswegen kann angenommen werden, dass die ANX!A1-Phoshorylierung in endosomalen Strukturen stattfand. ANX A1-defiziente Zellen zeigen einen langsameren EGF-Transport als ANX A1-exprimierende Zellen, obwohl biochemische Ansätze nicht gezeigt haben, dass ein verspäteter EGF-R-Abbau in ANX A1-defizienten Zellen erfolgt.

Zusammenfassend interagiert ANX!A1 mit dem EGF-R während seines gesamten innerzellulären Transports, welcher durch seinen Abbau in Lysosomen beendet wird. Dieses könnte bedeuten, dass ANX!A1 an der Aggregation von Membranen und an der Endosomenreifung beteiligt wäre.

5. MATERIALS AND METHODS

5.1 Chemicals

Cell culture media (Dulbecco's MEM/Nut Mix F-12 Ref. 21311-020 or Dulbecco's Mod Eagle Medium Ref. 41966-029), L-glutamine (BE17-605), non-essential amino acids (K0293), foetal calf serum (FCS) (A15-041), PBS with (B-L0500-I) or without calcium and magnesium (BE17-512F), trypsin (L2153) and penicillin/streptomycin (DE17-602E) solution were purchased from Gibco-BRL Invitrogen Cooperation, Biochrom AG Berlin, BioWhittaker Europe or PAA Laboratories GmbH. The antibiotics geniticin (G-9516) and tetracycline (T-7660) were purchased from Sigma. The growth factor EGF (354001) was bought from BD Bioscience.

The EGF receptor kinase inhibitor AG-1478 (658552), calpain II inhibitor (208724), calpain inhibitor I MG-101 (208719), proteasome inhibitor MG-132 (474790), pepstatin A (516482) and aprotinin (616398) were purchased from Calbiochem Merck, E64 (A2157), leupeptin (A2183), AEBSF (A1421) and PMSF (A099) from AppliChem and trypsin inhibitor (T-9253) and benzamidin (B-6560) from Sigma Albricht. The phosphatase inhibitors NaF (S-1929), $NaVO_3$ (S-6508), ß-glycerophosphate (G-6376) and sodium pyrophosphate (P-8010) were bought from Sigma.

Protein A (71-5015-85) coupled to sepharose, nitrocellulose membrane (RPN2020D), ECL protein revelation system (ECL RPN2106 or ECL Plus RPN2132), IPG buffer pH 3-10 (71-5007-36), CHAPS (17-1314-01) and immobiline drystrip pH 3-10NL (17-6001-12) were obtained from Amersham Pharmacia Biotech.

NH_4Cl (09718) was purchased from Fluka Biochemika. Deoxycholic acid (A2835), dithiothreitol (DTT) (A1101), N-ethylmaleinimid (NEM) (A2251), EDTA (A1104), EGTA (A0878), glycin (A1067), HEPES (A1069), sodium dodecyl sulfate (SDS) (A1112), Tween!20 (A1389), Tris (A1379) and ethanol (A3693) were bought from AppliChem. N,N,N',N'-tetramethyl-ethyldiamine (TEMED) (50406) was purchased from BioMol. Acrylamide mix (3029.1), ammoniumperoxodisulfat (APS) (9592.2), guanidin HCl (6069.1), urea (3941.2), imidazol (3899.1), NaCl (3957.2) and Triton X-100 (6683.1) were bought from Carl Roth GmbH. Saponin (S-7900), IGEPAL CA-630 *alias* NP-40 (I-3021), sodium azide (S-8032), iodoacetamide (I-6125) and bovine serum albumin (A-3294) were bought from Sigma Albricht. Mowiol (475904) was purchased form Calbiochem.

Prestained protein marker (#7708S) was bought from Biolabs, Coomassie®Plus protein Assay reagent kit (#23236) was purchased from Pierce and re-blot plus-strong (60512-S) or –mild (60513-S) from Chemicon International.

Texas-Red® conjugated transferrin (T-2875) and EGF (E-3480), Alexa Fluor® 488 conjugated dextran (D-22910), MitoTracker® Red CM-H$_2$XRos(M-7513) and Self-quenched BODYPY® Dye Conjugate of Bovine Serum Albumin (DQ^{TM} BSA) (D-12051) were purchased from Molecular Probes and tetramethylrhodamine B isothiocyanate phalloidin conjugate (phalloidin-TRITC) (P-1951) from Sigma.

5.2 Antibodies

Five different polyclonal and monoclonal anti-ANX!A1 antibodies made in different laboratories were used for immunoblotting (IB), immunoprecipitation (IP) and immunofluorescence (IF) studies (table 4). The ANX A1 sera were purified by affinity chromatography against their immunogens or using immobilized protein A before their application for immunofluorescence studies.

Antibodies directed against different members of the annexin family, cytoskeletal proteins, endosomal markers, the EGF and transferrin receptors and tyrosine phosphorylated proteins are listed in table 5.

Table 4: *Annexin A1 antibodies*

Antibody	Immunogen	Reactivity	Application	Reference
Sidney (rabbit)	Recombinant full-length human ANX A1 partially degraded at the N-terminus	Human, mouse, pig	IB, IP, IF	this work
r656 (rabbit)	Full-length pig ANX A1	Human, mouse, pig	IB, IP	Seemann *et al.* 1996
DE99603 (rabbit)	ANX A1 N-terminus, amino acids 15-26 (synthetic peptide)	Human, mouse	IB, IF	Dr U. Rescher ZMBE, Muenster
Dac 5 (mouse)	full-length ANX A1	human	IB, IP	Prof. Peter Kramer DKFZ, Heidelberg
αANX A1 TDL (mouse, IgG1)	ANX A1 N-terminus	Human and dog	IB, IF	Clone 29, A13920 Transduction Lab.

Table 5: List of Antibodies

Antibody	Immunogen	Reactivity	Application	Reference
Anti Annexin A2 HH7 (mouse)	ANX A2 N-terminus	Human, mouse	IB	Thiel et al. 1992
Anti Annexin A6 (rabbit)	ANX A6 full-length protein	Human, mouse	IB	Dr Russo-Marie Institut Cochin, Paris
Anti clathrin (mouse, IgG$_{2b}$/k)	Clathrin light chain	Cow, human, rat, yeast	IF	Clone con.1, Berkeley Antibody Company
Anti cytokeratin (mouse)	α-cytokeratin	Human	IF	Medac Novocastra
Anti EEA1 (mouse)	Human early endosome antigen 1	Human, dog, rat, mouse, chicken	IF	610456, clone 14 BD Biosciences
Anti EGF-R (rabbit)	Carboxy terminus of the human EGF-R	Mouse, rat, human	IB, IP, IF	1005 sc-03, Santa Cruz Biotechnology Inc.
Anti EGF-R (mouse, IgG$_{2a}$)	Cell surface epitope of human EGF- R	human	IF	528 sc-120, Santa Cruz Biotechnology Inc.
Anti GFP (mouse)	Green fluorescent protein	jellyfish	IB, IP	Prof. J. Wehland GBF
Anti Lamp-1 (mouse, IgG1)	Human Lamp-1	human	IF	Dept. of Pharmacology and Mol. Sciences, Baltimore
Anti LBPA (mouse)	Lysobisphosphatic acid	Human, mouse	IF	Prof. J. Gruenberg Geneva
Anti!phosphotyrosine (mouse Ig$_{2b\kappa}$)	Phosphotyramine-KLH		IB, IP, IF	05-321, Upstate Biotechnology
Anti TGN 46 (sheep)	Human TGN 46	Human, primate	IF	BD Transduction Laboratories
Anti transferrin receptor (IgG1, mouse)	Human transferrin receptor	Chicken, rat, mouse, chinese hamster	IB, IF	Clone H68.4 13-6800 Zymed
Anti tubulin YL1/2 (rat)	α-tubulin	human	IF	Prof. J. Wehland GBF
Anti ubiquitin (IgG1κ, mouse)	Bovine ubiquitin	Human, bovine, mouse, *C. elegans, Drosophilia*	IB	13-1600, !Zymed
Anti vimentin (mouse)	α-vimentin	Human, hamster	IF	Immunotech

The following secondary antibodies for immunofluorescence experiments were bought from Jackson ImmunoResearch Laboratories: TexasRed® conjugated goat anti-mouse IgG (H+L) (115-076-003), Cy™2 conjugated goat anti-mouse (H+L) (115-225-068), TexasRed® conjugated goat anti-rabbit IgG (H+L) (111-075-003), Cy™2 conjugated goat anti-rabbit IgG (H+L) (111-225-144), Cy™2 conjugated donkey anti-sheep IgG (H+L) (713-225-147), Rhodamin (TRITC) conjugated donkey anti-sheep IgG (H+L) (713-025-003) and TexasRed® conjugated goat anti-rat IgG (H+L) (112-075-062).

Horseradish peroxidase coupled secondary antibodies (anti-mouse NA9310V or anti-rabbit NA9340V) from Pharmacia Amersham Biotech were used for immunoblotting.

5.3 Molecular Biology

5.3.1 Bacterial strains and plasmids

Bacteria strains

The HB101 *E. coli* strain (BD Biosciences Clontech) was used for plasmid amplification and the BL21(DE3) *E. coli* strain (Invitrogen Life Technologies) for expression of recombinant ANX A1. Both strains were cultivated at 37 °C in LB medium (1!% trypton, 1!% yeast extract and 0.5!% NaCl).

Plasmids

The plasmid pGex-2T-anx a1, kindly donated by Dr F. Russo-Marie (Institut Cochin, Paris) encoded the gene for full-length human ANX A1 coupled to GST. A thrombin cleavage site was located between the fused proteins. It was used for ANX A1 expression and purification. The plasmid phegf-r-CFP, kindly given by Dr N. Zobiack (ZMBE, Institute for Medical Biochemistry, Münster), encoded the human EGF-R as a fusion protein with the cyano fluorescent protein. The plasmids pEGFP-N3 (6080-1, Clontech), panx a1 full-length-GFP and panx a1 core-GFP were kindly provided by Dr U. Rescher (Rescher *et al.* 2000). The plasmids panx!a1!Y21→A-EGFP and panx!a1!Y21→E-EGFP were made by Judith Austermann (Judith Austermann, 2003). The ANX!A1-GFP constructs were used to localize the different green-fluorescent ANX!A1 proteins (wt, truncated at the N-terminus, exchange of the potential EGF-R phosphorylation site) in eucaryotic cell systems. The plasmids panx a3 full-length-GFP and panx a6 full-length-GFP were kindly provided by Dipl. Biol. Daniela Ruhe and Dipl. Biol. Carsten Ludwig (ZMBE, Institute for Medical Biochemistry, Münster).

5.3.2 Preparation of competent *E. coli* for transformation by thermal shock

During the preparation of competent bacteria no antibiotics were applied. Bacteria were seeded on LB agar (1.5!% w/v agar in LB medium) and incubated over night. The colony of one bacterial clone was transferred into 5!ml LB medium and cultivated over night (preculture). 500!ml LB medium was inocculated with the pre-culture and cultivated until an optical density of 0.6 at 600!nm was reached. The bacteria were centrifuged for 20 min at 6,000 rpm (Beckmann rotor JA-10). The resulting pellet was resuspended in 125!ml $CaCl_2$-buffer (100!mM) and incubated at 4!°C for 20!min. The bacterial mass were centrifuged as before and resuspended in 12.5!ml $CaCl_2$-buffer containing 15!% glycerol. The now competent cells were shock-frozen in liquid nitrogen and kept at –80!°C.

5.3.3 Transformation of competent *E. coli* by thermal shock

Competent bacteria (50!μL) were defrosted on ice and mixed with 1!μg plasmid DNA. Subsequently, the bacteria were incubated on ice for 1!min then incubated at 37!°C for 5!min and again on ice for 2!min. 50!μl of LB medium containing the antibiotic corresponding to the resistance encoded by the applied vector was added to the transformed bacteria. The bacteria were cultivated at 37!°C for 30 min. Transformed *E. coli* were seeded on LB agar and incubated over night at 37!°C in antibiotic presence.

5.3.4 Amplification of plasmid DNA

For the amplification and analysis of plasmid DNA, the Plasmid Maxi Kit (12163) and the plasmid purification protocol for high copy plasmids developed by the company Quiagen, were used.

5.4 Protein Biochemistry

5.4.1 Protein purification

BL21(DE3) *E. coli* bacteria transformed with pGEX-2T-anx a1 were cultivated at 37!°C in 5!ml LB medium for 8!hrs in the presence of 100 μg/ml ampicilin (pre-culture). The pre-culture was diluted 1:100 in a 100 ml-over night culture (ON-culture). ANX A1 expression cultures of 500!ml were started with 5!ml of the ON-culture. Induction of ANX A1 expression was realized with 1!mM IPTG when optical density of the bacterial suspension was between 0.6 and 0.8 at 600!nm. After 2!hrs of further cultivation, the bacteria were harvested, centrifuged for 15!min at 6,000 g, resuspended in PBS and centrifuged again.

50 ml lysis buffer (50 mM Tris pH 8, 2 mM EDTA, 100 mM NaCl, 1!% NP-40, 10!% glycerol, 1!mM DTT, 20 μg/ml aprotinin, 2 μg/ml leupeptin and pepstatin, 40 μg/ml trypsin inhibitor and 1 mM PMSF) per 10!g of bacterial mass were used to resuspend the pelleted bacterial mass after centrifugation. 0.5 mg/ml of the enzyme lysozym (05934, BioMol) was added, followed by incubation on ice for 40 min, sonication and centrifugation at 150,000 g and 4!°C for 1 h and 30 min.

The following purification was carried out using the fast protein liquid chromatography system ÄKTAFPLC from Amersham Bioscience (18-1118-67). After centrifugation, the

supernatant was filtered and injected into a GSTrap Fast Flow Column prepacked with Glutathione Sepharose (17-5130, Amersham Bioscience). The column material was washed with 3 volumes of the column TBS buffer (50 mM Tris-HCl pH 8, 100 mM NaCl), 2 volumes of TBS Na# (50 mM Tris-HCl pH 8, 500 mM NaCl) and again with 3 volumes of TBS buffer. Then the column content was incubated for 1 h at room temperature with 500 international units of thrombin (Sigma) per 1 mg GST coupled protein. The thrombin solution with cleaved proteins was collected and EDTA and EGTA were added to a final concentration of 1 mM. Before applying to an ion exchanger, the buffer had to be changed from the Tris/NaCl buffer system to 50 mM MES pH 5.5 (buffer A) using a HiPrep 26/10 Desalting Column (17-5087-01, Amersham Bioscience). The sample was injected into the desalting column and washed with the buffer A until the salt of the TBS buffer was eliminated and the proteins left the desalting matrix in buffer A. The cation exchanger Resource S (17-1178-01, Amersham Bioscience) was used for the last purification step. The collected fractions were injected into the Resource S column. The ion exchanger was washed with buffer A before a gradient from 0 to 1 M NaCl (buffer B: 50 mM MES pH 5.5, 1 M NaCl) over three volumes of the column was applied. The resulting purified protein was analyzed by SDS polyacrylamid gel electrophoresis and immunoblotting. The protein was used for the production of polyclonal ANX A1 antibodies and for the purification of the ANX A1 sera by specific antigen affinity chromatography.

5.4.2 Production of anti ANX A1 antibodies by lymph node injection

The procedure was performed as described previously (Siegel *et al.* 1983). 200!µl of a 2.5!% solution of Evans Blue was injected subcutaneously between two toes of a rabbit one hour before surgery. The rabbit was anesthetized by very slowly injecting sodium pentobarbital (64.8!mg/ml) diluted to 25!% (v/v) with isotonic saline into the ear vein. The protein solution was diluted to 20-100!µg/ml and an equal volume of Freund's adjuvant complete was added and mixed thoroughly. The lymph nodes were exposed 20-30!min after anesthesia. In each lymph node, 0.1-0.4 ml of the prepared immunogen mixture was injected. The rest of the mixture was injected intradermally in the back along the spine. Three weeks later, approximately 100!µg of the immunogen protein was mixed with an equal volume of Freund's adjuvant incomplete and then injected into the subscapular cavity. One week later, 50!µg of the immunogen diluted in saline solution was injected again followed one day later by an ear

vein injection with the equivalent amount of protein. Ten days later, the rabbit was bled and the serum was collected after 20!min centrifugation at 1,300!g.

5.4.3 Purification of ANX A1 sera

The polyclonal anti ANX A1 sera, directed against full-length ANX A1 (Sidney and r656) and the N-terminal domain of ANX A1 (DE99603) were purified by specific antigen affinity chromatography or by protein A affinity chromatography.

5.4.3.1 Purification of ANX A1 sera by specific antigen affinity chromatography

Coupling of purified ANX A1 protein to activated CNBr⁻ sepharose
0.5 g cyanogen bromide-activated-Sepharose 4 B (CNBr⁻ sepharose) (C 9142, Sigma Aldrich) were swollen in 10 ml of 1 mM HCl at 4°C for 20 min and washed with coupling buffer (100 mM $NaHCO_3$ pH 8.3 and 500 mM NaCl). The ANX A1 serum was diluted (1:10) with coupling buffer and incubated with the prepared CNBr⁻-sepharose beads over night at 4!°C. At the end of the coupling procedure the beads were centrifuged and the quantity of coupled proteins analyzed by Bradford protein quantification. The free CNBr⁻-sides were saturated with ethanolamine solution (6.1!% in coupling buffer) for 2 hrs at room temperature. After the blocking procedure the beads were washed twice with acetate buffer (100 mM sodium acetate pH 4.0 and 500 mM NaCl), once with coupling buffer and stored in PBS containing 0.02!% NaN_3.

Purification of anti-ANX A1 sera
The ANX A1 containing beads were equilibrated with 10 ml buffer A (20 mM Tris HCl pH 7.5) and transferred into a 20 ml-Econo-Pac column (732-1010**,** Biorad). 1 ml of ANX A1 serum was mixed with 9 ml buffer A and filtered. The diluted serum was put on the ANX A1 beads and the flow through was applied again four times onto the column. The beads were washed with 10 ml buffer B (500 mM NaCl in buffer A). The elution of the bound antibodies was realized with 2 ml of buffer C (3.5 M $MgCl_2$ in buffer A) and the eluted protein solution dialyzed immediately against PBS containing 0.02!% NaN_3. The purified antibodies were stabilized by adding of 2 mg/ml BSA and stored at –20!°C.

Purification of the anti ANX A1 serum DE99603 directed against an N-terminal peptide
The purification of DE99603 was realized using the ÄktaFPLC system. The column HiTrap NHS-activated was bought from Amersham Bioscience (17-0716-01). The coupling of the

peptide Lys-Gly-Asn-Glu-Glu-Gln-Glu-Tyr-Val-Gln-Thr-Val (synthesized by Neosystem Strasbourg, part number SP012110) to the column material was done by the company Bioatlantic SARL (France).

The purification procedure was the same as described before for the purification of full-length ANX A1 serum with the difference that for all steps the pumps of the ÄktaFPLC system were used at a speed of 0.5!ml/min.

5.4.3.2 Purification of ANX A1 sera by protein A affinity chromatography

The purification of polyclonal ANX A1 sera by protein!A affinity chromatography was also carried out using the ÄktaFPLC system. The column HiTrap Protein A HP was bought from Amersham Bioscience (17-0402-03). 1!ml serum was mixed with 9!ml binding buffer (20!mM sodium phosphate pH 7) and was injected into the protein A sepharose column equilibrated with binding buffer at a flow rate of 1!ml/min. The flow through was incubated again twice with the column material. After a column wash with binding buffer and high salt buffer (500!mM NaCl in binding buffer) the bound proteins were first eluted with 3!ml elution buffer A (0.1!M citric acid NaOH pH 5) and then with 3!ml elution buffer B (0.1!M citric acid NaOH pH 3). The pH of the eluted fractions was directly neutralized with a 1!M Tris-solution of pH 9 and dialyzed against PBS containing 0.02!% NaN$_3$. The antibody solutions were tested by immunoblotting and indirect immunofluorescence studies.

5.4.4 Immunoprecipitation

Cells were EGF stimulated and the lysates prepared as described in chapters 5.5.2 and 5.5.3. The same amount total protein (in most cases 1!mg) of every sample was incubated with 1-4!µg antibody for at least 2!hrs or over night at 4!°C. Samples were centrifuged and the supernatant incubated for two hours with RIPA buffer equilibrated protein A sepharose. Finally, complexes containing protein A sepharose, IgG and directly and indirectly precipitated proteins were washed 3 times with RIPA buffer and subjected to immunoblotting analysis.

5.4.5 Immunoblotting

30-60!μg of total lysate protein or immunoprecipitated proteins were boiled for 5 min at 95 °C in Laemmli buffer (final concentration: 62.5!mM Tris-HCl pH!6.8, 2!% w/v SDS, 10!% glycerol, 300!mM β-mercaptoethanol and 0.1!% bromphenol blue) and separated by Tris/glycine SDS-polyacrylamide gel electrophoresis (electrophoresis buffer: 25 mM Tris, 192 mM glycin, 0.1% SDS) using 8-12!% polyacrylamide gels (table!6). Separated proteins were transferred to a nitrocellulose membrane for 1!hr at 100!V (transferring buffer: electrophoresis buffer containing 20!% ethanol). Before incubating the membrane with one of the primary antibodies of table 7, the membrane was saturated with 5!% nonfat milk or 3!% BSA in TBS-T buffer (50!mM Tris pH 7.5, 150!mM NaCl, 0.5!% Tween 20). HRP coupled secondary antibody at a dilution of 1:10,000 was used and the protein bands were revealed using the ECL or ECL Plus-System.

When immunoblotting had been performed with the ubiquitin antibody the membrane was incubated in denaturing buffer (6!M guanidine-HCl, 20!mM Tris-Hcl pH 7.5, 5!mM beta-mercaptoethanol and 1!mM PMSF) for 1!hr at 4°C followed by extensive PBS washing.

5.4.6 Membrane Stripping

In case immunblots had to be performed with several different antibodies, the membranes were either treated for 15 min at room temperature with re-blot reagent from Chemicon International or incubated for 30 min at 50!°C with stripping buffer (62.5!mM Tris pH 6.5, 2!% SDS, 100!mM ß-mercaptoethanol). After the stripping procedure had been performed the membrane was washed with water and TBS-T extensively before saturation and incubation with the antibody.

Table 6: Solutions for Tris/glycine SDS-polyacrylamide gel electrophoresis

	Stacking Gel (3!ml)	Separating Gels		
		8!% (5!ml)	10!% (5!ml)	12!% (5!ml)
H_2O	2.1	2.3	2.0	1.7
Acrylamid 30!%[*]	0.5	1.3	1.7	2.0
1 M Tris pH 6.8	0.38	-	-	-
1.5 M Tris pH 8	-	1.3	1.3	1.3
SDS 10 %	0.03	0.05	0.05	0.05
APS 10 %	0.03	0.05	0.05	0.05
TEMED	0.003	0.003	0.002	0.002

[*] commonly 29.2!% acrylamide and 0.8!% N, N'-methylene-bis-acrylamide

Table 7: Primary antibodies for immunoblotting

Antibody	Species	Dilution
DE99603	Rabbit	1:1,000
HP 1531	Rabbit	1:500
Dac 5	Mouse	1:1,000
α ANX A1 (TDL)	Mouse	1:1,000
Anti full-length ANX A1 serum	Rabbit	1:1,000
Anti ANX A2	Mouse	1:2,000
Anti ANX A6	Rabbit	1:1,000
Anti EGF-R	Rabbit	1:250
Anti GFP	Mouse	1:250
Anti phosphotyrosine antibody, clone 4G10	mouse	1:1,000
Anti S100A11	Rabbit	1:10
Anti transferrin-receptor	Mouse	1:1,1000
Anti ubiquitin (Ubi-1)	mouse	1-3 µg/ml

5.4.7 Isoelectric Focusing

Cells were incubated with 30 μM calpain II inhibitor (calpain inhibitor IV), or with the same volume DMSO for 2 hrs at 37 °C and then left unstimulated or EGF treated for 15 min. Lysates were made with 300 μl per petridish ($\varnothing$ = 10 cm) high urea buffer (8 M urea, 40 mM Tris-HCl pH 7.5, 10 mM EGTA, 20 mM DTT, 2 % Chaps, 0.5 % IPG solution and protease and phosphatase inhibitors as described in chapter 5.5.3).

300 μg of total protein was put on rehydrated Immobiline DryStrip pH range 3-10NL by the cup loading method with the following electrofocusing protocol: 0-50 V for 1 min, 50 V for 1 hr, 50-200 V for 30 min, 200 V for 2 hrs, 200-3500 in 1 hr and 30 min and 3500 V for 5 hrs. For the second dimension the strips were first incubated in equilibration buffer (6 M urea, 2 % SDS, 50 mM Tris-HCl pH 8.8 and 30 % glycerol) containing 1 % DTT and then in the same buffer containing 4.8 % iodoacetamid, each time for 15 min. The strips were bedded in recovery solution (0.5 % agarose, 25 mM Tris, 192 mM glycine, 0.1 % SDS and 0.03 % bromophenol blue) on 10 % acrylamide gels (table 6) and electrofocused proteins were separated and immunoblotted under normal SDS-PAGE conditions (chapter 5.4.5).

5.5 Cell biology

5.5.1 Cell lines

All cell lines were cultivated in the presence of 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell culture media, concentrations of decomplemented FCS (heated for 25 min at 56 °C) and special supplements were used as indicated in the following paragraphs.

HeLa wt/HeLa K44Adyn- cells
This special human epithelial HeLa cell line expressing a non-functional dynamin mutant under tetracycline control was kindly donated by Dr S. Schmid (Departement of Cell Biology, The Scripps Research Institute, La Jolla, C1A 92037 USA). Cells were grown at 37 °C and 5 % CO_2 in DMEM medium supplemented with 5 % FCS, 400 μg/ml geneticin and 1 μg/ml tetracycline. The expression of the non-functional dynamin mutant was induced by deprivation of tetracyclin for at least 48 hrs. The HeLa wildtype cells expressing functional

dynamin were cultivated in the presence and HeLa K44Adyn- cells in the absence of tetracyclin.

JaCro ANX A1-/- cells

This cell line with fibroblastic character made from lung tissue of the ANX A1 knock out mouse was kindly donated by James Croxtall (Department of Biochemical Pharmacology, William Harvey Research Institute, University of London – Croxtall *et al.* 2003). They were grown in DMEM/Nut Mix F-12 medium with 10!% FCS and non-essential amino acids.

5.5.2 EGF stimulation of eucaryotic cells

Cells were grown until 70-80 % confluence, then serum starved for at least 16!hrs and stimulated with 50!ng/mL EGF. As indicated the cells were previously incubated with different inhibitors: for 2!hrs with 30!μM calpain II inhibitor or for 15!min with 15!μM AG1478 at 37!°C.

5.5.3 Extraction of eucaryotic cells

Before cell extraction, the cell monolayers were washed with ice cold PBS and incubated on ice with RIPA buffer (50!mM Tris, 150!mM NaCl, 2!mM EDTA, 2!mM EGTA, 0.5!% DOC, 1!% NP-40, proteatase inhibitors: leupeptin, E64, pepstatin and aprotinin at a concentration of 10!μg/ml, 40!μl/ml trypsin inhibitor, 4!mM AEBSF, 10!μM benzamidin and MG-101, phosphatase inhibitors: 50!mM NaF, 10!μM NaVO$_3$, 2!mM sodium pyrophosphate and 10!mM β-glycerophosphate) for 30!min. If the ubiquitination of the EGF-R had to be studied in the following experiment, 10!nM of MG-132 and 10!mM NEM were also applied to inhibit ubiquitin-conjugating enzymes. Cell lysates were centrifuged for 20!min at 21,460!g and 4!°C. The total protein content of the supernatant was determined by means of Bradford protein quantification.

5.5.4 Transfection of eucaryotic cells with plasmid DNA

The plasmids panx!a1!full-length-EGFP and panx!a1!core-EGFP, panx!a1!Y21→A-EGFP and panx a1!Y21→E-EGFP, panx!a3-EGFP, panx!a6-EGFP and phEGF-R-CFP were used to transfect Jacro cells by electroporation and HeLa cells by the calcium phosphate method.

Electroporation

1/3 to 1/2 of the cells of one 10!cm Ø -petri dish cultivated until 80!% confluency were used for one transfection. Cells were trypsinized, centrifuged, resuspended with 500!µl of the corresponding complete medium and mixed with 10-15!µg of plasmid DNA in a special electroporation cuvette. Cells were porated by an electronic pulse at 250!V and 900!µF and seeded on cover slips in a 6!cm Ø -petri dish. The cell culture medium was changed 4!hrs after electroporation and the transfected cells were used for experiments 24!hrs after electroporation.

Calcium phosphate method

Cells were cultivated on cover slips in 6-well plates until 60!% confluence. 3!hrs before transfection the cell culture medium was replaced with 3 ml per well fresh medium. 1-3!µg DNA was diluted in 150!ml Hepes buffer (HBS: 20!mM HEPES pH 7, 140!mM NaCl, 5!mM KCl, 0.75!mM Na_2HPO_4, 0.1!% glycerol) and mixed with 10!µl $CaCl_2$ solution (2M). The mixture was incubated at room temperature for 30 min and added to the culture medium of one well. 6!hrs after the transfection the medium had to be replaced with fresh medium. After 24 to 36 hrs the transfected cells were used for microscopy analysis or immunoprecipitation.

5.5.5 Transfection of eucaryotic cells with siRNA duplexes

The following siRNA sequences were used for intracellular silencing of ANX!A1-synthesis (sense) or as control for the ANX!A1-silencing (invsense):
senseANX A1siRNA: 5'-rGrGrGrGrArCrArGrArCrGUrArArArCrGUrGTT-3'
invsenseANX A1siRNA: 5'-rGUrGrCrArArAUrGrCrArGrArCrArCrArGrGrGTT-3'

Cells were seeded on coverslips in 24-well plates and cultivated until 30-50!% confluency. 2!µl oligofectamine™ reagent (12252-011, Invitrogen life technologies) were mixed with 5.5!µl medium without antibiotics and incubated at room temperature for 5-10 min. 1.25 µl siRNA-duplex (20 µM) were diluted in 40!µl medium without antibiotics and added to the

oligofectamine mixture. SiRNA/oligofectamine complexes were formed within 20 min at room temperature and added to 200!µl fresh medium without antibiotics over the cell monolayer. After incubation for 4 hrs of incubation at 37!°C, 200 µl complete medium was added. Cells could be used for experiments 48!hrs after transfection.

5.5.6 Indirect immunofluorescence

Cells were grown on coverslips until 75!% confluency. The cell fixation was performed with 4!% (w/v) paraformaldehyde (PFA) in PBS for 20 min. The cells were then incubated with 50!mM NH_4Cl in PBS for 10!min and permeabilized with saponine (permeabilization medium: 0.05!% (w/v) saponine and 1!% BSA in PBS) for 5!min. The permeabilization medium was also used for antibody dilutions and the washing steps. The incubation of the cells with primary antibody (table 8) was performed at room temperature for 60!min. The cells were washed three times and incubated at room temperature for 45!min with a secondary antibody coupled to the fluorescent markers FITC, TRITC, Cy2 or TexasRed® at a dilution factor of 1:400. Lastly the cells were washed two times in permeabilization medium, twice in PBS and very briefly in water. Coverslips were mounted in Mowiol containing 4!% n-propyl gallate as antifade agent (P-3130, Sigma Aldrich) and analyzed using a epifluorescence (Leica DM RXA) or confocal laser scanning microscope (Zeiss LSM 510 Meta).

If cells transfected with ANX!A1!GFP-constructs were used for immuno-fluorescence studies, a different combined fixation/permeabilization technique was applied. For this method the cells were washed with cold PBS and incubated on ice with 0.2!% Triton X-100 in 4!% PFA/PBS Ca^{2+}/Mg^{2+} solution for 5!min. The cells were then washed once with PBS Ca^{2+}/Mg^{2+} and incubated with 4!% PFA for 15!min at room temperature. Afterwards the cells were incubated with 50!mM NH_4Cl in PBS for 10!min and the antibody diluted in PBS containing 2!% BSA, could be put on the cells without any further permeabilization step being necessary.

Table 8: Primary Antibodies used for immunofluorescence studies

Antibody	Dilution	Species
Anti full-length ANX A1[1], Sidney	1:50	Rabbit
Anti full-length ANX A1[2], Sidney	1:10	Rabbit
DE99603 [2]	1:2	Rabbit
Anti ANX A1 (TDL)	1:50	Mouse
Anti α-cytokeratin	1:10	Mouse
Anti clathrin, light chaine	1:100	Mouse
Anti EGF-R	1:20	Rabbit
Anti EEA1	1:100	Mouse
Anti LBPA	1:50	Mouse
Anti Lamp 1	1:20	Mouse
Anti TGN 46	1:100	Sheep
Anti transferrin receptor	1:100	Mouse
Anti α-tubulin	1:30	Rat
Anti α-vimentin	1:20	Mouse

1) purified by protein A affinity chromatography
2) purified by specific antigen affinity chromatography

5.5.7 Stimulation of eucaryotic cells with EGF-Texas Red®

JaCro or HeLa cells were cultivated on cover slips and serum starved over night before the stimulation experiment. EGF coupled to the fluorescent dye TexasRed® (stock solution 200!µg/ml) was diluted to a final concentration of 2!µg/ml in cell culture medium without serum containing 1!% BSA. Cells were incubated for 5, 30 and 60 min in the EGF-TexasRed® solution at 37!°C. Fixation and permeabilization procedures were the same as described in chapter 5.5.6.

5.5.8 Clustering of the EGF receptor

HeLa wt cells were transfected with different constructs encoding ANX A1, ANX A3 or ANX A6 coupled to GFP. The cells were serum starved over night and then incubated for 15!min at 37!°C with anti EGF-R antibody directed against the EGF binding domain (528 sc-120), diluted 1:100 in medium without FCS containing 1!% BSA. The cells were washed with PBS and then incubated for 15!min with the secondary antibody conjugated with the fluorescent dye Texas Red® (dilution factor 1:100). The cells were washed, fixed and permeabilized by the combined PFA/Triton-method as described in chapter 5.5.6.

5.5.9 Labeling of different intracellular organelles and proteins of the cytoskeleton

In most cases ANX A1-GFP transfected cells were used for the ANX A1 localization studies. After labeling the cells were fixed and permeabilized as described previously.

Golgi complex

For the labeling of the Golgi complex an antibody directed against a glycoprotein designated TGN46 (table 8) that was known to be primarily localized in the Trans-Golgi network was used.

Lysosomes

The cells were incubated for 1!hr with 0.1!mg/ml DQ^{TM} BSA ($\lambda_{abs-max}$ 590!nm, $\lambda_{emm-max}$ 620!nm) under normal cell culture conditions. Afterwards, the cells were washed with warm PBS and the culture was performed over night under normal conditions in the absence of DQ^{TM} BSA.

Early endosomes and recycling compartment

Starved cells were incubated with 50!µg/ml transferrin-TexasRed$^{®}$ ($\lambda_{abs-max}$ 595!nm, $\lambda_{emm-max}$ 615!nm) for 5, 30 and 60!min respectively in serum-free cell culture medium. Subsequently, the cells were washed and fixed immediately.

Pinocytosis studies

1!mg/ml dextran-Alexa Fluor$^{®}$ 568 ($\lambda_{abs-max}$ 578!nm, $\lambda_{emm-max}$ 603!nm) was applied to living cells for 5, 30 and 60!min under normal cell culture conditions. The labeling was completed as described for transferrin-treated cells.

Cytoskeleton

   Actin

Fixed and permeabilized cells were incubated with 1!µg/ml phalloidine-TRITC (λ_{abs} 540-545!nm, λ_{emm} 570-573!nm) for 1!hr, washed with PBS and then mounted in Mowiol containing 4!% n-propyl gallate as antifade agent.

   Tubulin, vimentin and cytokeratin

In order to visualize the three cytoskeleton proteins α-tubulin, α-vimentin and α-cytokeratin, the antibodies indicated in table 8 were used and the immunofluorescence technique of the chapter 5.5.6 was applied.

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Curriculum Vitae

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Work Experience

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09/01/1999 – 07/15/2000	**DEA Internship** INRA, Jouy-en-Josas, France 'Protein kinases C and non-classical action of estrogen' Supervisor: Dr Lieberherr
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Publications and posters

Debret, R., Btaouri, H., Duca, L., Rahmann, I., **Radke, S,** Haye, B., Sallenave, J. M., Antonicelli, F. (2003) Annexin A1 processing is associated with caspase-dependent apoptosis in BZR cells. FEBS Letters 546: 195-202.

Presentation of the poster 'The role of annexin A1 (ANX A1) in EGF stimulated cell proliferation' at the ELSO Meeting in Nice 2002 and ECS Meeting in Brussel 2002.

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