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The Role of the Small GTPase Rab11 in Glucose Transport in H9c2 Cardiomyocytes

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ABSTRACT

Members of the Rab family of Ras-like small GTPases have been proposed to participate in the insulin-regulated redistribution of glucose transporter GLUT4. Recent studies suggested that Rab11 was colocalized with GLUT4 in rat heart and may be involved in the movement of GLUT4 to the plasma membrane in response to insulin. However, the exact role for this small GTP-binding protein in GLUT4 translocation and the underlying mechanisms remain unknown.

Here, a wild type cardiomyocyte line H9c2 (H9) and a stable clone H9K6 (K6) overexpressing GLUT4 were used to further investigate the role of Rab11 in glucose transport. First, reverse transcription-polymerase chain reaction (RT-PCR) was employed to observe the influence of insulin on the transcription of Rab11 gene. Then, transient transfection strategy was established to study the effects of Rab11 on glucose transport. Finally, wortmannin and bisindolylmaleimide (BIM) were used to explore possible functional associations of Rab11 with PI 3-K (phosphatidylinositol 3-kinase) and PKC (Protein Kinase C).

The results from RT-PCR confirmed the presence of Rab11 mRNA in K6 cells. Insulin was able to upregulate the transcription of Rab11 gene by about 90%. 2-Deoxy-glucose uptake assay revealed that: (1), overexpression of Rab11 increased basal glucose uptake by 40% ± 6% in H9 and 30 ± 8% in K6 cells, (2), under this conditions, however, insulin-stimulated glucose uptake were 20% in H9 and 45% in K6 cells over basal level compared with 40% and 70% in the controls transfected with blank vectors. (3), the effects of Rab11 on glucose uptake could be blocked completely by PKC inhibitor, BIM, whereas wortmannin, the PI 3-K inhibitor had no effects. Western-blot demonstrated that Rab11 did not affect the total expression of GLUT4, indicating the increase of glucose uptake is probably due to the enhanced translocation of GLUT4 to the plasma membrane. BIM treatment immediately after transfection had no influence on the expression of Rab11.

These results indicate that Rab11 may be involved in the translocation of GLUT4, but its precise role in insulin signal cascade is still not determined. Although Rab11 does not participate in the PI 3-K-dependent pathway for glucose transport, it might be functionally associated with PKC in regulating glucose uptake.

Die Rolle des niedermolekularen GTP-bindenden Proteins Rab11 beim Glukosetransport in H9c2-Herzmuskelzellen.

(Tianyun Jiang)

ABSTRAKT

Es wird vermutet, daß Mitglieder der niedermolekularen G-Proteine der Rab-Subfamilie an der Umverteilung des Glukosetransporters GLUT4 teilhaben. Neuere Studien deuten auf eine Lokalisation von Rab11 in GLUT4-Vesikeln im Herzmuskel der erwachsenen Ratte hin und möglicherweise ist Rab11 an der Translokation von GLUT4 von einem intrazellulären Pool zur Plasmamembran nach Insulinstimmulation beteiligt. Unklar bleiben allerdings die genaue Rolle der niedermolekularen GTPasen in der GLUT4-Translokation und die zugrunde liegenden Mechanismen.

In der vorliegenden Arbeit wurden eine Herzmuskelzelllinie des Wildtyps H9c2 (H9) und ein stabiler Klon H9K6 (K6), der GLUT4 überexprimiert, als Modelle benutzt, um die Rolle von Rab11 im Glukosetransport zu untersuchen. Zuerst wurde RT-PCR angewandt, um den Einfluß von Insulin auf die Rab11 mRNA-Expression zu beobachten. Dann wurde die transiente Transfektionstechnik etabliert, um den Effekt von Rab11 auf den Glukosetransport zu studieren. Wortmannin und BIM wurden schließlich benutzt, um ein mögliches funktionales Zusammenwirken von Rab11 mit PI 3-K und PKC zu erforschen.

Die Ergebnisse der RT-PCR konnten die Anwesenheit von Rab11 mRNA in K6 Zellen nachweisen. Insulin kann die Transkription des Rab11 Gens um ungefähr 90% überregulieren. Messungen der 2-Deoxy-D-Glukoseaufnahme zeigen: (1) Bei einer Über-expression von Rab11 steigt die Glukoseaufnahme um 40 ± 6% in den H9 und um 30 ± 8% in den K6 Zellen. (2) Unter den gleichen Bedingungen beträgt jedoch die insulinstimulierte Glukoseaufnahme in H9-Zellen nur 20%, sowie 45% in K6 Zellen in Bezug auf den Basalwert. Wohingegen in pCMV-transfizierten Kontrollzellen nach Insulinstimulus ein Anstieg der Glukoseaufnahme um 40% bzw 70% gemessen werden konnte. (3) Der Effekt von Rab11 auf die Glukoseaufnahme konnte mit dem PKC Inhibitor BIM komplett blockiert werden, wohingegen Wortmannin, ein PI 3-K-Inhibitor, keinen Effekt zeigte. Der Western-Blot zeigt, daß Rab11 nicht die totale Expression von GLUT4 beeinflußen kann, und somit die erhöhte Glukoseaufnahme auf die gesteigerte Translokation von GLUT4 zu der Plasmamembran zustandekommt. Die sofortige Behandlung der transfizierten Zellen mit BIM für zwei Tage hat keinen Einfluß auf die Rab11-Expression.

Unsere Daten zeigen, daß Rab11 möglicherweise an der Translokation von GLUT4 beteiligt ist, jedoch konnte seine präzise Rolle in der Insulinsignalkaskade nicht genau bestimmt werden. Außerdem unterstützen die gezeigten Daten die Annahme, daß Rab11 funktionell mit der PKC an der Regulation der Glukoseaufnahme beteiligt ist.

INTRODUCTION

Glucose homeostasis is very important in mammalian metabolism. Insulin plays a pivotal role in maintaining this homeostasis by increasing the rates of glucose transport into muscle, adipose and other glucose-utilizing tissues. This action of insulin can be accounted for primarily by translocation of glucose transporters from intracellular pool to plasma membrane (Cushman *et al.,* 1980, Suzuki *et al.,* 1980). Glucose transporter family consists of at least six isoforms (GLUT1~6), which differ in expression in different tissues, kinetic characteristics and substrate specificity. New members are still being identified all the time. GLUT1 and GLUT4 represent the major glucose carriers in cardiac myocytes. Now it is generally accepted that GLUT4 translocation is the major mechanism for insulin stimulated glucose uptake in target tissues, while GLUT1 is mainly responsible for constitutive glucose transport (Birnbaum *et al.,* 1992; Bell *et al.,* 1993; Rea & James, 1997).

The signal cascades leading to GLUT4 translocation initiate at the binding of insulin to the specific receptor, which is an α2/ß2 tetramer (Van Obberghen *et al.,* 1981). The binding of insulin to α subunit activates the tyrosine kinase of the receptor, leading to autophosphorylation of tyrosine residues in several regions of intracellular ß subunit and the tyrosine phosphorylation of insulin receptor substrates (IRS proteins) (Kasuga *et al.,* 1983; Sun *et al.,* 1991). The phosphorylated IRS-1 binds to the two SH2 (Src homology 2) domains in p85α regulatory subunit of phosphatidylinositol 3-kinase (PI 3-K), resulting in activation of p110α subunit (Hiles *et al.,* 1992; Dhand *et al.,* 1994). The physiologically important product of PI 3-K is phosphatidylinositol 3,4,5-triphosphate (PIP_3). Interaction of PIP_3 with PH domains (Pleckstrin Homology domain) of a number of signal molecules results in translocation and/or activation of these molecules. Several protein kinases, such as PKB and some PKC isoforms appear to serve as downstream molecules that carry the signals initiated by the activation of PI 3-K (Dhand *et al.,* 1994; Alessi *et al.,* 1998).

PKC has been studied for a long time as a mediator of insulin stimulated glucose transport (Farese *et al.,* 1992; Yu *et al.,* 1992). Phorbol ester which stimulates PKC can mimic the effects of insulin on glucose uptake (Cherqui *et al.,* 1987). Depletion of PKC by chronic preincubation with a high concentration of PMA or by pharmacological blockers inhibits insulin stimulated glucose uptake in rat heart (Tanti *et al.,* 1989) and adipose tissues

(Avignon *et al.,* 1995). Moreover, a parallel translocation of PI 3-K and some of PKC isoforms in response to insulin was recently observed (Alessi *et al.,* 1998). Atypical PKC isoforms, PKCζ and PKCλ which are not activated by diacylglycerol (DAG) or phorbol ester, have been suggested to be activated by PI 3-K and play a role in insulin regulated glucose uptake (Standaert *et al.,* 1997; Kotani *et al.,* 1998).

Although the early steps of the signal pathway leading to GLUT4 translocation have been relatively well understood, the downstream components coupling the upstream events with the GLUT4 translocation machinery remain largely unknown. GLUT4, like other membrane integral proteins, continually recycles between plasma membrane and intracellular storage sites through a series of discontinuous tubular and vesicular structures. As revealed by immunoelectron microscopy, GLUT4 is localized to several elements of the constitutive recycling pathway including the trans-Golgi-network (TGN), clathrin-coated vesicles and endosomes. However, the vast majority of GLUT4 is found in small vesicles and tubulo-vesicular structures, which are different from the above constitutive recycling pathways. Insulin triggers the recruitment of these sequestered GLUT4-containing vesicles to the constitutive recycling pathway and stimulates the exocytotic branch of this pathway, resulting in much more GLUT4 on the cell surface (Satoh *et al.,* 1993; Rea & James 1997; Pessin *et al.,* 1999)

Vesicle traffic involves targeting, docking and fusion between different membrane structures. These processes are precisely modulated based on a complex network of interaction between regulatory molecules and mechanical components. According to the widely accepted SNARE hypothesis, all membrane traffic events require a high affinity match between ligands in transport vesicles (termed v-SNAREs) and receptors in the target membrane (termed t-SNAREs) (Söllner *et al.,* 1993; Pevsner *et al.,* 1994; Hay & Scheller 1997). Two SNARE (soluble NSF attachment protein receptor) proteins, VAMP2 (vesicle associated membrane protein 2) and Syntaxin 4, acting as v-SNARE and t-SNARE respectively, are required for insulin-regulated GLUT4 translocation (Cain *et al.,* 1992; Martin *et al.,* 1996; Volchuk *et al.,* 1996; Tellam *et al.,* 1997). Under the basal condition, the interaction of Syntaxin 4 with VAMP2 was precluded by the binding of Munc-18c to Syntaxin 4 (Tellam *et al.,* 1997). Insulin stimulates the dissociation of Munc-18c from Syntaxin 4, thereby allows the association of these SNARE proteins and the subsequent translocation of GLUT4 to the plasma membrane (Pessin *et al.,* 1999).

The Rab family of Ras-like small GTP-binding proteins is known to play essential roles in vesicle traffic. Rab proteins have been primarily implicated in vesicle docking as regulators of SNARE paring. They may facilitate assembly of SNARE complex by catalyzing dissociation of the members of Sec1/Munc-18 family which prevents t-SNAREs from the binding of v-SNAREs (Schimmoller *et al.,* 1998; Novick & Zerial 1997). Although Rab proteins are not stable components of SNARE complex, it can not be excluded that they may transiently interact with SNARE molecules and activate them (Lupashin & Waters, 1997). Currently, accumulated evidence suggested more extensive roles of Rab proteins in regulating membrane traffic. They may promote vesicle formation, recruit docking complex and directly link transport vesicles to the cytoskeleton (Echard *et al.*, 1998; Guo *et al.,* 1999; Chavrier *et al.*, 1999). However, the precise modes of the action of Rab proteins are still poorly understood. To date, more than 30 Rabs have been identified (Lazar *et al.,* 1997). Individual Rab proteins have characteristic subcellular distributions, suggesting specialized functions of different Rabs in discrete transport steps (Schimmoller *et al.,* 1998). For example, Rab1 and Rab2, locating in endoplasmic reticulum and Golgi apparatus respectively, are involved in vesicle transport between these compartments (Tisdale *et al.,* 1992). Rab6, associated with medial and trans-Golgi cisternae, as well as the trans-Golgi network (TGN), is necessary for intra-Golgi transport (Martinez *et al.,* 1994).

Increasing evidence suggested that Rab proteins might also be implicated in the traffic of GLUT4. Like other GTP-binding proteins, Rabs cycle between GTP-binding active and GDP-binding inactive forms (Novick & Zerial 1997). Baldini *et al.* reported that guanosine 5'-[γ-thio] triphosphate (GTP-γ-S) stimulated the translocation of GLUT4 in permeablized adipocytes (Baldini *et al.,* 1991). They also observed an increased expression of a new Rab3 isotype in differentiating 3T3-L1 cells, temporally coincident with the expression of GLUT4, although it remains to be clarified whether this small GTPase is involved in insulin actions (Baldini *et al.,* 1992). Using radioactive overlay technique, several low molecular weight GTP-binding proteins in the 21-27 kDa range have been identified as components of GLUT4-containing vesicles (Cormont *et al.,* 1991). Three members of Rab family including Rab3c, Rab4 and Rab5 have been detected in rat heart (Uphues *et al.,* 1994, 1995; Uphues & Eckel 1996), among them Rab4 was investigated extensively. Current evidence supports the participation of Rab4 in insulin-stimulated GLUT4 translocation. It has been reported that Rab4 was associated

with GLUT4-containing vesicles (Cormont *et al.,* 1993) and redistributed to plasma membrane in response to insulin (Uphues *et al.,* 1994). Transient coexpression of Rab4 with a epitope-tagged GLUT4 in rat adipocytes led to the enhanced retention of GLUT4 in basal states, but the same effects on insulin dependent release of GLUT4 can be observed only when Rab4 was overexpressed at ~200 folds of endogenous level (Cormont *et al.,* 1996). Moreover, electroporation of Rab4 or synthetic peptide corresponding to the Rab4 hypervariable C-terminal domain inhibited insulin stimulated GLUT4 translocation (Shibata *et al.,* 1997; Vollenweider *et al.,* 1997). Previous studies in our laboratory also agree with the above findings (Dransfeld *et al.,* 2000). However, these data indicated that it is more likely for Rab4 to participate in intracellular sequestration of GLUT4 and biogenesis of the GLUT4 storage compartments, but not in the exocytotic movement to the plasma membrane (Rea & James, 1997).

Recently, we have reported a 24 kDa GTPase different from Rab4 was associated with GLUT4-containing vesicles isolated from rat heart. This small GTP-binding protein was identified as Rab11 in further experiments. We also demonstrated that insulin increased the abundance of Rab11 in GLUT4-containing vesicles and triggered a recruitment of this protein to plasma membrane (Kessler *et al.,* unpublished data). These findings indicated the possible involvement of Rab11 in insulin-stimulated GLUT4 translocation.

Rab11 has been shown to be localized at sorting endosomes, recycling endosomes, trans-Golgi network (TGN) and post-Golgi secretary vesicle membrane (Goldenring *et al.,* 1997). Several lines of evidence indicated its functions in regulating vesicle traffic through the endosome system and from the trans-Golgi network to the plasma membrane. For example, Ullrich *et al.* showed that Rab11 is required for the transport of transferrin through the pericentriolar recycling endosome (Ullrich *et al.,* 1996). Rab11 was also able to regulate the direct delivery of transferrin from the pericentriolar-recycling compartment to the cell surface (Ren *et al.,* 1998). Moreover, overexpression of dominant negative mutant Rab11 S25N which does not bind GTP impairs recycling of transferrin receptor from endosome to TGN and the plasma membrane (Chen *et al.,* 1998). Transferrin receptors are also components of GLUT4-containing vesicles (Morris *et al.,* 1998). These data, together with the above findings of our group lead us suppose that Rab11 may be involved in the movement of GLUT4 to the plasma membrane.

As mentioned previously, recycling of GLUT4, like that of other integral membrane proteins, is mediated by a series of discontinuous tubular and vesicular structures.

Although the vast majority of GLUT4 (~60%) is found in small vesicles and tubulo-vesicular structures which are sequestered from constitutive recycling endosome system, insulin stimulation allows these separated vesicles adjacent to the endosomes enter the constitutive recycling pathway and thereby results in movement to the plasma membrane. Given the particular importance of Rab11 in vesicle recycling, we proposed a model that at least two different members of the Rab family, Rab4 and Rab11 are involved in GLUT4 translocation, and their functions maybe different. Rab4 participates in the endocytosis of GLUT4, whereas Rab11 is implicated in the insulin dependent exocytosis of GLUT4 (Kessler *et al.,* unpublished data). However, in contrast to Rab4, much less information is presently available concerning the involvement of Rab11 in GLUT4 translocation.

In spite of the essential roles of Rab proteins in vesicle traffic, the underlying mechanisms by which they accomplish their functions remain largely unknown. It has been shown that GRP1, a molecule that specifically binds to phosphatidylinositol 3,4,5-triphosphate, the main products of PI 3-K, contains a domain that is possibly involved in GTP exchange on small GTP-binding protein (Klarlund *et al.,* 1997). Recent studies demonstrated that guanine-nucleotide exchange on Rab4 was stimulated by insulin in a wortmannin-sensitive manner, providing evidence for the first time that Rab4 lies downstream of the insulin-activated PI 3-K (Shibata *et al.,* 1997). A dependence of Rab proteins on PI 3-K activity has also been suggested from studies of other trafficking proteins. For example, wortmannin was able to block Rab9 regulated exit of recycling from prelysosomal compartments (Riederer *et al.,* 1994). The inhibitory effects of wortmannin on endosome fusion in a cell-free system can be overcome by the addition of constitutively active Rab5 (Li *et al.,* 1995). In addition, EEA1, which binds to PIP3 in the process of endosome fusion, was recently identified as an effector of Rab5. This provides the first molecular link between PI 3-K and Rab proteins in regulating membrane traffic (Simonsen et al 1998).

Phosphorylation/dephosphorylation may also represent an important mechanism in modulating the actions of Rab proteins. It has been reported that thrombin induces phosphorylation of Rab3B, Rab6 and Rab8, and the phosphorylation of these Rab proteins may be implicated in the secretory processes of activated platelets (Karniguian *et al.,* 1993). The phosphorylation of Rab6 was mediated by PKC and resulted in translocation of Rab6 from platelet particulate fraction to the cytosol (Fitzgerald & Reed

1999). In adipocytes, insulin was able to stimulate the phosphorylation of Rab4 concomitant with the translocation of GLUT4 to the plasma membrane (Cormont *et al.,* 1994; Vollenweider *et al.,* 1997). Recent studies on MDCK cells implied that dominant negative mutant of Rab11 slightly inhibited reassembly of stress fibers and focal adhesions induced by PKC activator, TPA (Imamura *et al.,* 1998). These data suggest that phosphorylation of Rab proteins may be an important mechanism through which the cells response to various extracellular stimuli.

Aims of the study

The Rab family of Ras-like small GTP-binding proteins is known to play pivotal roles in vesicular traffic. The translocation of GLUT4-containing vesicles is also regulated by the members of Rab family. The precise role of Rabs and the molecular mechanisms by which they exert their functions are still not well understood.

It has been well documented that Rab11 is required in exocytotic branch of vesicle recycling, such as the vesicle traffic through the endosomes and from TGN to the plasma membrane. Recently, Rab11 has been found to associated with GLUT4-containing vesicles. Insulin stimulation results in an increased abundance of Rab11 in GLUT4-containing vesicles and redistribution of this protein to the plasma membrane, indicating a role of Rab11 in insulin-stimulated GLUT4 translocation. In order to further elucidate the roles of Rab11 in GLUT4 translocation and the underlying mechanisms, this study was initiated to clarify the following questions:

1. Do H9c2 cardiac myoblasts express GLUT4?

2. Does insulin influence the expression of Rab11?

3. What is the role of Rab11 in glucose uptake of cultured myoblasts derived from embryonic rat heart? How does Rab11 exert its functions? Could Rab11 alter the expression of GLUT4.

4. Are there any functional linkages of Rab11 with PI 3-K and / or PKC?

MATERIALS AND METHODS

1. Materials

Chemicals

Acetate acid	Roth, Karsruhe
Ampicillin	Sigma, München
Bacto-Agar	Difco, Detroit, USA
BIM	Sigma, München
Boric acid	ICN Biomedicals, Aurora, USA
BSA	Boehringer, Mannheim
CIAP	Promega, Madison
Calciumchlorid-dihydrate	Merck, Darmstadt
Cytochalasin B	Sigma, München
DMEM (1000 mg/l D-Glucose, with L-Glutamine, Sodium-pyruvat, 25 mM HEPES)	Gibco, Berlin
DMSO	Sigma, München
DNase I	Gibco, Berlin
DTT	Sigma, München
EDTA	Sigma, München
EGTA	Sigma, München
EtBr	Sigma, München
ExcelGel buffer strips	Pharmacia Biotech, USA
ExcelGel gradient 8~18%	Pharmacia Biotech, USA
FCS	Gibco, Berlin
Geneticin (G418)	Gibco, Berlin
L-[1-^{14}C]-Glucose (55mCi/mmol)	Amersham, Braunschweig
2-Deoxy-D-[1-^{14}C]-Glucose (55mCi/mmol)	Amersham, Braunschweig
Glycerin	Roth, Karlsruhe
Hepes	Serva, Heidelberg
HRP-conjugated anti-rabbit IgG	Promega, Madison
Insulin	Novo, Bagsvaerd, Denmark
Potassium chloride (KCl)	Merck, Darmstadt
Magnesiumchlorid-Hexahydrat ($MgCl_2 \cdot 6H_2O$)	Fluka, Neu-Ulm
M-MLV Reverse Transcriptase	Gibco, Berlin
Nonessential amino acid	Gibco, Berlin
Not I	Boehringer, Mannheim
Di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$)	Merck, Darmstadt
Oligo $(dT)_{12-18}$ primer	Gibco, Berlin
Protease inhibitor cocktail	Boehringer, Mannheim
Penicilin (5000U/ml)/ Streptomycin (500U/ml)	Gibco, Berlin
Petroleum benzin	Riedel de Haen, Seelze
PMSF	Sigma, München

Rat Rab11 primers	MWG-Biotech, Germany
RNase OUT Ribonuclease Inhibitor	Gibco, Berlin
SDS	Sigma, München
Sodium azide (NaN_3)	Sigma, München
Sodium chloride (NaCl)	Fluka, Neu-Ulm
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$)	Merck, Darmstadt
Sodium hydroxide (NaOH)	Merck, Darmstadt
SuperFect Transfection Reagent	Qiagen, Hilden
T4-DNA ligase	Promega, Madison
TRIzol Reagent	Gibco, Berlin
Tris	ICN Biomedicals, Aurora, USA
Trypan blue solution (0.4%)	Sigma, München
Trypsin (0.5g/l)-EDTA (0.2g/l)	Gibco, Berlin
Tryptone	Difco, Detroit, USA
Tween-20	Sigma, München
Wortmannin	Sigma, München
Yeast extract	Difco, Detroit, USA

Molecular weight markers

λDNA (Eco RI/Hind III)	Boehringer, Mannheim
100 bp DNA Ladder	Gibco, Berlin
Wide Range Color Markers (M.W. 65~205 kDa)	Sigma, München
10 kDa Protein Ladder	Gibco, Berlin

Kit systems

BCA protein Assay Kit	Pierce, Rockford, USA
Plasmid Maxi Kit Tip 500	Qiagen, Hilden
QIAquick PCR purification Kit	Qiagen, Hilden
QIAquick Gel Extraction Kit	Qiagen, Hilden
Rat ß-Actin Control Amplimer Set	Clontech, Heidelberg
Taq PCR Core Kit	Qiagen, Hilden

Cells and plasmid

E.coli, DH5α	Clontech, Heidelberg
H9c2 cell line	ATCC, Maryland, USA
pCMVß	Clontech, Heidelberg

2. Methods

2.1 Culture of the cells

DMEM complete medium		PBS (pH 7.4)	
DMEM medium	435 ml	NaCl	140 mM
FCS	10%	Na_2HPO_4	10 mM
non-essential amino acid	1%	KH_2PO_4	1.8 mM
Penicillin	100 U/l	Cell stock medium	
Streptomycin	100 μg/ml	DMEM medium	70%
		FCS	20%
		DMSO	10%

H9c2 (H9) rat heart muscle cell line was initially purchased from American Type Culture Collection (ATCC) and stored with cell stock medium containing 10% DMSO in liquid nitrogen before use. The cells were recovered at 37°C and seeded at once in pre-warmed DMEM complete medium supplemented with 10% fetal calf serum, 1% nonessential amino acids, streptomycin (100μg/ml) and penicillin (100 U/ml), then incubated at 37°C with a humidified atmosphere of 5% CO_2. 24 h after recovery, the medium was changed to remove remaining DMSO. For long time culture, the medium was changed every 48~72 h. Myoblasts were maintained in continuous passages by subculture the confluent cells in the late logarithmic phage. The medium was removed and the monolayer was washed once with PBS, then digested with pre-warmed Trypsin-EDTA solution for 2~5 min at 37°C. The reaction was stopped by two volume of complete medium and the detached cells were centrifuged at 1500 rpm for 10 min. Cell number was determined and 2.5×10^5 cells were plated into 75 cm^2 flask.

For freezing of the cells, the cell number was adjusted to $1\sim2\times10^6$/ml in stock medium. Aliquoted in cryotubes, the cells were put in the cryobox, which was deposited overnight at -80°C so that the temperature of the cell suspension would decrease 1°C every minute.

H9K6 (K6) cells stably expressing GLUT4, which were derived from H9 cells were also recovered and continuously passaged in DMEM complete medium. After adhesion, Geneticin (G418) was added to a final concentration of 0.8mg/ml for selection.

2.2 Western-blot

2.2.1 Lysis of the cells

RIPA buffer	
Tris-HCl	50 mM, pH 7.4
NP-40	1%
Na-deoxycholate	0.25%
NaCl	150 mM
EDTA	1 mM
Na_3VO_4	1 mM
NaF	1 mM
Protease inhibitor cocktail	1 tablet/50 ml RIPA

Cells were lysed with RIPA buffer according to a widely accepted protocol. Protease inhibitor cocktails were added and all the procedures were performed at 4°C or on ice to avoid degradation of the proteins. After the medium was aspirated, the cells were washed once with ice cold PBS. Proper volume of RIPA buffer was added to the cell surface. The cells were detached from the bottom of the plates with a cell scraper. The cell lysates were tumbled at 4°C for 2 h and then centrifuged at 14,000 rpm for 15 min at 4°C to pellet the high molecular chromosomal DNA and cell debris. The supernatants containing proteins were transferred to a new tube and stored at –70°C. An aliquot of samples was quantified with BCA protein assay.

2.2.2 Protein assay

Protein concentration of the samples was determined with the BCA protein Assay Kit (Pierce). BCA protein assay is a detergent-compatible formulation based on bicinchoninic

acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines well-known reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{2+}) using a unique reagent containing bicinchoninic acid. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentration over a board working range of 20 μg/ml to 2,000μg/ml. BSA in a list of dilutions is used as protein standards. The samples were quantified by comparing the absorbance with that of BSA.

2.2.3 SDS-PAGE

Laemmli stock (pH 6.8)		2×Laemmli buffer	
Tris	6.06g	SDS	1.0g
SDS	0.4g	EDTA	3mg
NaN_3	0.01g	Bromophenol blue	10mg
adjust pH to 6.8		Laemmli stock	2.5 ml
add ddH_2O to 100 ml		87% Glycerin	20ml
		add ddH_2O to 50 ml	

When proteins are denatured with excess SDS, the detergent binds to the polypeptides at a constant mass ratio (1.4 g SDS per gram polypeptide) and the polypeptide is organized into a rod-like structure. The bound SDS molecular each contributes a strong negative charge which effectively swamps the intrinsic charge of the polypeptide. The SDS polypeptide complexes have the same charge/mass ratio. Electrophoretic migration is thus approximately proportional to the molecular weight of the polypeptide chain.

A horizontal electrophoresis apparatus (Multiphore II, Pharmacia Biothech) with cooling system was used. Precast polyacrylamide gradient gel (ExcelGel, gradient 8-18%, Pharmacia Biothech) and buffer strips (ExcelGel SDS buffer strip, Pharmacia Biothech) designed for horizontal electrophoresis are supplied ready to use. The buffer system in the buffer strips, together with the gel buffer, forms a discontinuous buffer system.

The cooling system was switched on 15 min before the separation. About 1 ml of insulating fluid (Petroleum benzin) was pipette onto the plate of Multiphor II. Carefully avoiding air bubbles, the gel was positioned on the cooling plate so that the polarity of the gel corresponds to that of the plate. Apply the cathodic and anodic SDS buffer strips to their respective sides of the gel. The sample application pieces (Pharmacia Biothech) were placed on the gel surface at about 5 mm from the cathodic buffer strip. For each sample, 20 µg of protein was denatured at 95°C for 10 min in 1×Laemmli buffer containing 2.6 M DTT and then applied to the sample application pieces. The running condition was 600V/50mA/30W. The electrophoresis is complete and stopped when the bromophenol blue front has reached the anodic buffer strip.

2.2.4 Transfer of proteins to PVDF membrane

Anode buffer I		Anode buffer II	
Tris	300 mM	Tris	25 mM
Methanol	20% (v/v)	Methanol	20% (v/v)
Cathode buffer			
6-aminohexan acid		40mM	
SDS		0.01%	
Methanol		20% (v/v)	

The protein separated by SDS-PAGE can be transferred to PVDF membrane using a semidry-blot apparatus. While the gel is running, 18 layers of Whatman paper and a piece of PVDF membrane were prepared according to the size of the gel. Six layers of Whatman paper wetted by Anode buffer I was laid on the anode plate of the blot apparatus. Before it was equilibrated in Anode buffer II, the PVDF membrane needs to be activated by immerging in methanol for a few seconds and washing with distilled water. When electrophoresis was complete, the buffer strips were removed. The gel was equilibrated in Anode buffer II for 5 min and separated from the plastic support. The PVDF membrane was laid on the surface of the gel and three layers of whatman paper which was first immerged in Anode buffer II was put on the membrane. Then, these layers were placed on the plate of blot apparatus and the plastic support was removed

from the gel. 9 layers of Whatman paper wetted with cathode buffer was laid on the gel. Air bubbles between different layers should be carefully avoided. The cathode plate was then placed on the top of these layers. The blot parameters are 0.8mA/cm membrane/20V/5W.

2.2.5 Immunodetection

When the blot was complete, the membrane was blocked in blocking solution (5% milk powder and 0.05% Tween-20 in PBS) at room temperature for at least 1 hour with continuous shaking, then the blots were incubated with proper primary antibody solution at 4°C overnight with gentle shaking. The primary antibody was diluted in blocking solution at 1:500 for GLUT4 and 1:1,000 for Rab11. The membrane was washed five time with 30 ml of PBS containing 0.05% Tween-20 times at 10 min intervals and then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody for 1 hour at room temperature. The dilution rate of the secondary antibody is 1:2,500 for GLUT4 or 1:10,000 for Rab11. Then the same wash was perform as for the primary antibody. The blots were visualized by Enhanced Chemluminescence (ECL). SuperSignal West Pico working solution was prepared by adding equal volume of the Luminol/Enhancer Solution and Stable Peroxide Solution. The blots were immerged in the working solution for 5 min and detected on a Lumi imager system. The signals were quantified using image analysis software (Boehringer Mannheim).

2.3 Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

2.3.1 Precautions for RNase contamination

All plasticwares used for RNA were treated with 0.1% DEPC solution at RT for 24 h and rinsed two times with RNase free sterile water before autoclaved for 50 min at 1 bar. Glasswares were baked at 240°C for at least 8 h. RNase free sterile water was prepared by treatment of distilled water with 0.1% DEPC for 24 h at RT and then autoclaved to sterilize and vaporize DEPC. All solutions were prepared in the DEPC treated water using RNase free glasswares. Solution and items of glasswares and plasticwares to be used only for experiments with RNA were marked distinctively and stored in a separate place.

2.3.2 Isolation of total RNA

The confluent cells were left untreated or treated with 1 µM insulin for 10 min, 30 min, 1 h, 2 h, 4 h, 8 h and 16 h respectively, then, TRIzol Reagent was applied to isolate total RNA from the culture cells. The reagent, a mono-phase solution of phenol and guanidine isothiocyanate is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (Chomczynski & Sacchi 1987). Cell monolayers grown in 3.5 cm diameter dishes was washed once with ice-cold PBS and then lysed directly by adding 1 ml of Trizol Reagent to their surface. Passed several times through a pipette, the homogenized lysates were incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added to the lysates and mixed by shaking vigorously for 15 seconds. Then the mixtures were incubated at room temperature for 2 to 3 min and centrifuged at 12000×g for 15 min at 4°C. The colorless upper aqueous phases containing RNA were transferred to a fresh tubes. 0.5 ml isopropyl alcohol was added to precipitate RNA. After incubated at room temperature for 10 min, RNA was pelletted by centrifugation at 12,000×g for 10 min at 4°C. The supernatants were removed. The RNA pellet was washed once with 1 ml of 75% ethanol, briefly dried and dissolved in RNase-free water. Quantification of the RNA was performed with an Ultra Violet spectrophotometer. The concentration was calculated according to the following formula:

$$\text{RNA concentration } (\mu g/ml) = OD260 \times 40 \times \text{dilution factor.}$$

All the RNA samples used for RT-PCR had OD260/OD280 ratios between 1.8 to 2.0.

2.3.3 Reverse Transcription of RNA (RT)

Complementary DNA (cDNA) was synthesized using a reverse transcription system. 1µg RNA was treated by DNase I in the total volume of 10 µl to avoid possible DNA contamination. After the reaction was terminated, a reverse transcription reaction was set up in the same tube, which contains 1 µg of total RNA, 8 µl 5×RT buffer, 8 µl dNTP (dATP, dGTP, dCTP, dTTP, 2.5 mM each), 2µl M-MLV reverse transcriptase (200U/µl), 1 µl oligo $d(T)_{12\text{-}18}$ primer (1 µg/µl), 0.5 µl RNase (40U/µl) and 4µl DTT (100mM). The cDNA was synthesized at 37°C for 1 hour, then heated at 70°C for 10 min to inactivate reverse transcriptase. The products were stored at –70°C before use.

2.3.4 Amplification of cDNA by PCR

To amplify the coding region of Rab11 gene, we selected a pair of specific primers: sense 5'-AAC GCG GCC GCA TGG GCA CCC GCG ACG AC-3', antisense 5'-AAC GCG GCC GCT TAG ATG TTC TGA CAG CAC-3'. The primers contained Not I linkers for subcloning. The following reagents were added in order to a sterile 0.5 ml microcentrifuge tube: 5 µl of 10×PCR buffer, 4 µl of dNTPs (mixture of dATP, dGTP, dCTP, and dTTP, 2.5mM each), 2 µl of each primer (10 µM each, 0.3 µl of Taq DNA polymerase (5U/µl). 5 µl of template cDNA and 34 µl of ddH_2O. The total reaction volume was 50 µl. The reaction was performed in a thermoblock and incubated for 5 min at 94°C prior to PCR cycles. The program used was 1 min for denaturation at 94°C, 1 min of annealing at 53°C, and 1.5 min of extension at 72°C for 35 cycles, followed by an further extension at 72°C for 5 min.

ß-actin which has an invariable expression level in different cells was used as an internal control for semi-quantity PCR. For this purpose, the ß-actin mRNA was also amplified by PCR with the same cDNA as template. The primers for the 764 bp rat ß-actin mRNA were: sense 5'-TTG TAA CCA ACT GGG ACG ATA TGG-3', antisense 5'-GAT CTT GAT CTT CAT GGT GCT AGG-3'. The annealing temperature was 60°C.

2.3.5 Agarose gel electrophoresis

5×TBE		6×DNA gel loading buffer	
Tris base	54 g	Bromophenol blue	0.25% (w/v)
Boric acid	27.5 g	Xylene Cyanol	0.25% (w/v)
0.5 M EDTA (pH 8.0)	20 ml	Ficoll	15%
add ddH_2O to 1 l			

DNA molecules are negatively charged in neutral pH value and migrate to the anode in an electronic field. The migration is proportional to the molecular weight. Agarose gel electrophoresis was used to separate DNA samples according to their molecular weight. 10 µl of PCR products were mixed with 2 µl 6×DNA gel loading buffer. The samples were loaded on 1.5% agarose gel containing 0.5 µg/µl ethidium bromide. The gel was run

in 0.5×TBE buffer at 80 mA for 1 hour and visualized with a UV transillumitor. The signals were quantified using image analysis software (Boehringer Mannheim).

2.4 Construction of Rab11 expression vector

2.4.1 Cloning of Rab11 cDNA by RT-PCR

Rab11 mammalian expression vector were constructed by cloning the PCR-amplified Rab11 cDNA into the Not I site of pCMV plasmid, which is a expression vector designed for the expression of genes in mammalian cells from the human cytomegalovirus immediate early gene promoter.

Total RNA isolated from rat heart was used as template for cDNA synthesis. A pair of specific primers containing a Not I linker (as described in Chapter 2.3) were designed for PCR-amplification and cloning of Rab11 cDNA. The PCR products were purified with QIAquick PCR purification Kit (Qiagen), then digested with Not I. Meanwhile, the pCMV parental plasmid was linearized with the same restriction enzyme. The cut cDNA and vectors were fractionated by agarose electrophoresis and eluted from the gel with QIAquick Gel Extraction Kit (Qiagen). The linearized original pCMV vectors were treated with Alkaline Phosphatase Calf Intestinal to avoid self-ligation. The digested Rab11 cDNA and the pCMV vectors were ligated with T4-DNA ligase (Promega, Madison). The ligation products were used directly to transform *E.coli* strain DH5α.

2.4.2 Restriction and sequence analysis of pCMV-Rab11

Plasmid DNA from several transformants were extracted by mini-preparation and digested with Not I to detect whether the Rab11 cDNA was inserted in the vector. A reaction containing 2 µg plasmid, 2 U Not I, and 0.1 volume of 10×Reaction buffer was incubated at 37°C for 1 hour and the fragments were separated by agarose gel electrophoresis. The positive clones were further conformed by sequence analysis from both of the two directions. The sequencing primers were: pCMV-5' 5'-AGG CCT GTA CGG AAG TGT TAC-3', pCMV-3' 5'-AGT TGT GGT TTG TCC AAA CTC-3'.

2.4.3 Transformation of *E.coli* bacteria

LB liquid medium		LB-agar
Tryptone	10g	15g Bacto-agar / l LB
Yeast extact	5g	liquid medium
NaCl	10g	
adjust pH to 7.0		LB selective medium
add ddH_2O to 1 l		Ampicillin 100µg/ml in
		LB medium

The *E.coli* host strain should be made competent to facilitate the introduction of exogenous DNA. A single clone picked from freshly streaked LB plate was seeded into 400 ml LB liquid medium and incubated at 37°C with vigorous shaking until OD600 reached 0.45~0.55. The fresh culture was put on ice for 10 min and then centrifuged at 4°C for 5 min at 6000 rpm. Resuspended with 200 ml of cool $CaCl_2$ (75mM), the bacterial pellets were incubated at 4°C for 20 min and centrifuged at 4°C for 5 min at 6000 rpm. The pellets were resuspended with 20 ml 75mM $CaCl_2$ and incubated at 4°C for 15 min before adding 4.2 ml of 87% glycerin solution for long time storage at –70°C.

100 ng plasmid DNA and 40 µl of the competent cells was mixed and incubated on ice for 40 min and heat shocked at 42°C for exactly 90 seconds. The mixture was plated on LB selective medium containing 100 µg/ml ampicilin and grown overnight at 37°C.

2.4.4 Plasmid amplification and purification

Buffer P1		Buffer P2	
RNase	100 µg/ml	NaOH	200 mM
Tris-HCl	50 mM	SDS	1%
EDTA	10 mM (pH 8.0)		
		TE buffer (pH 8.0)	
Buffer P3		Tris-HCl	10 mM
Potassium acetate	3 M	EDTA	1 mM

QBT buffer (pH 7.0)		QC buffer (pH 7.0)	
NaCl	750 mM	NaCl	1 M
MOPS	50 mM	MOPS	50 mM
Ethanol	15%	Ethanol	15%
QF buffer (pH 8.5)			
NaCl	1.25 M	Tris-HCl	50 mM
Ethanol	15%		

A single colony picked fom the above plate was inoculated to a starter culture of 2 ml LB liquid medium containing 100 μg/ml ampicilin and grown at 37°C for 8 hour with vigorous shaking.

Large-scale extraction was performed with Plasmid Max Kit 500 (Qiagen). A protocol based on the optimized alkaline lysis method was provided by the manufacturer. Briefly, the starter culture was diluted 1:500 into 100 ml of selective medium and grown at 37°C for 12~16 h with vigorous shaking. The bacteria were harvested by centrifugation and resuspended in 10 ml of buffer P1. 10 ml of buffer P2 was added to the cell suspension. Mixed gently but thoroughly by inverting 4~6 times, it was incubated at room temperature for 5 min to allow the proceeding of the lysis. Then 10 ml of prechilled buffer P3 was added, mixed immediately but gently by inverting 4~6 times and incubated on ice for 20 min. Then, the sample was centrifuged at 20,000×g for 30 min at 4°C. The supernatants were transferred to a new tube and recentrifuged at 20,000×g for 15 min at 4°C. The supernatants containing plasmid DNA were applied to a Qiagen-tip 500 equilibrated with 10 ml buffer. The liquid was allowed to enter the resin by gravity flow. The tip was washed twice with 10 ml buffer QC. Plasmid DNA was eluted with 15 ml buffer QF, precipitated with 10.5 ml (0.7 volumes) room-temperature isopropanol. Mixed by inverting for several times, the plasmid DNA were pelleted by centrifuging immediately at 15,000×g for 30 min at 4°C. DNA pellets were washed with 5 ml of room-temperature 70% ethanol and centrifuged at 15,000×g for 10 min. The supernatants were carefully decanted without disturbing the pellet. Air-dried for 5~10 min, the pellets were redissolved in 500 μl TE buffer (pH 8.0).

The plasmid DNA was quantified with a Ultra Violet spectrophotometer. The

concentration was calculated according to the following formula:

$$\text{DNA concentration } (\mu g / ml) = OD260 \times 50 \times \text{dilution factor.}$$

All the plasmids used in the following experiments had OD260/OD280 ratio between 1.8 to 2.0.

2.5 Transient transfection of the cells

When the cells are transiently transfected, the DNA is introduced into the nucleus of the cells, but does not integrate into chromosome. This means many copies of the gene of interest are present, leading to high levels of expressed proteins. For this purpose, SuperFect transfection reagent was used. 1.5×10^5 cells were seeded on six-well plates the day before transfection, so that the cells would be 40~60% confluent on the day of transfection. Because serum and antibiotics will interfere with complex formation and will significantly decrease transfection efficiency, the reaction should be performed in serum free medium containing no antibiotics. Thus, 3 µg of DNA dissolved in TE buffer (pH 7.4) was diluted with cell growth medium without serum proteins and antibiotics to a total volume of 150 µl. The solution was mixed and spinned down for a few seconds to remove drops from the tube. 30µl of SuperFect Reagent was added to the DNA solution and mixed by vortexing for 10 seconds. The mixture was incubated for 10 min at room temperature to allow complex formation. While complex formation took place, the growth medium was removed from the wells and cells were washed twice with 2 ml PBS. After complex formation, 1 ml of complete medium containing serum and antibiotics was added to the reaction tube. Mixed by pipetting up and down twice, the total volume was immediately transferred to the cells in the six-well plates. The cells were incubated with the complexes for 2~3 h at 37°C and 5% CO_2. Before fresh complete medium was added, the medium containing the remaining complexes was removed from the cells by gentle aspiration and the cells were washed once with 2 ml PBS. The medium was changed every 24 h.

In some groups, 100 nM wortmannin (wort) or bisindolylmaleimide (BIM) dissolved in DMSO was added to the medium immediately after transfection. The controls were treated with the same concentration of the vehicle. The final concentration of DMSO was 0.01%, which was believed to never affect glucose uptake. New wortmannin or BIM was supplemented after changing the medium.

2.6 2-Deoxy-D-[1-^{14}C]-glucose uptake assay

T-buffer			
NaCl	134 mM	KCl	5.4 mM
$CaCl_2$	1.8 mM	$MgSO_4$	0.8 mM
Hepes	20 mM		

2-Deoxy-glucose uptake was measured in H9c2 cells and K6 cells transiently transfected with Rab11 cDNA and treated with different reagents. The cells seeded in six-well plates were washed twice with PBS buffer. In wortmannin or BIM treatment groups, the cells were washed three additional times to remove the reagent completely and then incubated in serum free medium for at least 2.5 h to avoid the influence of FCS in the complete medium. Then the cells were incubated in parallel without insulin (basal) and with 1 µM of insulin in T-buffer for 45 min. Transport reactions were performed by adding 100 µl 2-Deoxy-D-[1-^{14}C]-glucose/well. The final concentration of the glucose was 7.3 µM/l (0.4 µCi per well). Hexose uptake was assayed for 30 min at 37°C. The reaction was stopped by aspirating the medium and washing the cells twice with ice cold 25 µM of cytochalasin B dissolved in PBS buffer. 1ml of 1M NaOH was added to the dry cell surface to lyse the cells. The radioactive cell lysates were incubated at 70°C for 30 min. 500µl of the lysates were neutralized by 100 µl acetic acid before adding to 10 ml scinticocktail for ß-counting. Another 50 µl of the lysates was applied for protein test.
Carrier-independent uptake was determined in parallel incubation of the cells with L-[^{14}C]-glucose (2~5% of total uptake) and subtracted from all determinations.

2.7 Statistical analysis

Data are presented as mean value ± SEM. For the number of each experiment indicated in the legends to the figures. Statistical significant was assessed using Student't test. Statistical significance was set at $p \leq 0.05$. Non-linear regression analysis was performed by computer program (Graph Pad Prism).

RESULTS

1. Detection of GLUT4 in H9c2 myoblasts

It has been reported that GLUT4 expression is developmentally regulated. Both L6 myogenic and 3T3-L1 adipogenic lines undergo differentiation in culture, from myoblasts to myotubes in the case of L6, from fibroblasts to adipocytes in the case of 3T3-L1. In these cell lines, GLUT4 expression occurs on and after differentiation into myotubes or adipocytes (Ueyama *et al.*, 1999). However, in contrast to L6 and 3T3-L1 cells, previous studies in our laboratory showed myoblasts from a cardiomyocyte line H9c2 also express appreciable amount of GLUT4 when highly passaged. To further confirm this finding, Western-blot was used to detected GLUT4 expression in H9c2 cells. K6 cells stably expressing GLUT4 were used as a positive control.

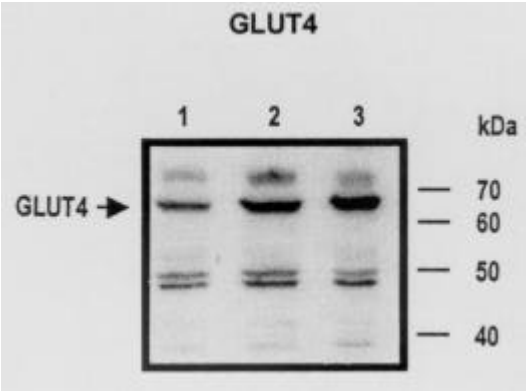

Fig. 1. Detection of GLUT4 in wild type H9c2 cardiomyocytes.
Myoblasts from wild type H9c2 and K6 stably overexpressing GLUT4 were lysed in RIPA buffer. 20 µg proteins were separated by SDS-PAGE using 8-18% gradient gels, transferred to PVDF membrane and immunoblotted with antiserum against GLUT4. Lane 1, low passage H9c2 cells, lane 2, high passage H9c2 cells, lane 3, K6 cells.

Low passage (3~5 passage) and high passage (20~25 passage) H9c2 cells and K6 cells were lysed with RIPA buffer. 20 µg proteins from each group were separated by SDS-PAGE, transferred to PVDF membrane and probed with anti-GLUT4 antiserum. The

results revealed that low passage H9c2 cells expressed a very low level of GLUT4, while high passage H9c2 cells had a relatively higher GLUT4 level which was comparable to

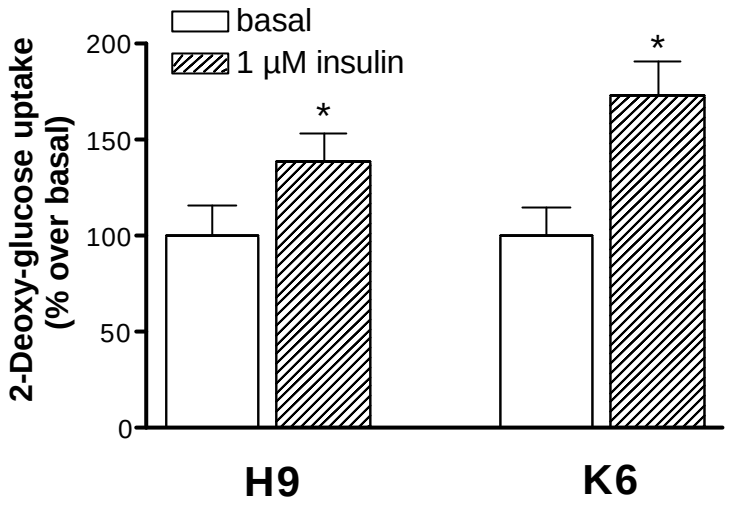

Fig. 2. Insulin responsiveness of high passage H9c2 myoblasts.
High passage H9c2 and K6 myoblasts were incubated in serum free medium for 2.5 h before stimulated with 1 μM insulin for 45 min. 2-Deoxy-glucose uptake was then monitored by incubating the cells with 0.2 μCi/ml of 2-Deoxy-D-[l-^{14}C]-glucose for 30 min as detailed in "Methods". Data are shown as mean value±SEM of three separate experiments. * indicated the difference between basal and insulin stimulated glucose uptake was statistically significant with $p<0.05$.

that of K6 cells. Fig. 1. is a typical blot from three separate experiments with the same results. We next examined insulin responsiveness in the high passage H9c2 cells. The results of 2-Deoxy-glucose uptake revealed that insulin stimulation caused a mean increase of 38% over basal control in high passage H9 cells compared with 70% in K6 cells (Fig. 2.). This indicated the possibility for the use of high passage H9c2 myoblasts in studying glucose transport.

2. Insulin increases mRNA transcription of Rab11 gene

Insulin is known to regulate expression of many genes of metabolic importance in mammalian cells. This action involves activation of transcription as well as the process of mRNA translation. In order to investigate whether Rab11 gene is also the target of

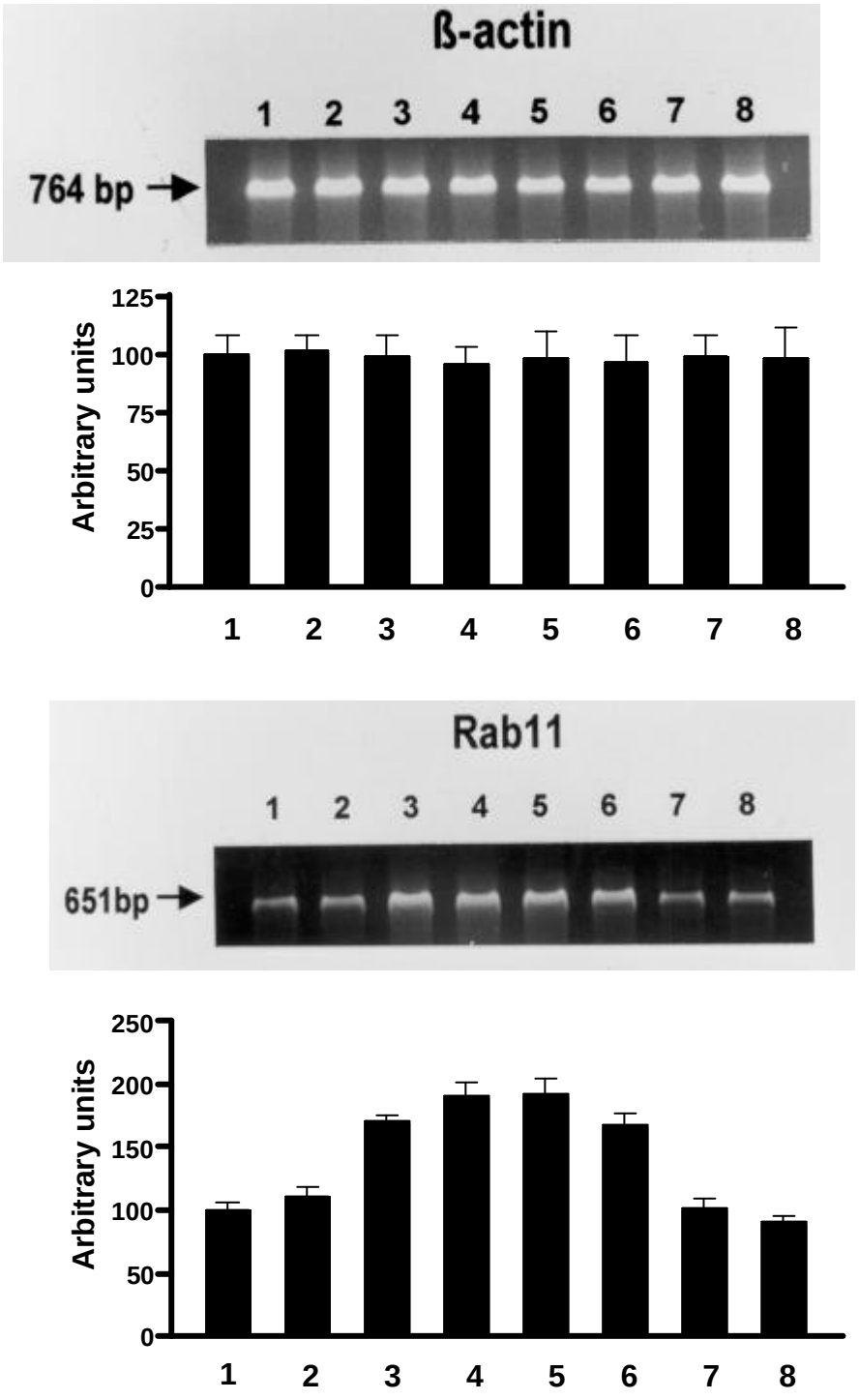

Fig. 3. Influence of insulin on Rab11 mRNA expression.
Total RNA from K6 cells was reversely transcribed with M-MLV reverse transcriptase. The resulting cDNA was used as template for PCR amplification. PCR products were electrophoresed through 1.5% agarose gels containing 0.5 µg/µl ethidium bromide. Size of the fragments was determined by comparing with DNA standards run on the same gel. The expected PCR fragments of 764 bp ß-actin and 651 bp Rab11 were obtained. The signal density was measured with image analysis software. Lane 1, untreated control, lane 2-8, insulin treated for 10 min, 0.5 h, 1 h, 2 h, 4 h, 8 h, 16 h, respectively. The data were presented as mean value ± SEM (n=3).

insulin, RT-PCR was employed to detect and quantify the expression of Rab11 in K6 cells stably transfected with GLUT4.

The confluent cells were left untreated or exposed to 1 μM insulin for different time, total RNA was then isolated with Trizol Reagent according to a modification of the single-step RNA isolation method. The complementary DNA (cDNA) was synthesized by reverse transcription of the total RNA. PCR was performed with a pair of specific primers selected from the cDNA sequence of Rab11. ß-actin, a house-keeping gene, was used as an internal control for semi-quantification. The RT-PCR products of ß-actin were standardized to ensure that equal amount of RNA was used in each group. Under this condition, the transcription level of Rab11 gene could be compared among different groups. As shown in Fig. 3. K6 cells had a basal level of Rab11 transcription, when exposed to 1 μM of insulin, it increased in a time-dependent manner. At 10 min, there was no significant change of Rab11 transcription. At 30 min, however, transcripts for Rab11 increased dramatically and then reached 1.9 folds of control level at 60 min. It remained a high level until returned to basal level at 8 h.

3. Transient transfection of Rab11 gene

3.1 Detection of Rab11 in H9c2 and K6 cells

Previous studies in our laboratory revealed that considerable amount of Rab11 was expressed in rat heart. In this study, we used Western-blot to analyze the expression of Rab11 in H9c2 and K6 cardiomyocyte lines. The cells were lysed with RIPA buffer, 20μg proteins from each sample were separated by SDS-PAGE using 8~18% gradient gels and transferred to PVDF membrane. Immunodetection was performed with anti-Rab11 antiserum. The blots were visualized by ECL on Lumi imager system using image analysis software. The results revealed that Rab11 is barely expressed in both H9c2 and K6 cells at the protein level (Fig. 4.). Therefore, it is necessary to establish a model to study the role of Rab11 in glucose transport. We decided to overexpress this gene in H9 and K6 cells using a transient transfection strategy.

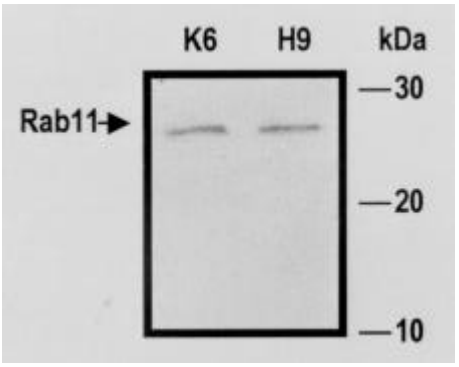

Fig. 4. Detection of Rab11 in H9 and K6 cells.
20 µg proteins from wild type H9 and K6 cells were separated by SDS-PAGE using 8-18% gradient gels and immunoblotted with anti-serum against Rab11.

3.2 Identification of the plasmids used for transfection

In order to overexpress Rab11 gene, RT-PCR technique was used to construct a mammalian expression vector containing encoding region of Rab11 cDNA. Because the quality of the plasmid is critical for high transfection efficiency, the plasmids DNA should be of high purity and the contamination of RNA and proteins should be removed. For this purpose, Qiagen plasmid Kits were used to purify the plasmid DNA. The products were then quantified with spectrophotometry at a wavelength of 260 nm. All the plasmids used in the experiments had OD260/OD280 ratios between 1.8 to 2.0, indicating that the quality of the plasmids is reliable.

As Rab 11 cDNA was inserted in the Not I site of the vector, 2 µg plasmids were digested by this enzyme and then visualized by agarose gel electrophoresis. The resulting fragment should be 651 bp as shown in Fig. 5. Sequence analysis confirmed that one copy of Rab11 cDNA was inserted into the vector with the right direction.

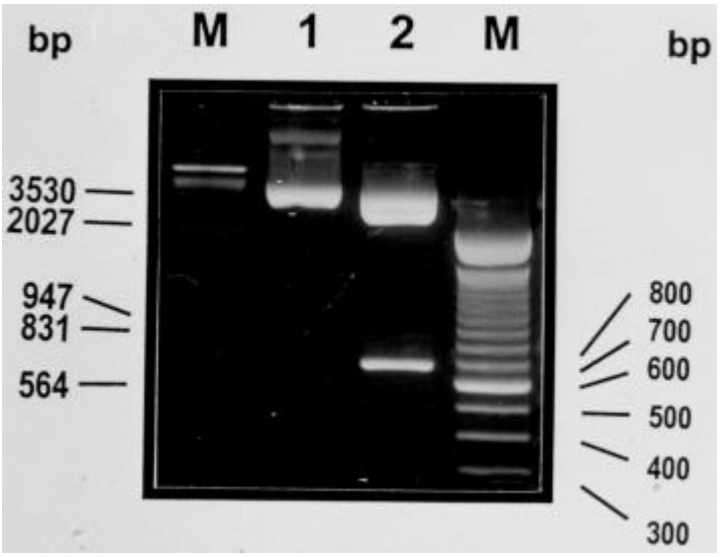

Fig. 5. Restriction analysis of pCMV-Rab11.

2 µg plasmids were digested with Not I and run on a 1% agarose gel. The resulting fragment was 651 bp as predicted. Left marker, λ DNA cut with Hind III and Eco RI, right marker, 100 bp DNA ladder, lane 1, undigested pCMV-Rab11, lane 2, pCMV-Rab11 digested with Not I.

3.3 Overexpression of Rab11 gene

According to the results of the above experiments, both H9 and K6 cells express only very small amount of Rab11. In order to study the role for Rab11 in glucose transport, we decided to establish a model by transient transfection of the cells with pCMV mammalian expression vector containing Rab11 cDNA. K6 cells seeded in six well plates were transiently transfected with 3 µg of pCMV plasmids containing Rab11 cDNA using SuperFect transfection reagent. The control (C) group was transfected with pCMV parental vector without Rab11 cDNA, while the blank group (B) were left without any treatment. The expression of Rab11 gene was assayed by Western-blot after two-days' incubation. The proteins were harvested by lysis of the cells with RIPA buffer and then separated by SDS-PAGE. Immunodetection was performed with anti-Rab11 antiserum. Fig. 6. showed that in consistence with the above results, there were very weak signals in

blank and control groups, indicating very small amount of proteins encoded by Rab11 gene in nontransfected cells. After two days of transfection, the expression of Rab11 increased dramatically.

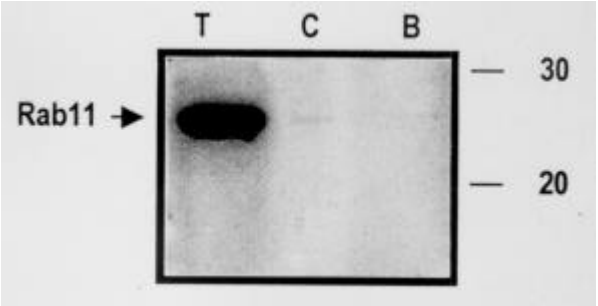

Fig. 6. Overexpression of Rab11 in K6 myoblasts.
K6 myoblasts seeded on 6 well-plates were left untreated (B), transiently transfected with 3 μg pCMV (C) or pCMV-Rab11 (T). After culture for 48 h, 20 μg proteins from each group were separated by SDS-PAGE, transferred to PVDF membrane and detected with antiserum against Rab11.

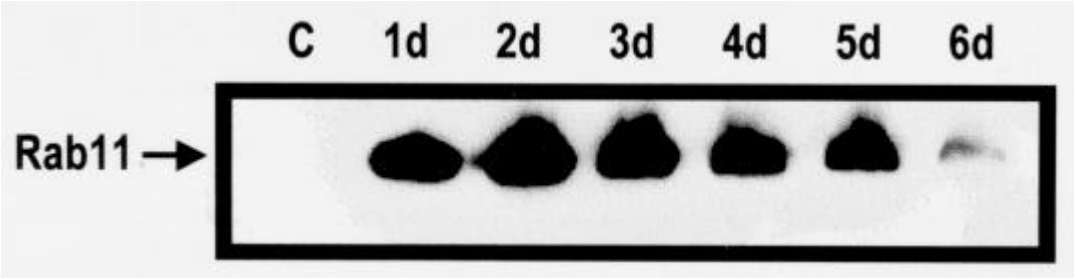

Fig. 7. Kinetic analysis of Rab11 overexpression.
K6 cells were transfected with 3 μg Rab11 and harvested after culture for 1~6 days (1d~6d). 20 μg proteins were used for Western-blot. The control (C) was transfected with pCMV parental plasmid without Rab11.

In order to study the kinetics of the expression of Rab11 in transiently transfected cells, the cells were incubated for different time after transfection and the expression level was

investigated with Western-blot. The results demonstrated that 24 h post-transfection, there was already considerable expression of this gene. At about the second or the third day post-transfection, the expression level reached a peak, then went down gradually. At the sixth days of transfection, the expression level became much lower.

4. The effects of Rab11 on 2-Deoxy-D-[1-^{14}C]-glucose uptake

4.1 Rab11 increases glucose uptake in H9c2 and K6 cells

It has been well documented that Rab11 is associated with vesicle recycling from the endosomes to apical plasma membrane. Recent work in our laboratory provided the evidence that Rab11 is colocalized with GLUT4 and exhibits insulin responsiveness, suggesting its potential in modulating the translocation of GLUT4. In order to investigate possible regulatory effects of Rab11 on GLUT4 traffic, glucose transport was measured in cells overexpressing Rab11.

Since transfection is a kind of stress for the cells, which may result in changes in metabolism of transfected cells, we first compared the glucose uptake levels between blank controls without transfection and the experimental controls transfected with parental pCMV vector without Rab11 cDNA. Fig. 8. showed mean value ± SEM from two separate experiments, the results indicated that there was no difference between the two groups. Thus it can be concluded that transfection itself has no influence on glucose transport.

High passage H9c2 and K6 cells were seeded in 6 well-plates and transfected with 3 μg of pCMV plasmids encoding Rab11 cDNA. Control groups were transfected with the same amount of pCMV parental plasmids. According to the above results, the expression of Rab11 reached a peak at the second day of transfection, therefore, 2-Deoxy-D-[1-^{14}C]-glucose uptake was assayed after 2 days of transfection. Fig. 9. (panel A) demonstrated that in H9c2 cells, insulin stimulation caused an increase in glucose uptake by about 40% over basal level in control groups. Transfection with Rab11 resulted in a significant elevation of basal glucose uptake by 40±6% ($p<0.05$). However, the responsiveness of the Rab11 transfected cells to insulin was depressed by about 20%. Similar results were observed in K6 cells. As shown in Fig. 9. (panel B), overexpression of Rab11 also induced a 30±8% ($p<0.05$) increase in basal glucose uptake. Under this condition, insulin

was able to stimulate glucose uptake by 45%. There was no significant difference in insulin stimulated glucose uptake between Rab11 and controls.

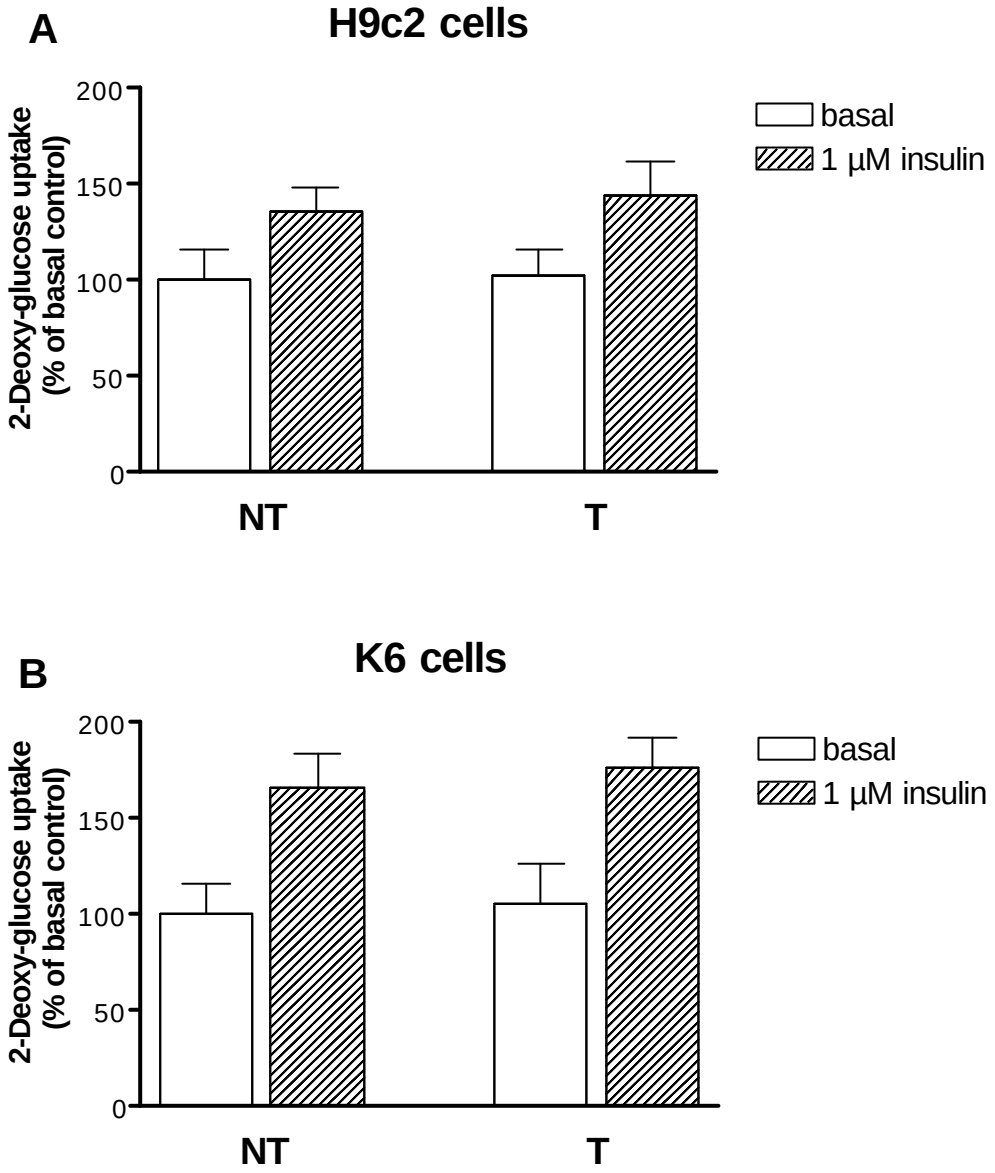

Fig. 8. The effects of transient transfection on glucose uptake.

The cells were left untreated or transfected with empty vectors. 2-Deoxy-glucose uptake was measured after 2 days. The results were presented as mean value ± SEM (n=2). There are no significant differences between the two groups. Panel A, H9 cells, panel B, K6 cells, NT, nontransfected controls, T, transfected groups.

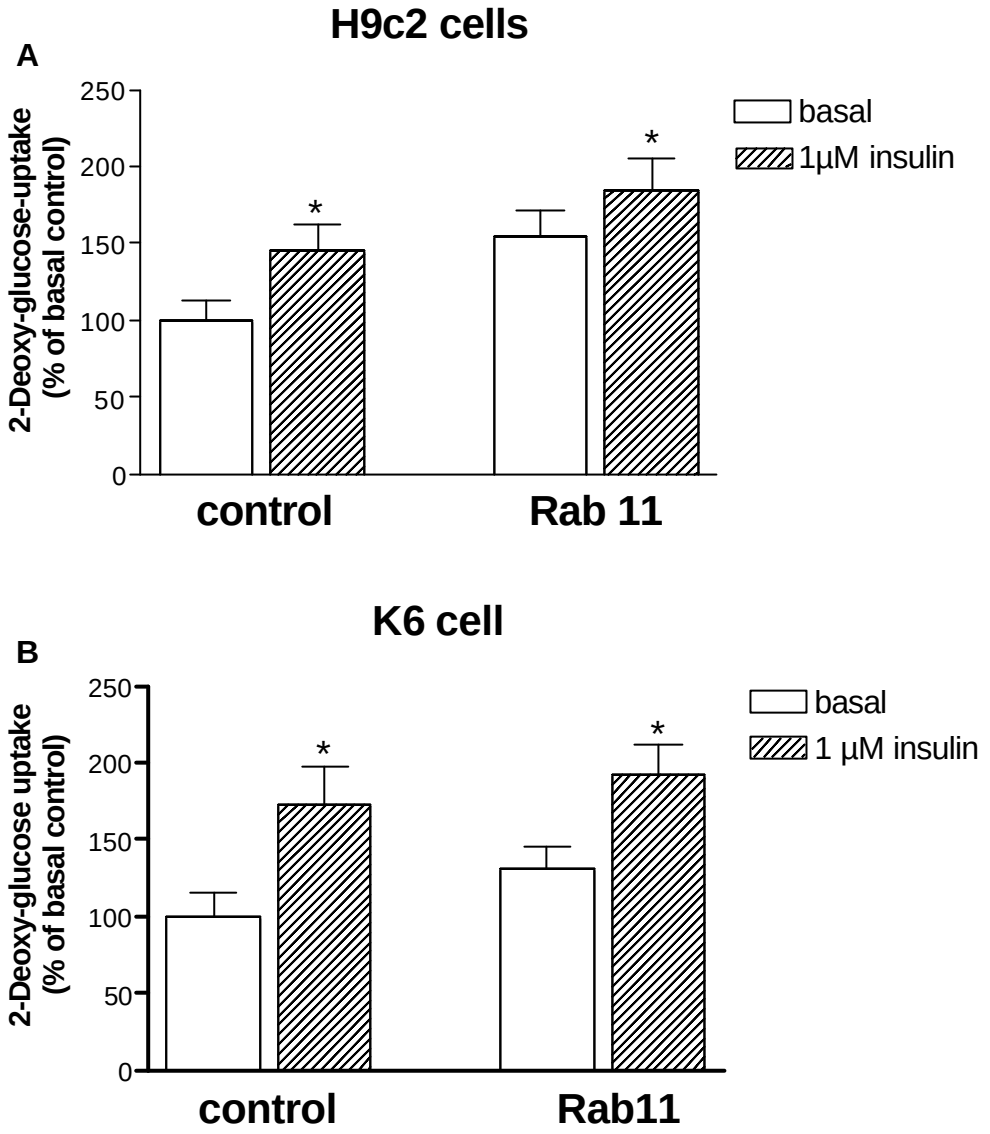

Fig.9. The effects of Rab11 on glucose uptake.

H9 (panel A) and K6 (panel B) cells were transiently transfected with pCMV-Rab11 and empty pCMV vectors, respectively. After 2 days, the cells were serum starved for 2.5 h, and incubated with and without 1 μM insulin for 45 min, 2-Deoxy-glucose uptake was measured as described previously. The data were shown as mean value ± SEM (n=3). The differences between basal and insulin stimulation conditions were significant (*$p<0.05$).

In order to investigate the effects of Rab11 on the ability of insulin to trigger glucose transport, we compared the magnitude of insulin stimulatory effects in controls and Rab11 groups. As seen in Fig. 10., in the control groups, insulin promotes 40% increase in H9c2 cells and 70% increase in K6 cells compared with basal state. However

overexpression of Rab11 caused significant decrease in insulin responsiveness in both cell lines, with only 20% in H9c2 and 45% in K6 cells. All the results were obtained from at least three separate experiments and presented as mean value ± SEM.

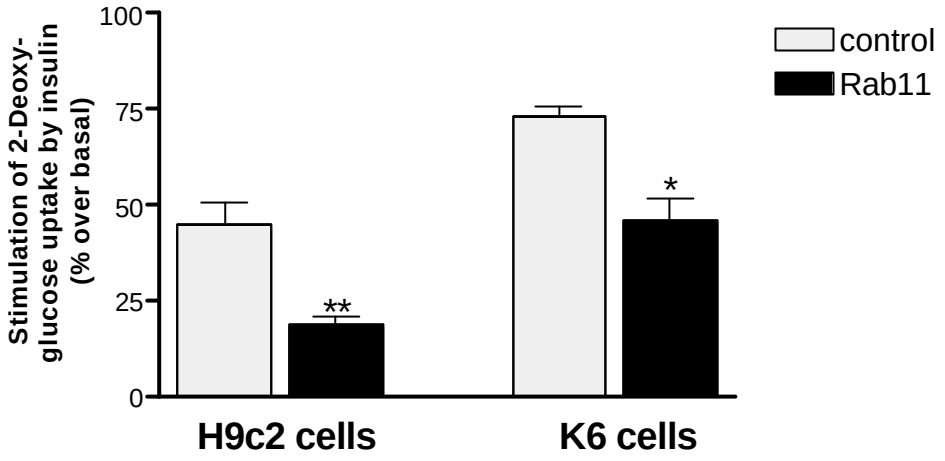

Fig. 10. Alterations of insulin responsiveness by Rab11.

The insulin responsiveness was compared between control and Rab11 groups. In the control groups, insulin promotes 40% increase in H9c2 cells and 70% increase in K6 cells over basal state. Overexpression of Rab11 caused significant decrease in the magnitude of insulin stimulatory effects in both cell lines, with only 20% in H9c2 and 45% in K6 cells. All the results were obtained from at least three separate experiments and presented as mean value ± SEM. There were significant differences between control and Rab11 groups (*$p<0.05$, **$p<0.01$).

4.2 The effect of Rab11 on the expression of GLUT4

The above results demonstrated that overexpression of Rab11 results in increased glucose uptake under basal and insulin stimulation conditions. A possible explanation of these observations was that there might be more available glucose transporters in the cells overexpressing Rab11. Given the important role of GLUT4 in insulin regulated glucose uptake, this glucose carrier might be a target site of many regulators. Here, Western-blot was used to analyze the level of GLUT4 expression. The cells were transfected with 3 μg blank vector or recombinant plasmids containing Rab11 cDNA. The blank controls were left without any treatment. Two days after transfection, the cells were lysed with RIPA buffer. The proteins were separated by SDS-PAGE and transferred to PVDF membrane

before immunodetection. Fig. 11. showed a typical experiment from three separate experiments with similar results. The result revealed that there was no difference in the amount of GLUT4 in different groups.

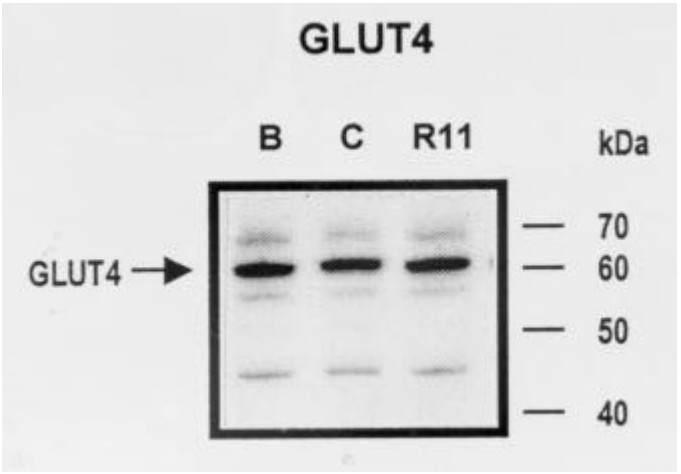

Fig. 11. The effects of Rab11 on the expression of the GLUT4 gene.
The expression of GLUT4 in K6 cells was analyzed with Western-blot using 20 μg proteins from the following groups: untreated cells (B), the cells transfected with empty pCMV (C), and the cells transfected with pCMV-Rab11 (R11).

4.3 Wortmannin cann't block the effects of Rab11

PI 3-K plays a central role in a variety of biological functions of insulin, including the redistribution of GLUT4. Recent reports proposed that several Rab proteins may be downstream molecules of PI 3-K. In this study, an inhibitor of PI 3-K, wortmannin was used to clarify whether PI 3-K is involved in the effects of Rab11. 100 nM wortmannin dissolved in DMSO was added to the medium immediately after transfection of the H9c2 cells with Rab11. The final concentration of DMSO is 0.01%, which is believed to never affect glucose uptake. 24 h later, the medium was changed and new wortmannin was added to avoid the potential influence of the degradation of this reagent. The cells were incubated for an additional 24 h. Before glucose uptake was assayed, the cells were

washed intensively to remove trace of wortmannin in order to avoid acute effects of wortmannin on insulin signal transduction.

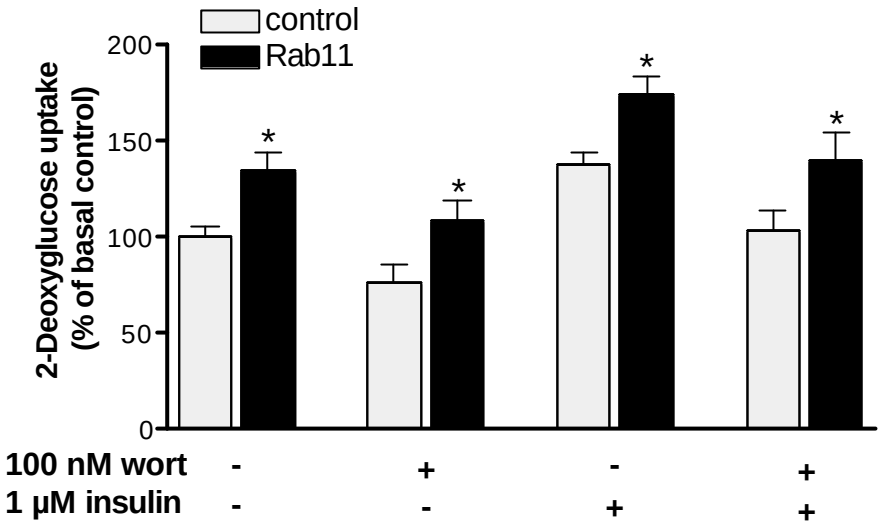

Fig. 12. The effects of wortmannin on Rab11-induced increase of glucose uptake.
The H9 cells were treated with 100 nM wortmannin immediately after transfection. The same concentration of vehicle was given to the controls. 2-Deoxy-glucose uptake assay was performed after two days. The data were presented as mean value±SEM from at least three separate experiments. * indicated the differences between Rab11 and controls were significant with $p<0.05$.

Fig. 12. demonstrated that chronic treatment of wortmannin decreased basal and insulin-stimulated glucose uptake in control and Rab11 groups. In control cells, wortmannin caused 20% and 25% decrease in basal and insulin-stimulated glucose uptake. Similar results were obtained from Rab11 groups with 30% and 25% decrease under these two conditions.

To get a further insight into the role of PI 3-K in Rab11 induced increase of glucose uptake, we then compared the inhibitory rate of wortmannin in control and Rab11 overexpression cells. As revealed in Fig. 13., under basal state, the mean inhibitory rate is 27% and 22% in control and Rab11 groups respectively. The results were similar under the insulin stimulation state. The difference of inhibitory rate between these two groups was not statistically significant ($p=0.11$), indicating the effect of wortmannin was not specific for Rab11. It can be concluded that Rab11 induced increase in glucose uptake can

not be abolished by PI 3-K inhibitor. Thus, PI 3-K may not involved in the effects of Rab11.

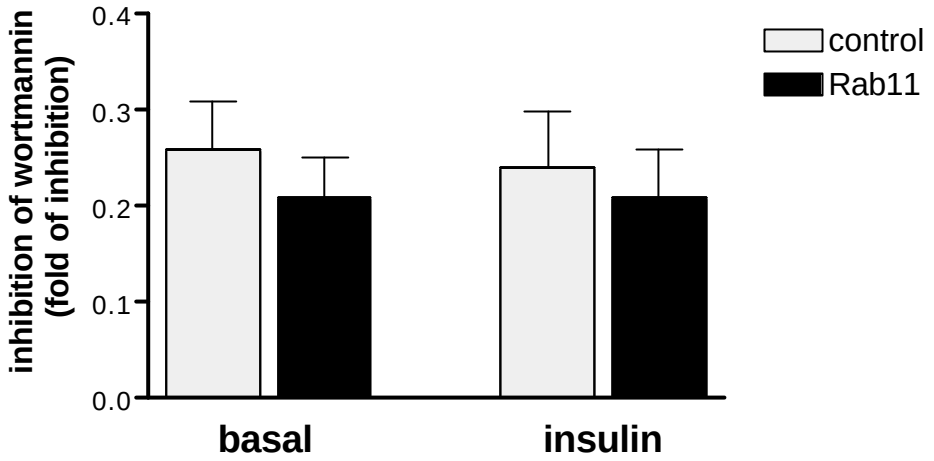

Fig. 13. Inhibitory effects of wortmannin on glucose uptake.

Inhibitory effects of wortmannin on glucose uptake were compared between control and Rab11 groups. The results indicated that there was no significant difference between these two groups under basal and insulin stimulation condition.

4.4 Involvement of PKC in the effects of Rab11

4.4.1 PKC is involved in Rab11 induced increase of glucose uptake

It has been reported that several members of Rab family can be phosphorylated by a large range of protein kinases (Karniguian *et al.*, 1993; Cormont *et al.*, 1994; Vollenweider *et al.*, 1997). PKC has been identified as components in insulin signal pathway leading to GLUT4 translocation. Recent research revealed that Rab6 could be phosphorylated by PKC. This PKC-dependent phosphorylation results in translocation of Rab6 from the platelet particulate fraction to the cytosol (Fitzgerald & Reed 1999). These findings led us suppose that there may also be functional associations between PKC and Rab11. In this present study, the PKC inhibitor, BIM was used to investigate the possible role of PKC in the function of Rab11. The BIM treatment was performed according to the same protocol as that for wortmannin, except that 1 μM BIM was used.

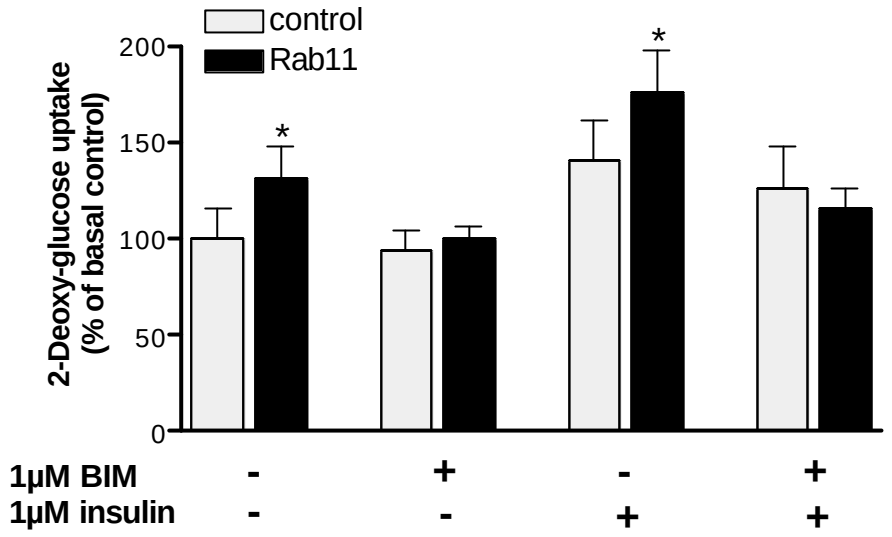

Fig. 14. The effects of PKC inhibitor, BIM on the action of Rab11.
1 μM BIM was added to the cells immediately after transfection. The controls were only treated with the same concentration of vehicle. The cells were incubated for two day before glucose uptake was assayed. The data are presented as mean value±SEM from at least three separate experiments. * indicated the differences between Rab11 and controls were significant with $p<0.05$.

The results showed that in control groups, glucose uptake under basal and insulin stimulation conditions were only slightly inhibited by BIM treatment, which were not statistically significant. However this inhibition was much stronger in the cells overexpressing Rab11 with 31% and 36% decrease in basal and insulin-stimulated glucose uptake. As seen from Fig. 14., the effects of Rab11 were completely blocked with BIM. These results proposed that Rab11 might affect glucose uptake via a PKC-dependent pathway.

4.4.2 The effect of PKC inhibitor on the expression of Rab11 gene

Another explanation of the above findings would be that PKC might be required for the expression of Rab11. Thus Western-blot was used to find out whether BIM had any influence on the expression of Rab11 in H9c2 cells. Transfected cells were treated with BIM according to the above protocol. The blank controls were left untreated or treated

with the same concentration of the vehicle. The results demonstrated that there were no detectable changes in the expression of Rab11 (Fig. 15.). This result suggested that PKC might be involved in the action of Rab11 through mechanisms other than altering the expression of Rab11 gene.

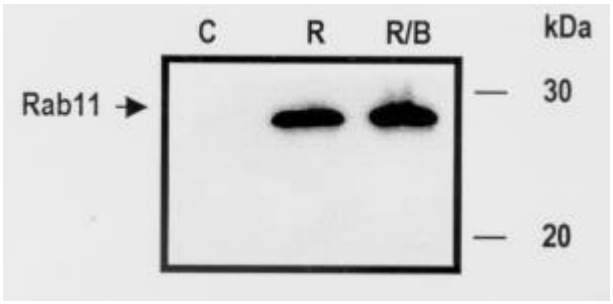

Fig. 15. The influence of BIM on the expression of Rab11 in H9 cells.
The transfected cells were exposed to 1 μM BIM as detailed in "Methods". The expression of Rab11 was analyzed with Western-blot using 20 μg proteins from the following groups: controls transfected with pCMV parental plasmids (C), Rab11+DMSO (R), Rab11+BIM (R/B).

DISCUSSION

1. Insulin and the expression of Rab11

Polypeptide hormone insulin mediates an array of biological effects including stimulation of glucose uptake, glycogen, lipid and protein synthesis, antilipolysis, modulation of cellular growth and differentiation. Many of these effects occur very rapidly through rapid signal transduction. Alongside these rapid nongenomic effects, insulin also regulates expression of key genes of metabolism pathways (Flier & Hollenberg 1999).

Among all the diverse actions of insulin, one of the most critical is the regulation of glucose homeostasis by triggering glucose uptake from circulation. It has been well established that this action of insulin results from a redistribution of glucose transporters, mainly GLUT4 from intracellular storage sites to the plasma membrane (Rea & James 1997; Holman & Kasuga 1997). Rab proteins play a key role in regulating the transportation of biological macromolecules. Accumulating evidence supports the idea that Rab proteins are involved in traffic of GLUT4-containing vesicles. Previous work in our laboratory suggested that Rab11 is colocalized with GLUT4 and may be involved in insulin-stimulated redistribution of GLUT4 (Kessler *et al.*, unpublished data). However, the precise role of Rab11 in GLUT4 translocation and its association with insulin signal cascade remains to be elucidated.

Here, RT-PCR was used to analyze the expression of Rab11 in H9K6 rat heart muscle cells stably expressing GLUT4. We found that this cell line had a basic expression of Rab11, insulin treatment caused a moderate increase in Rab11 expression by about 90%, indicating insulin was able to activate the expression of this gene. This finding raises the possibility of functional relations of Rab11 with insulin signal pathway.

Insulin is known to regulate many important genes in mammalian metabolism (Taha & Klip 1999; Flier & Hollenberg 1999). This action involves activation of transcription as well as the processes of mRNA translation. At transcriptional level, insulin signaling induces increased transcription of ADD-1/SREBP-1c gene. ADD-1 (adipocyte determination and differentiation factor 1), previously identified as a transcription factor involved in adipogenesis, now is considered as major regulator of key genes in fatty acid and triglyceride metabolism and also named SREBP-1c (sterol regulatory element binding protein 1c). In addition to activating the expression of this transcription factor, insulin regulates its activity by phosphorylation and unknown altering processing.

ADD-1/SREBP-1c then acts on promoters in insulin target genes to modulate their expression. Insulin may also affect the function and/or expression of peroxisome proliferator-activated receptor-γ (PPAR-γ), another transcription factor through which it regulates gene expression (Flier & Hollenberg 1999). Thus, a possible explanation of our results is insulin may active the transcription of Rab11 through one of the above transcription factors. It is noteworthy that in our experiments, the upregulation of Rab11 mRNA expression occurred at 0.5 hour after insulin treatment. This rapid effect is more likely due to the activation of certain transcription factors than the increase in the amount of them.

In addition to activating the expression of specific genes, insulin also plays important roles in the overall regulation of gene expression in sensitive cells. This hormone acutely stimulates a global increase in gene expression of responsive cells. Therefore, another possibility is that this increase in the transcription of Rab11 is merely the result of unspecific changes in the overall gene expression. This possibility is less likely in accordance with the data from ß-actin. More experiments are needed to elucidate the significance of the insulin-induced upregulation of Rab11 gene expression and the underlying mechanisms.

2. The role of Rab11 in glucose uptake

It has been well established that insulin-stimulated glucose uptake is achieved mainly by recruitment of GLUT4 to the plasma membrane. Under the basal condition, most of the GLUT4 molecules exist in the putative retention pool, insulin induces the movement of GLUT4 from the retention pool to the recycling pathway and results in subsequent translocation to the plasma membrane (Rea & James 1997; Pessin *et al.,* 1999; Holman & Kasuga 1997). Rab11 has emerged as an important regulator of vesicle traffic through the recycling endosomal system. It has been reported that Rab11 is required for vesicle traffic from TGN to plasma membrane (Goldenring *et al.,* 1997; Chen *et al.,* 1998). Recent work in our laboratory demonstrated that Rab11 was associated with GLUT4-containing vesicles isolated from rat heart. We also observed that insulin induced an increased abundance of Rab11 in GLUT4-containing vesicles and recruitment of Rab11 to the plasma membrane. These data indicate the possible role of Rab11 in insulin-regulated GLUT4 translocation (Kessler *et al.,* unpublished data).

In order to obtain further understanding of potential roles of rab11 in GLUT4 translocation, we examined the effects of Rab11 on glucose uptake of wild type H9c2 rat cardiomyocyte line and a stable clone (H9K6) overexpressing GLUT4. It is wildly accepted that GLUT4 expression is developmentally regulated in L6 skeletal muscle cell line and 3T3-L1 adipogenic cell line. It expresses during and after differentiation from myoblasts or fibroblasts into myotubes or adipocytes (Ueyama *et al.,* 1999). Earlier work of our laboratory indicated that in contrast to L6 and 3T3-L1 cells, H9c2 myoblasts at high passage also express appreciable amount of GLUT4. Here, we first compared the expression of GLUT4 in low passage and high passage H9c2 cells. In agreement with our previous studies, although low passage H9 cells have only marginal expression of GLUT4, the high passage cells expressed this glucose transporter at a level comparable to that of K6 cells. Then we analyzed the insulin sensitivity of these high passaged H9 cells. The results of 2-Deoxy-glucose uptake assay revealed that these cells also exhibit insulin-stimulated glucose transport by about 38% over basal state in comparison to 70% in K6 cells. These results indicated that high passage H9 cells could also be used to study the regulation of GLUT4 translocation.

Using Western-blot we found that both wild type H9c2 cells and K6 cells expressed marginal amount of Rab11, although considerable amount of Rab11 mRNA was detected in K6 cells by RT-PCR. The reason of this discrepancy between mRNA transcripts and protein amount is still obscure. Therefore, we decided to establish a model for studying the role of Rab11 in glucose transport by transient transfection of these cells with Rab11 gene cloned in a mammalian expression vector. Western-blot demonstrated that transient transfection resulted in significant increase of Rab11 expression, indicating high transfection efficiency of this gene. We further studied the kinetics of Rab11 expression in transfected cells. As revealed by Western-blot, the highest expression level was observed at the second day post-transfection. Therefore, the relative experiments were performed at this time point.

The results demonstrated that overexpression of Rab11 led to a significant increase in basal glucose uptake by about 40% and 30% in H9c2 and K6 cells, respectively. Taking into account the transfection efficiency of 60%~70% as revealed in our previous experiments (Dransfeld *et al.,* 2000), it can be assumed that these effects of Rab11 may be even stronger. We estimate that basal glucose uptake in Rab11 transfected cells was increased by about 60% in H9c2 cells and 50% in K6 cells. However, under this

condition, the effects of insulin was depressed significantly from 40% to 20% in H9 cells and from 70% to 45% in K6 cells. These data indicated that the changes in glucose uptake induced by Rab11 were not addictive to the effects of insulin.

Based on the notion that glucose transport rate is proportional to the amount of the transporters on the cell surface, together with the particular significance of GULT4 in glucose uptake of insulin sensitive tissue (Suzuki *et al.,* 1980; Cushman *et al.,* 1980), we suppose that this increase in glucose uptake might be attributed to elevated amount of GLUT4 on the plasma membrane. This hypothesis can be interpreted by two possibilities. First, overexpression of Rab11 might up-regulate the total expression of GLUT4, resulting in more of these transporters on the cell surface. Second, overexpression of Rab11 may induce the movement of more GLUT4 from the intracellular storage pool to the plasma membrane in the basal state, thus decrease the amount of recruitable GLUT4 in these cells and result in depressed insulin responsiveness. To explore these possibilities, we first compared the expression of GLUT4 in the cells transfected with Rab11 and controls transfected with the blank vectors. Western-blot revealed that there was no difference of GLUT4 expression between these two groups. It can be concluded that total GLUT4 expression was not affected by Rab11. It is more likely that Rab11 induces the redistribution of GLUT4 to the cell surface under the basal condition.

Moreover, we can not exclude the possibility that Rab11 may affect the distribution of GLUT1, which is mainly responsible for basal glucose uptake. Thus another explanation of the above findings would be that overexpression of Rab11 results in more GLUT1 on the plasma membrane under the basal state. Since GLUT1 is only weakly responsive to insulin, the accumulation of GLUT1 on the cell surface enhances the background glucose uptake, which decreases insulin responsiveness.

3. PI 3-K and Rab proteins

PI-3 K plays a central role in the insulin signaling cascade. The PI 3-K-dependent pathway is necessary in mediating most of the biological activities of insulin. At present, substantial evidence support the idea that the activation and/or appropriate targeting of class 1a PI 3-K is required for insulin-stimulated glucose uptake and GLUT4 translocation (Hara *et al.,* 1994; Okada *et al.,* 1994; Clarke *et al.,* 1994; Kotani *et al.,* 1995; Tanti *et al.,* 1996). However, the specific downstream targets for PI-3 K still remain to be elucidated.

There are now examples of Rab proteins acting downstream of PI 3-K. Its activity may be required for the function of Rab9 and Rab5 (Riederer *et al.*, 1994; Li *et al.*, 1995). Furthermore, recent studies demonstrated that activation of PI 3-K by insulin directly leads to an increase in the GTP loading of Rab4. PI 3-K is linked to GLUT4-containing vesicles and insulin stimulation can increase the abundance of it on these vesicles (Heller-Harrison *et al.*, 1997). Previous studies in our laboratory showed that Rab11 is similar to PI 3-K in this regard (Kessler *et al.*, unpublished data), providing the possible functional association of Rab11 with PI 3-K.

In the present study, we investigated the potential involvement of PI 3-K in Rab11 induced increase in glucose uptake. The cells were incubated with PI 3-K inhibitor, wortmannin immediately after transfection with Rab11. To avoid the acute effect of wortmannin on insulin signaling, the cells were washed intensively before 2-Deoxy-glucose uptake was assayed. Our results demonstrated that wortmannin inhibited glucose uptake to the same extent in control and Rab11 overexpression groups. It is apparent that the inhibitory effects of wortmannin are not specific for Rab11. These results indicated that Rab11 may not be functionally related to PI 3-K.

Although PI 3-K may be required for insulin-stimulated GLUT4 translocation, it is not clear whether its activity is sufficient in this process. Several lines of evidence suggested the existence of PI 3-K-independent pathways. For example, it had been reported that depletion of PI 3-K by wortmannin could not block GLUT4 translocation triggered by insulin through the activation of trimeric GTP-binding proteins (Kanzaki *et al.*, 2000). Synthesized short peptides containing two tyrosine-phosphorylated YMXM motifs, which is necessary for the activation of PI 3-K stimulated PI 3-K as strong as insulin. However, the ability of this peptide to increase glucose uptake was only 20% of insulin (Herbst *et al.*, 1995). Earlier studies demonstrated that insulin was able to change the subcellular distribution of Rab5 independently of PI 3-K activation (Cormont *et al.*, 1996). These findings, together with the above observation raised the possibility that Rab11 may be involved in PI 3-K-independent pathway in insulin signal transduction. However, with the present data, we can not draw a definite conclusion on whether Rab11 acts as a signaling component of insulin-regulated glucose transport.

4. PKC and Rab proteins

Multiple studies using various pharmacological reagents suggested that protein kinase C was a mediator of insulin-stimulated glucose transport (Farese *et al.*, 1992; Yu *et al.*, 1992; Avignon *et al.*, 1995). In addition, PLCγ which catalyzes the production of DAG and inositol 1,4,5-triphosphate (IP_3), two second messengers involved in activation of PKC and the release of calcium from intracellular stores, has also been found as an active participant in insulin-stimulated glucose uptake (Kayali *et al.*, 1998). However, the exact mechanism by which insulin activates PKC and its linkage to the GLUT4 translocation machinery are still obscure.

The Rab family of small GTP-binding proteins is know to play crucial roles in vesicle traffic. Like other GTPases, their functions are superimposed onto the cycling between a GDP-bound inactive and a GTP-bound active conformation, which is regulated by three types of modulators: GDP/GTP exchange protein (GEP), GTPase activating protein (GAP) and GDP dissociation inhibitor (GDI) (Schimmoller *et al.*, 1998; Novick & Zerial 1997). Increasing evidence suggested that phosphorylation of Rab proteins may also be an important mechanism through which the functions of Rab proteins were modified by various extracellular signals. Members of Rab family were found to be phosphorylated differentially by different protein kinases including ERK-1/2, cdc2 kinase and PKC (Bailly *et al.*, 1991; Karniguian *et al.*, 1993; Chiariello *et al.*, 1999). In platelets, thrombin induced a PKC-dependent phosphorylation of Rab6. This phosphorylation led to translocation of Rab6 from platelet particulate fractions to the cytosol. Moreover, PKC phosphorylation of Rab6 increased its affinity for GTP by 3 fold compared with that for GDP by 1.6 fold without altering the GTPase activity (Fitzgerald & Reed 1999). Rab4 can be phosphorylated on serine residues by insulin-activated extracellar-signal-regulated kinase ERK1 (Corment *et al.*, 1994). More interestingly, in MDCK cells, transient expression of dominant negative mutant of Rab11 slightly inhibited reorganization of stress fibers and focal adhesions induced by PKC activator, TPA (Imamura *et al.*, 1998). These findings collectively suggested that phosphorylation may be an important mechanism through which Rab proteins exert their regulatory functions in vesicle traffic.

In an effort to explore the functional relations between Rab11 and PKC, the effects of Rab11 on glucose uptake were examined under the condition that the activity of PKC was inhibited by BIM. The results revealed that the addition of PKC inhibitor, BIM was able to completely block the effects of Rab11.

There are several plausible interpretations of the above results. First of all, it should be clarified whether PKC is necessary for the expression of Rab11 under our experimental conditions. For this purpose, the cells were exposed to PKC inhibitor immediately after transfection with Rab11. Western-blot was performed 48 h post-transfection. The results demonstrated that there is no significant difference between BIM treated group and untreated controls. This result excluded the possibility that BIM blocked the effects of Rab11 by down-regulating its expression.

Second, Rab11 may be directly phosphorylated by PKC in a way similar to that of Rab6, this PKC-dependent phosphorylation of Rab11 may result in translocation and/or alteration of GTP/GDP-bound states. Unfortunately, till now, there are no data available in this respect.

Third, PKC may induce phosphorylation of the upstream regulators of Rab11 or the molecules involved in GDP/GTP cycling of Rab11. This hypothesis was supported by the earlier findings that a 55 kDa GDI was able to be phosphorylated and therefore regulated the cycling of Rab proteins between membrane and cytosol (Steele-Mortimer *et al.*, 1993). Recent studies showed that insulin promotes phosphorylation and activation of GGTase, the enzyme that catalyzes geranylgeranylation, the first step of post-translation modification of Rab proteins and the prerequisite to their activation (Goalstone *et al.*, 1999). PKC might be a good candidate responsible for these phosphorylation reactions.

Finally, the downstream effectors of Rab11 may also serve as targets of PKC. In this respect, Fujita *et al.* recently showed that recombinant Munc-18 was phosphorylated by PKC in a cell free system and this PKC-dependent phosphorylation of Munc-18 could inhibit its interaction with Syntaxin (Fujita *et al.*, 1996). As mentioned before, Munc-18c emerged as a negative regulator of the formation of SNARE complex required for GLUT4 translocation. Rab proteins are proposed to accelerate the assembly of SNARE complex probably by catalyzing the dissociation of the member of Sec1/Munc-18 family from t-SNAREs (Rea & James 1997; Pessin *et al.*, 1999). It can be assumed that Rab11 may accomplish such functions by directly or indirectly acting on phosphorylated Munc-18 protein and promote release of these proteins from t-SNARE molecules. That is to say, phosphorylation of Munc-18 might be the prerequisite for the function of Rab11. Moreover, with current data, we still can not rule out the possibility that Rab11 may lie upstream of PKC. More work will be necessary to elucidate the precise functional interaction of Rab11 and PKC.

CONCLUSIONS

The data from the present study lead to the following conclusions concerning the roles of the small GTP-binding protein Rab11 in glucose uptake.

1. mRNA transcription level of Rab11 is upregulated by insulin as revealed by RT-PCR, suggesting possible involvement of Rab11 in insulin-mediated biological effects.

2. Overexpression of Rab11 increases glucose uptake in wild type H9c2 cardiomyocyte line and K6 stable clone overexpressing GLUT4 without changing the total amount of GLUT4 in these cells. These findings suggested that Rab11 might play a role in GLUT4 translocation.

3. PI 3-K inhibitor, wortmannin can not block the effects of Rab11 on glucose uptake, indicating that Rab11 may not participate in PI 3-K-dependent pathway.

4. Rab11 induced increase in glucose uptake can be completely blocked with PKC inhibitor, BIM. Expression of Rab11 was not modified by depletion of PKC activity with BIM. These results indicate possible functional associations between Rab11 and PKC.

REFERENCES

Alessi D. R., C. P. Downes: The role of PI 3-kinase in insulin action. *Biochim. Biophys. Acta.* 1436: 151-164, 1998

Avignon A., M. L. Standaert, K. Yamada, H. Mischak, B. Spencer, R.V. Farese: Insulin increases mRNA levels of protein kinase G-alpha and -beta in rat adipocytes and protein kinase C-alpha, -beta and -theta in rat skeletal muscle. *Biochem. J.* 308: 181-187, 1995

Bailly E., M. McCaffrey, N. Touchot, A. Zahraoui, B. Goud, M. Bornens: Phosphorylation of two small GTP-binding proteins of the Rab family by p34cdc2. *Nature.* 350: 715-718,1991

Baldini G., R. Hohman, M. J. Charron, H. F. Lodish: Insulin and non- hydrolyzable GTP analogs induce translocation of GLUT4 to the plasma membrane in alpha- toxin-permeablized rat adipose cells. *J. Biol. Chem.* 266: 4037-4040, 1991

Baldini G., T. Hohl, H. Y. Lin, H. F. Lodish: Cloning of a Rab3 isotype predominately Expressed in Adipocytes. *Proc. Natl. Acad. Sci. USA.* 89: 5049-5052, 1992

Bell G. I., C. F. Burant, J. Takeda, G. W. Gould: Structure and function of mammalian facilitave sugar transporters. *J. Biol. Chem.* 268:19161-19164, 1993

Birnbaum M. J.: The insulin-sensitive glucose transporter. *Int. Rev. Cytol.* 137A: 239-297, 1992

Cain C. C., W. S. Trimble, G. E. Lienhard: Members of the VAMP family of synaptic vesicle proteins are components of glucose transporter-containing vesicles from rat adipocytes. *J. Biol. Chem.* 267: 11681-11684, 1992

Chavrier P., B. Goud: The role of ARF and Rab GTPase in membrane transport. *Curr. Opin. Cell. Biol.* 11: 466-475, 1999

Chen W., Y. Feng, D. Y. Chen, A. Wandinger-Ness: Rab11 is required for trans-Golgi network to plasma membrane transport and a preferential target for GDP dissociation inhibitor. *Mol. Biol. Cell.* 9: 3241-3257, 1998

Cherqui G., M. Caron, D. Wicek, O. Lascols, J. Capeau, J. Picard: Decreased insulin responsiveness in fat cells rendered protein kinase C-deficient by a treatment with a phorbol ester. *Endocrinology.* 120: 2192-2194, 1987

Chiariello M., C. B. Bruni, C. Bucci: The small GTPase Rab5a, Rab5b and Rab5c are differentially phosphorylated in vitro. *FEBS Lett.* 453: 20-24, 1999

Clarke J. F., P. W. Young, K. Yonezawa, M. Kasuga, G. D. Holman: Inhibition of the translocation of GLUT1 and GLUT4 in 3T3-L1 cells by the phosphatidylinositol 3-kinase inhibitor, wortmannin. *Biochem. J.* 300: 631-635, 1994

Cormont M, J.-F. Tanti, A. Zahraoui, E. Van Obberghen, A. Tavitian, Y. Le Marchand-Brustel: Insulin and okadaic acid induce Rab4 translocation in adipocytes. *J. Biol. Chem.* 268: 19491-19497, 1993

Cormont M, J.-F. Tanti, A. Zahraoui, E. Van Obberghen, Y. Le Marchand-Brustel: Rab4 is phosphorylated by the insulin-activated extracellular-signal-regulated kinase ERK1. *Eur. J. Biochem.* 219: 1081-1085, 1994

Cormont M, E. Van Obberghen, M. Zerial, Y. Le Marchand-Brustel: Insulin induces a change in Rab5 subcellular localization in adipocytes independently of Phophatidylinositol 3-kinase activation. *Endocrinology.* 137: 3408-3415, 1996

Cormont M., J.-F. Tanti, T. Gremeaux, E. Van Obberghen, Y. Le Marchand-Brustel: Subcellular distribution of low molecular weight guanosine triphosphate-binding proteins in adipocytes: colocalization with the glucose transporter GLUT4. *Endocrinology.* 129: 3343-3350, 1991

Cormont M., M-N. Bortoluzzi, N. Gautier, M. Mari, E. Van Obberghen, Y. Le Marchand-Brustel: Potential role of Rab4 in the regulation of subcellular localization of GLUT4 in adipocytes. *Mol. Cell. Biol.* 16: 6879-6886, 1996

Cushman S. W., L. J. Wardzala: Potential mechanism of insulin action on glucose transport in the isolated rat adipose cell: apparent translocation of intracellular transport system to the plasma membrane. *J. Biol. Chem.* 255: 4758-4762, 1980

Dhand R., K. Hara, I. Hiles: PI 3-kinase structure and functional analysis of intersubunit interactions. *EMBO. J.* 13: 511-521, 1994a

Dransfeld O., I. Uphues, S. Sasson, A. Schurmann, H. G. Joost, J. Eckel: Regulation of subcellular distribution of GLUT4 in cardiomyocytes: Rab4A reduces basal glucose transport and augments insulin responsiveness. *Exp. Clin. Endocrinol. Diabetes.* 108: 26-36, 2000

Echard A, F. Jollievet, O. Martinez, J. J. Lacapere, A. Rousselet, I. Janoeix Lerosey, B. Goud: Interaction of a Golgi-associated Kinesin-like protein with Rab6. *Science.* 279: 580-585, 1998

Farese R., M. L. Standaert, T. Arnold, B. Yu, T. Ishizuka, J. Hoffman, M. Vila, D. R. Copper: The role of protein kinase C in insulin action. *Cell. Signalling.* 4: 133-143. 1992

Fitzgerald M. L., G. L. Reed: Rab6 is phosphorylated in thrombin-activated platelets by a protein kinase C-dependent mechanism: effects on GTP/GDP binding and cellular distribution. *Biochem. J.* 342: 353-360, 1999

Flier J. S., A. N. Hollenberg: ADD-1 provides major new insight into the mechanism of insulin action. *Proc. Natl. Acad. Sci. USA.* 96: 14191-14192, 1999

Fujita Y., T. Sasaki, K. Fukui, H. Kotani, T. Kimura, Y. Hata, T. C. Sudhof, R. H. Scheller, Y. Takai: Phosphorylation of Munc-18/n-Sec1/rbSec1 by protein kinase C. *J. Biol. Chem.* 271: 7265-7268, 1996

Goalstone M. L., J. W. Leitner, I. Golovchenko, M. R. Stjernholm, M. Cormont, Y. Le. Marchand-Brustel B. Draznin: Insulin promotes phosphorylation and activation of geranylgeranyltransferase II. *J. Biol. Chem.* 274: 2880-2884, 1999

Guo W., D. Roth, C. Walch-Solimena, P. Novick: The exocyst is an effector for Sec 4p, targeting secretory vesicles to sites of exocytosis. *EMBO. J.* 18: 1071-1080, 1999

Hara K., K. Yonezawa, H. Sakaue: Phosphatidylinositol 3-kinase activity is required for insulin-stimulated glucose transport but not Ras activation in CHO cells. *Proc. Natl. Acad. Sci. USA.* 91: 7415-7419, 1994

Hay J. C., H. R. Scheller: SNAREs and NSF in targeted membrane fusion. *Curr. Opin. Cell. Biol.* 9: 505-12, 1997

Heller-Harrison R. A., M. Morin, A. Guilherme, M. P. Czech: Insulin-mediated targeting of phosphatidylinositol 3-kinase to GLUT4-containing vesicles. *J. Biol. Chem.* 271: 10200-10204, 1996

Herbst J. J., G. C. Andrews, L. G. Contillo, D. H. Singleton, P. E. Genereux, E. M. Gibbs, G. E. Lienhard: Effect of the activation of phosphatidylinositol 3-kinase by a thiophosphotyrosine peptide on glucose transport in 3T3-L1 adipocytes. *J. Biol. Chem.* 270: 26000-26005, 1995

Hiles, I. D., M. Otsu, S. Volinia, M. J. Fry, I. Gout, R. Dhand, G. Panayotou, F. Ruiz-Larrea, A. Thompson, N. F. Totty: Phosphatidylinositol 3-kinase: structure and expression of the 110 kd catalytic subunit. *Cell.* 70: 419-429, 1992

Holman G. D., M. Kasuga: From receptor to transporter: insulin signalling to glucose transport. *Diabetologia.* 40: 991-1003, 1997

Imamura H., K. Takaishi, K. Nakano, A. Kodama, H. Oishi, H. Shiozaki, M. Monden, T. Sasaki, Y. Takai: Rho and Rab small G proteins coordinately reorganize stress fibers and focal adhesions in MDCK cells. *Mol. Biol. Cell.* 9: 2561-2575, 1998

Ishizuka T., D. R. Cooper, T. Arnold, H. Hernandez, R.V. Farese: Downregulation of protein kinase C and insulin-stimulated 2-deoxyglucose uptake in rat adipocytes by phorbol esters, glucose, and insulin. *Diabetes.* 40: 1274-1281, 1991

Kanzaki M., R. T. Wastson, N. O. Arteyev, J. E. Pessin: The trimeric GTP-binding protein (G_q/G_{11}) α subunit is required for insulin-stimulated GLUT4 translocation in 3T3L1 adipocytes. *J. Biol. Chem.* 275: 7167-7175, 2000

Karniguian A, A. Zahraoui, A. Tavitian: Identification of small GTP-binding Rab proteins in human platelets: Thrombin-induced phosphorylation of Rab3B, Rab6, and Rab8 proteins. *Proc. Natl. Acad. Sci. USA.* 90: 7647-7651, 1993

Kasuga M., Y. Fujita-Yamaguchi, D. Blithe, C. R. Kahn: Tyrosine-specific protein kinase activity is associated with purified insulin receptor. *Proc. Natl. Acad. Sci. USA.* 80: 2137-2141, 1983

Kayali A. G., J. Eichhorn, T. Haruta, A. J, Morris, J. G. Nelson, P. Vollenweider, J. M. Olefsky, N. J. G. Webster: Association of the insulin receptor with phospholipase C-γ (PLCγ) in 3T3-L1 adipocytes suggests a role for PLCγ in metabolic signaling by insulin. *J. Biol. Chem.* 273: 13808-13818, 1998

Klarlund J. K., A. Guilherme, J. J. Holik, J. V. Virbasius, A. Chawla, M. P. Czech: Signaling by phosphoinositide-3,4,5-triphosphate through proteins containing pleckstrin and Sec7 homology domains. *Science.* 275: 1927-1930, 1997

Kotani K., A. J. Carozzi, H. Sakaue, K. Hara, L. J. Robinson, S. F. Clark, K. Yonezawa, D. E. James, M. Kasuga: Requirement for phosphoinositide 3-kinase in insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes. *Biochem. Biophy. Res. Commun.* 209: 343-348, 1995

Kotani K., W. Ogawa, M. Matsumoto, T. Kitamura, H. Sakaue, Y. Hino, K. Miyake, W. Sano, K. Akimoto, S. Ohno, M. Kasuga: Requirement of atypical protein kinase C for insulin stimulation of glucose uptake but not for Akt activation in 3T3-L1 adipocytes *Mol. Cell. Biol.* 18: 6971-6982, 1998

Lazar T., M. Gotte, D. Gallwitz: Vesicle transport: how many Ypt/Rab-GTPases make an eukaryotic cell? *Trends. Biochem. Sci.* 22: 468-472, 1997

Li G., C. D'Souza-Schorey, M. A. Barbieri, R. L. Roberts, A. Klippel, L. T. Williams, P. D. Stahl: Evidence for phosphatidylinositol 3-kinase as a regulator of endocytosis via activation of Rab5. *Proc. Natl. Acad. Sci. USA.* 92: 10207-10211, 1995

Lupashin V. V., M. G. Waters: t-SNARE activation through transient interaction with a Rab-like guanosine triphosphatase. *Science.* 276: 1255-1258, 1997

Martin S., J. Tellam, C. Livingstone, J. W. Slot, G. W. Gould, D. E. James: The glucose transporter (GLUT4) and vesicle-associated membrane protein-2 (VAMP-2) are segregated from recycling endosomes in insulin-sensitive cells. *J. Cell. Biol.* 134: 625-635, 1996

Martinez O., A. Schmidt, J. Salamero, B. Hoflack, M. Roa, B. Goud: The small GTP-binding protein Rab6 functions in intra-Golgi transport. *J. cell. Biol.* 127: 1575-1588, 1994

Morris N. J., S. A. Ross, W. S. Lane, S. K. Moestrup, C. M. Petersen, S. R. Keller, G. E. Lienhard: Sortilin is the major 110-kDa protein in GLUT4 vesicles from adipocytes. *J. Biol. Chem.* 273: 3582-3587. 1998

Novick P., M. Zerial: The diversity of Rab proteins in Vesicle transport. *Curr. Opin. Cell Biol.* 9: 496-504, 1997

Okada T., Y. kawano, T. Sakakibara, O. Hazeki, M. Ui: Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. Studies with a selective inhibitor Wortmannin. *J. Biol. Chem.* 269: 3568-3573, 1994

Pessin J. E., D. C. Thurmond, J. S. Elmendorf, K. J. Coker, S. Okada: Molecular basis of insulin-stimulated GLUT4 vesicle trafficking. Location! Locatoin! Location! *J. Biol. Chem.* 274: 2593-2596, 1999

Pevsner J., S. C. Hsu, J. E. Braun, N. Calakos, A. E. Ting, M. K. Bennett, R. H. Scheller: Specificity and regulation of a synaptic vesicle docking complex. *Neuron*. 13: 353-361, 1994

Rea S., D. E. James: Moving GLUT4: The biogenesis and trafficking of GLUT4 storage vesicles. *Diabetes.* 46: 1667-1677, 1997

Ren M., G. Xu, J. Zeng, C. De Lemos-Chiarandini, M. Adesnik, D. D. Sabatini: Hydrolysis of GTP on Rab11 is required for the direct delivery of transferrin from the pericentriolar recycling compartment to the cell surface but not from sorting endosome. *Proc. Natl. Acad. Sci. USA.* 95: 6187-6192, 1998

Riederer M. A., T. Soldati, A. D. Shapiro, J. Lin, S. R. Pfeffer: Lysosome biosynthesis requires Rab9 function and receptor recycling from endosomes to the trans-Golgi network. *J. Cell. Biol.* 125: 573-582, 1994

Satoh S., H. Nishimura, A. E. Clark, I. J. Kozka, S. J. Vannucci, I. A. Simpson, M. J. Quon, S. W. Cushman, G. D. Holman: Use of bismannose photolabel to elucidate insulin-regulated GLUT4 subcellular trafficking kinetics in rat adipose cells. Evidence that exocytosis is a critical site of hormone action. *J. Biol. Chem*. 268: 17820-17829, 1993

Schimmoller F., I. Simon, S. R. Pfeffer: Rab GTPase, directors of vesicle docking. *J. Biol. Chem.* 273: 22161-22164, 1998

Shibata H, W. Omata, I. Kojima: Insulin stimulates guanine nucleotide exchange on Rab4 via a wortmannin-sensitive signaling pathway in rat adipocytes. *J. Biol. Chem.* 272:14542-14546,1997

Shibata H, W. Omata, Y. Suzuki, S. Tanaka, I. Kojima: A synthetic peptide corresponding to the Rab4 hypervariable carboxyl-terminal domain inhibits insulin action on glucose transport in rat adipocytes. *J. Biol. Chem.* 271:9704-9709, 1996

Simonsen A, R. Lippe, S. Christoforidis, J-M Gaullier, A. Brech, J. Callaghan, B-H. Toh, C. Murphy, M. Zerial, H. Stenmark: EEA1 links PI (3) K function to Rab5 regulation of endosome fusion. *Nature*. 394: 494-498, 1998

Söllner T, M. K. Bennett, S. W. Whiteheart, R. H. Scheller, J. E. Rothman: A protein assembly-disassembly pathway *in vitro* that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell*. 75: 409-18, 1993

Söllner T., S. W. Whiteheart, M. Brunner, H. Erdjument-Bromage, S. Geromanos, P. Tempst, J. E. Rothman: SNAP receptors implicated in vesicle targeting and fusion. *Nature*. 362: 318-324, 1993

Standaert M. L., L. Galloway, P. Karnam, G. Bandyopadhyay, J. Moscat, R. V. Farese: Protein kinase C-zeta as a downstream effector of phosphatidylinositol 3-kinase during insulin stimulation in rat adipocytes. Potential role in glucose transport. *J. Biol. Chem.* 272: 30075-30082, 1997

Steele-Mortimer O., J. Gruenberg, M. J. Clague: Phosphorylation of GDI and membrane cycling of Rab proteins. *FEBS Lett*. 329: 313-318, 1993

Sun X. J., P. Rothenberg, C. R. Kahn: Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. *Nature*. 352: 73-77, 1991

Suzuki K., T. Kono: Evidence that insulin causes the translocation of glucose transport

activity to the plasma membrane from an intracellular storage site. *Proc. Natl. Acad. Sci. USA.* 77: 2542-2545, 1980

Taha C., A. Klip: The insulin signaling pathway. *J. Membrane Biol.* 169: 1-12, 1999

Tanti J.-F., T. Gremeaux, S. Grillo, V. Calleja, A. Klippel, L. T. Williams, E. Van Obberghen, Y. Le Marchand-Brustel: Overexpression of a constitutively active form of phosphatidylinositol 3-kinase is sufficient to promote Glut 4 translocation in adipocytes. *J. Biol. Chem.* 271: 25227-25232, 1996

Tanti J.-F., N. Rochert, T. Gremeaux, E. Van Obberghhen, Y. Le. Marchand-Brustel: Insulin-stimulated glucose transport in muscle. Evidence for a protein-kinase-C-dependent component which is unaltered in insulin-resistant mice. *Biochem. J.* 258: 141-146, 1989

Tellam J. T., S. L. Macaulay, S, McIntosh, D. R. Hewish, C. W. Ward, D. E. James: Characterization of Munc-18c and Syntaxin 4 in 3T3-L1 adipocytes. *J. Biol. Chem.* 272: 6179-6186,1997

Tisdale E. J., J. R. Bourne, R. Khosravi-Far, C. J. Der, W. E. Balch: GTP-binding mutants of Rab1 and Rab2 are potent inhibitors of vesicular transport from the endoplasmic to the Golgi complex. *J. Cell. Biol.* 119: 749-761, 1992

Ueyama A., K. L. Yaworsky, Q. H. Wang, Y. Ebina, A. Klip: GLUT4-myc ectopic expression in L6 myoblasts generates a GLUT4-specific poll conferring insulin sensitivity. *Am. J. Physiol.* 277: E572-E578, 1999

Ullrich O., S. Reinsch, S. Urbe, M. Zerial, R. G. Parton: Rab11 regulates recycling through the pericentriolar recycling endosome. *J. Cell. Biol.* 135, 913-924, 1996

Uphues I, Y. Chern, J. Eckel: Insulin-dependent translocation of the small GTP-binding protein rab3C in cardiac muscle: studies on insulin-resistant Zucker rats. *FEBS. Lett.* 377: 109-112, 1995

Uphues I., J. Eckel: Insulin-dependent glucose transport in cardiac muscle of obese Zucker rats: Studies on the role of small GTP-binding proteins. *Exp. Clin. Endocrinol. Diabetes.* 104: (Suppl. 2) 111-112, 1996

Uphues I., T. Kolter, B. Goud, J. Eckel: Insulin-induced translocation of the glucose transporter GLUT4 in cardiac muscle: studies on the role of small-molecular-mass GTP-binding proteins. *Biochem. J.* 301: 177-182, 1994

Van Obberghen E., M. Kasuga, A. LeCam, A. Itin, J.-A. Hedo, L. C. Harrison: Biosynthetic labeling of the insulin receptor: studies of subunits in cultured human IM-9 lymphocytes. *Proc. Natl. Acad. Sci. USA.* 78: 1052-1056, 1981

Volchuk A, Q. Wang, S. Ewart, Z. Liu, L. He, M. K. Bennett, A. Klip: Syntaxin 4 in 3T3-L1 adipocytes: regulation by insulin and participation in insulin-dependent glucose transport. *Mol. Biol. Cell.* 7: 1075-1082, 1996

Vollenweider P., S. S. Martin, T. Haruta, A. J. Morris, J. G. Nelson, M. Cormont, Y. Le Marchard-Brustel, D. W. Rose, J. M. Olefsky: The small guanosine triphosphate-binding protein Rab4 is involved in insulin-induced GLUT4 translocation and actin filament rearrangement in 3T3-L1 cells. *Endocrinology* 138: 4941-4949, 1997

Yu B., M. Standaert, T. Arnold, H. Hernandez, J. Watson, K. Ways, D. R. Cooper, R. V. Farese: Effects of insulin on diacylglycerol/protein kinase-C signalling and glucose transport in rat skeletal muscles in vivo and in vitro. *Endocrinology.* 130: 3345-3355, 1992

APPENDIXES

Abbreviations

BIM	bisindolylmaleimide I
bp	base pair
BSA	bovine serum fraction V
cDNA	complementary DNA
CIAP	calf Instestinal alkaline phosphatase
DEPC	diethyl pyrocarbonate
DMSO	dimethyl suloxide
DTT	dithiothreitol
ECL	enhanced chemluminescence
EtBr	ethidium bromide
EDTA	ethylene diaminetetraacetic acid
EGTA	ethylene glycol-bis (ß-aminoethl ether)-N,N,N',N'-tetraacetic acid
FCS	fetal calf serum
g	gram
GLUT	glucose transporter
GTP-γ-S	5'-[γ-thio] triphosphate
h	hour (s)
Hepes	N-2-hydroxyethypiperazine-N#-2-ethanesulfonic acid
HRP	horseradish peroxidase
IRS	insulin receptor substrate
M	mole/l
min	minute (s)
mRNA	messenge RNA
ml	mililiter
NSF	N-ethylmaleimide-sensitive fusion protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PKC	protein Kinase C
PI 3-K	phosphatidylinositol 3-kinase
PIP_3	phosphatidylinositol 3,4,5-triphosphase
PMSF	phenylmethylsulfonylfluorid
RNase	ribonuclease
RT-PCR	reverse transcription-polymerase chain reaction
SH2	Src Homology 2
SNARE	soluble NSF attachment protein receptor
SDS	sodium dodecyl sulfate
TGN	trans-Golgi network
Tris	tris (hydroxymethl) aminomethane
UV	ultra-violet
µl	microliter
VAMP	vesicle associated membrane protein

List of figures

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Ich versichere, daß ich die vorliegende Dissertation selbst verfaßt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Ich versichere, daß die vorliegende Dissertation nicht von einer anderen medizinischen Fakultät abgelehnt worden ist.

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