Chapter 5

PRAS40 contains a nuclear export signal

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Submitted for publication
Abstract

Alterations in signalling via protein kinase B (PKB/Akt) and mammalian target of rapamycin (mTOR) have been demonstrated in type 2 diabetes and human malignancies. Proline-rich Akt substrate of 40 kDa (PRAS40), originally identified as PKB/Akt substrate, has been implicated as a direct regulator of mTOR complex (mTORC) 1. Interestingly, phosphorylated PRAS40 has been found in the nucleus, whereas the components of the mTORC1 complex have a more cytosolic localisation. This raises the possibility that PRAS40 shuttles between the cytosol and the nucleus. In the current study, we examined the localisation of PRAS40 in A14 fibroblasts and rat skeletal muscle. In subcellular fractionation experiments, PRAS40 is predominantly found in the cytosolic fraction. Insulin stimulation increased the levels of phosphorylated PRAS40-Thr246 in both the cytosol and the nucleus of A14 fibroblasts and rat skeletal muscle. Within the PRAS40 protein, we identified a putative nuclear export signal (NES). Incubation of A14 fibroblasts with the nuclear export inhibitor leptomycin B (LMB) resulted in nuclear accumulation of overexpressed human PRAS40 (hPRAS40). Mutation of the NES mimicked the effects of LMB treatment on hPRAS40. Finally, A14 cells expressing the hPRAS40 NES-mutant showed increased phosphorylation of the mTORC1 substrate p70 S6 kinase (S6K) and PKB/Akt after insulin stimulation compared to cells expressing wild-type hPRAS40. L6 myotubes expressing the hPRAS40 NES-mutant showed enhanced basal- and insulin-stimulated glucose uptake. In conclusion, we have identified a NES within PRAS40, and nuclear accumulation of PRAS40 may sensitize insulin action mediated through PKB/Akt and mTORC1.
Introduction

The protein kinase mammalian target of rapamycin (mTOR) is the shared catalytic subunit of mTOR complex 1 (mTORC1) and mTORC2 (1;2). In addition to mTOR, mTORC1 consists of the regulatory associated protein of mTOR (raptor) and mLST8 (also known as Gβl) (1;2). Activation of mTORC1, which is sensitive to inhibition by rapamycin, regulates protein translation via phosphorylation of p70 S6 kinase (S6K) and the translation inhibitor eukaryotic initiation factor 4E binding protein 1 (4EBP1) (1;2). The mTORC2 complex is insensitive to rapamycin and contains rapamycin-insensitive companion of TOR (rictor), protor, mLST8 and Sin1 (1;2). Active mTORC2 has been implicated in the phosphorylation of the hydrophobic motif of AGC-kinases, including Ser473 of protein kinase B (PKB/Akt) (3;4).

Deregulated activity of mTORC1 and PKB/Akt has been implicated in the pathogenesis of insulin resistance (1). Overactivation of the mTORC1/S6K-pathway in humans and rodent models of insulin resistance has been linked to inhibition of insulin signalling (5-10). The inhibition of insulin signalling is accomplished by phosphorylation on multiple serine residues of insulin receptor substrate 1 (IRS1), which inhibits the phosphatidylinositol 3’-kinase (PI3K) dependent activation of PKB/Akt.

Interestingly, a substrate of PKB/Akt, proline-rich Akt substrate of 40 kDa (PRAS40) has been identified as a binding partner in mTORC1 (11-15). PRAS40 directly binds to mTORC1 through Phe129 in its TOR signalling (TOS) motif (13-15). In response to extracellular stimuli, such as insulin or amino acids, PRAS40 is phosphorylated on multiple residues (11-13;16). These residues include the mTORC1-dependent phosphorylation of Ser183 and Ser221 and the PKB/Akt-mediated phosphorylation of Thr246 (15-17). After phosphorylation of PRAS40, the protein dissociates from mTORC1 (13-15) and binds 14-3-3 chaperones (13;16;17).

The function of PRAS40 within the mTORC1 complex is not completely clear. Overexpression of PRAS40 lowers the basal phosphorylation of S6K and 4EBP1, suggesting that PRAS40 can function as substrate inhibitor of mTORC1 (11-15;17). Accordingly, knockdown of PRAS40 enhanced phosphorylation of S6K after amino acid stimulation (15). However, silencing of PRAS40 has also been reported to lower insulin-stimulated phosphorylation of S6K and 4EBP1, indicating that PRAS40 is also required for proper signalling by mTORC1 (13;14;17).

The components of the mTORC1 complex are predominantly found in the cytosol, although individual partners can be detected in both cytosol and nucleus (18). Intriguingly, PRAS40 was originally identified as a nuclear phosphoprotein in Hela cells (19) and staining of cultured cells and target tissues with phospho-specific antibodies showed nuclear localisation of phosphorylated PRAS40-Thr246 (20). This raises the possibility that
PRAS40 traffics between the cytosol and the nucleus, and that subcellular localisation of PRAS40 may contribute to the regulation of mTORC1 activity.

In the current study, we examined the subcellular localisation of PRAS40 in vitro in insulin sensitive A14 fibroblasts and in vivo in rat skeletal muscle. We identified a nuclear export signal (NES) in PRAS40 and examined the functionality of the NES using the nuclear export inhibitor leptomycin B (LMB), and by mutation of the NES in PRAS40. Finally, we determined whether nuclear accumulation of PRAS40 affects the activation of PKB/Akt and mTORC1-signalling pathways by insulin.

Materials and methods

**Materials.** Tissue culture media were obtained from Invitrogen (Carlsbad, CA, USA). Wortmannin was purchased from Calbiochem (Darmstadt, Germany). Bovine insulin was purchased from Sigma Aldrich (St. Louis, MO, USA). LMB was obtained from Biomol international (Plymouth Meeting, PA, USA).

**Plasmid generation.** cDNA encoding human PRAS40 (hPRAS40) was kindly provided by Dr. R.A. Roth (Stanford University, Stanford, CA, USA) and cloned behind a CMV promoter in a plasmid which also contains green fluorescent protein (GFP) behind an IRES (21). Via mutagenesis PCR, a stop codon was introduced at the end of the coding sequence of hPRAS40, and leucine at position 225 and 227 were mutated into alanine. This resulted in constructs encoding wild-type hPRAS40 and mutant L225A/L227A-hPRAS40. Both constructs were verified by sequence analysis. Plasmids were used for transient transfections or to generate lentiviruses as described (21).

**Animals.** Rat skeletal muscle was obtained from a previously published study, in which male adult Wistar WU rats were fed a high-fat diet (HFD) or low-fat diet (LFD) for 7 weeks (20). The investigation conformed to the guide for the care and use of laboratory animals, as published by the National Institute of Health (NIH publ. no 85-23, revised 1996) and the regulations of the institutional animal care and use committee. Following a 6 hr fast, rats received an intraperitoneal injection with saline or insulin (10 U/kg body weight; Actrapid 100 U/ml; Novo Nordisk, Alphen aan den Rijn, The Netherlands) 30 min before being sacrificed by decapitation. Skeletal muscles were rapidly removed, snap frozen in liquid nitrogen-cooled isopentane and stored at −80°C until further analysis.

**Cell culture.** A14 cells are NIH3T3 fibroblasts overexpressing the human insulin receptor (22). Cells were grown on Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin. L6 myoblasts were obtained from ATCC (CRL-1458), and were grown on α-MEM supplemented with 10% FBS, penicillin, and streptomycin. Prior to insulin stimulation, cells were serum starved for 6 hrs on medium supplemented with 0.5% FBS, penicillin and streptomycin. Where indicated serum starvation was combined with 10 nM LMB.
Transient transfections. A14 cells grown in 6-well plates were transfected with 500 ng of expression vector using Fugene 6 reagent (Roche Biochemicals, Indianapolis, IN, USA) according to the manufacturer’s protocol.

Subcellular fractionation. Nuclear proteins were extracted from A14 fibroblasts and rat skeletal muscle as described previously (23). Protein content was determined using the BCA protein assay kit. Purity of the cell fractions was checked by Western blotting, using antibodies against α/β-tubulin (#2148, Cell Signalling Technology, Danvers, MA, USA) and lamin A/C (#2032, Cell Signalling Technology, Danvers, MA, USA) as cytosolic and nuclear markers, respectively.

Immunofluorescence. Immunofluorescence was carried out as described previously (20). In short, cells were grown on coverslips and fixed in 3.7% formaldehyde. Residual cross-linking was quenched using NH₄Cl and cells were permeabilised using 0.1% Triton X-100. Coverslips were blocked (0.2% BSA) and incubated overnight with hPRAS40 antibodies (AHO131, Invitrogen, Carlsbad, California, USA) followed by 2 h with Cy3-conjugated anti-mouse IgG (Jackson Immunochemicals, West Grove, PA, USA) prior to mounting with Vectashield containing 4’,6-diamidino-2-phenylindole (DAPI). The fluorophore-conjugated secondary antibodies used were Cy3-conjugated anti-mouse IgG (Jackson Immunochemicals, West Grove, PA, USA). Pictures were taken with a CCD CoolSnap K4 camera (Photometrics, Tucson, AZ, USA) on a Leica DM 5500 B microscope. Confocal pictures were taken with on a Leica TCS SP2 system using a Dm IRBE confocal microscope with maximal magnification. Pictures were analyzed with Leica Confocal Software (version 2.5).

Analysis of insulin signalling. Cells were lysed in 100 mM Tris.HCl (pH 6.8), 3% SDS and 10% glycerol. Protein content was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Expression and phosphorylation of proteins was studied by SDS-PAGE and Western blotting as described (20). Antibodies against total PRAS40 (#2691) and phospho-specific PKB/Akt-Thr308 (#9275), PKB/Akt-Ser473 (#4051), S6K-Thr389 (#9205) were from Cell Signalling Technology (Danvers, MA, USA). Antibodies against hPRAS40 (AHO1031) and phospho-specific PRAS40-Thr246 (44-1100G) were from Invitrogen (Carlsbad, CA, USA). Phospho-specific antibodies against PRAS40-Ser183 were from IBL Hamburg GmBH (Hamburg, Germany). Antibodies against PKBβ/Akt2 (07-372) were from Upstate (Billerica, MA, USA). Total S6K antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bound antibodies were visualized by enhanced chemiluminescence and quantified by densitometry analysis of scanned films (ImageJ 1.34S; National Institutes of Health, Bethesda, MD, USA).

Analysis of 2-deoxyglucose uptake. Glucose uptake was performed on 12-well plates of differentiated L6 myotubes obtained from L6 myoblasts, which were transduced at 20%
confluence at an MOI of 2 with lentiviral vectors encoding either wild-type hPRAS40 or mutant L225A/L227A-hPRAS40 as described (21). Three days after transduction, cells were split, grown to confluence and differentiated into myotubes by culturing them for 7 days on α-MEM supplemented with 2% horse serum and antibiotics. Cells were serum-starved for 4 hrs, followed by a 45 min incubation in 50 mM HEPES, 138 mM NaCl, 1.85 mM CaCl₂, 1.3 mM MgSO₄, 4.8 mM KCl (pH 7.4) at 37°C, and stimulation with insulin. 10 minutes after the addition of insulin, 2-deoxyglucose (2-DG) uptake was initiated by the addition of 0.012 μCi 2-deoxy-D[1-14C]glucose. After 10 min, the assay was terminated by 3 washes with ice-cold phosphate-buffered saline and the addition of 1% SDS, 0.2 M NaOH. Incorporated radioactivity was determined by liquid scintillation counting.

**Statistical analysis.** Data are expressed as mean ± SEM. Differences between groups were determined using the independent student’s two-tailed *t*-test in SPSS version 16.0. *p*<0.05 was considered statistically significant.

**Figure 1.** Localisation of PRAS40 in A14 fibroblasts (A) and rat skeletal muscle (B). A14 cells were serum starved during 6 hrs, and were left untreated (-) or stimulated with 100 nM insulin for 5 min (+). Male Wistar rats were put on a control low-fat diet (LFD) or high-fat diet (HFD) for 7 weeks. Following a 6 hr fast, rats received an intraperitoneal injection with saline or insulin (10 U/kg body weight), and were sacrificed 30 min later. Cytosolic and nuclear extracts from A14 fibroblasts and rat skeletal muscles were prepared as described in the experimental procedures. 10 μg of protein lysate was loaded on a SDS-PAGE gel. Western blot analysis was performed using phospho-specific antibodies against PRAS40-Thr246. Purity of the fractions was determined by reprobing the western blots with antibodies against tubulin and lamin A/C, respectively. Representative blots are shown of three independent experiments. C. Quantification of Western blots for pPRAS40-Thr246 obtained in panel B. Data are expressed as mean ± SEM (*n*=3); *: *p*<0.05 versus saline.
Results

**Subcellular localisation of PRAS40.** Subcellular fractionation of A14 fibroblasts showed that phosphorylated PRAS40-Thr246 is predominantly localized to the cytosol (Fig. 1A). Insulin stimulation increased the cytosolic level of phosphorylated PRAS40-Thr246 (Fig. 1A). Furthermore, phosphorylated PRAS40-Thr246 was detected in nuclear extracts of insulin-treated cells (Fig. 1A).

We also determined the localisation of PRAS40 in vivo in skeletal muscle from control LFD-fed and insulin-resistant HFD-fed rats. Following subcellular fractionation, the cytosolic fraction of rat skeletal muscle contained phosphorylated PRAS40-Thr246 (Fig. 1B). No differences were observed between muscles from saline-injected LFD- or HFD-fed rats (Fig. 1B). Insulin injection increased the level of phosphorylated PRAS40-Thr246 by 6.5-fold in the cytosol and 3.2-fold in the nucleus of LFD-fed rats (Fig. 1B and 1C). In skeletal muscle from HFD-fed rats, insulin-stimulated PRAS40-Thr246 was markedly lower in the cytosolic fraction, and no significant nuclear accumulation of phosphorylated PRAS40-Thr246 was observed (Fig. 1B and 1C).

**Figure 2.** PRAS40 contains a nuclear export signal. A. Alignment of the nuclear export signal (NES) of PRAS40 with the NES found in human activating transcription factor 2 (ATF2), inhibitor of nuclear factor κB (IκBα), the rev protein of simian human immunodeficiency virus (rev), and cAMP-dependent protein kinase inhibitor α (PKIA). In the consensus sequence for the leucine-rich NES above the aligned sequences represents X any amino acid, and can L be substituted for by any other large hydrophobic amino acid (24). B. Alignment of the NES of human PRAS40 with the PRAS40 homologues from other species.

**PRAS40 contains a functional nuclear export signal.** The presence of phosphorylated PRAS40-Thr246 in the nuclear fraction after insulin stimulation prompted us to analyze the PRAS40 protein for motifs regulating trafficking between the cytosol and the nucleus. A 10 amino acid sequence in the carboxyterminus of the PRAS40 protein (amino acid 218-227 of hPRAS40), matched the sequence for a leucine-rich NES (24) (Fig. 2A). The NES in PRAS40 is identical between Homo sapiens, Bos taurus, Rattus norvegicus, and Mus musculus, slightly altered in Xenopus tropicalis and Danio rerio, and conserved to a lesser extent in the putative Drosophila melanogaster ortholog Lobe (Fig. 2B).
In order to verify the functionality of the NES, we used the nuclear export inhibitor leptomycin B (LMB) (25) in A14 cells expressing hPRAS40. In the absence of LMB, immunofluorescence labelling of A14 cells with antibodies recognizing total hPRAS40 showed a predominant cytoplasmic localisation of hPRAS40 (Fig. 3A). Incubation with LMB, induced nuclear accumulation of hPRAS40 (Fig. 3A). Quantification of the immunofluorescence stainings using confocal microscopy showed that the percentage of nuclear hPRAS40 increased from 0.7% in the basal state to 22% after LMB incubation (Fig. 3B). Due to the broad inhibitory effect of LMB on proteins carrying a NES, we next mutated the NES in PRAS40 by replacing the critical leucine residues at positions 225 and 227 by alanine (L225A/L227A). Immunofluorescent staining of A14 cells expressing mutant L225A/L227A-hPRAS40 showed a predominant nuclear localisation of the protein (Fig. 3A). Quantification of the immunofluorescence staining showed that 65% of mutant L225/L227A-hPRAS40 was found in the nucleus (Fig. 3B).
Figure 3. Nuclear accumulation of hPRAS40. A. A14 fibroblasts were transfected with wild-type hPRAS40 or mutant L225A/L227A-hPRAS40. Transfected cells are positive for green fluorescent protein (GFP; green). Cells were untreated (basal) or incubated with LMB (6 hrs, 10 nM), fixed, permeabilised and incubated with anti-hPRAS40. Bound PRAS40 antibody was visualized with anti-mouse conjugated Cy3 secondary antibodies (red). DNA was stained with DAPI (blue). Photographs are representative of three independent experiments. The scale bar equals 50 μm. B. Quantification of fluorescence detected in the nucleus and cytoplasm for the hPRAS40 antibody in A14 cells transfected with wild-type hPRAS40 that were kept untreated (open bars), treated with LMB (gray bars), or transfected with mutant L225A/L227A-hPRAS40 (black bars). Data are expressed as mean ± SEM (n=3); *: p<0.05 versus wild-type.

Nuclear accumulation of PRAS40 and insulin action. Expression of mutant L225/L227A-hPRAS40 had no significant effect on basal phosphorylation of Ser183 and Thr246 compared to cell expressing wild-type hPRAS40 (Figs. 4A-C). Insulin-mediated phosphorylation of PRAS40 on Ser183 and Thr246 showed a slight trend to be increased in cells expressing mutant L225A/L227A-hPRAS40 as compared to cells expressing wild-type hPRAS40 (Figs. 4A-C). Pretreatment of cells expressing hPRAS40 with LMB also displayed increased phosphorylation of Ser183 and Thr246 (data not shown).

To further investigate this point, we next determined whether nuclear accumulation of hPRAS40 affects insulin-induced phosphorylation of S6K on Thr389, which, like Ser183 on PRAS40, is regulated by mTORC1 (15). Insulin stimulated phosphorylation of Thr389 of S6K by 3.7-fold in A14 cells expressing wild-type hPRAS40 versus 4.3-fold in cells expressing mutant L225A/L227A-hPRAS40 (Fig. 4A/D). We also examined the insulin-induced phosphorylation of PKB/Akt, the main kinase phosphorylating PRAS40 on Thr246, in cells expressing mutant L225A/L227A-hPRAS40. Insulin-induced phosphorylation of PKB/Akt on both Thr308 (Fig. 4A/E) and Ser473 (Fig. 4A/F) was slightly increased in A14 cells expressing mutant L225A/L227A-hPRAS40 when compared to wild-type hPRAS40. Finally, we analyzed L6 myotubes expressing wild-type or mutant L225A/L227A-hPRAS40 for insulin-stimulated glucose uptake. As shown in Fig. 4G, both basal and insulin-stimulated glucose uptake were elevated in L6 myotubes expressing mutant L225A/L227A-hPRAS40. Comparable data were obtained when glucose uptake was determined in L6 myotubes pretreated with LMB (data not shown).
Figure A shows the relative phosphorylation levels of various proteins under different conditions.

- **hPRAS40**
  - WT vs. L225A/L227A
  - Insulin treatment

- **pPRAS40-Ser183**
- **pPRAS40-Thr246**
- **PRAS40**
- **pS6K-Thr389**
- **S6K**
- **pPKB/Akt-Thr308**
- **pPKB/Akt-Ser473**
- **PKB/Akt2**

B, C, D, E, F, and G show the relative phosphorylation (AU) for specific proteins under different conditions.

- **pPRAS40-Ser183**
- **pPRAS40-Thr246**
- **pS6K-Thr389**
- **pPKB/Akt-Thr308**
- **pPKB/Akt-Ser473**

G also includes the measurement of glucose uptake.

- **Glucose uptake**
  - WT vs. L225A/L227A
  - 2-DOG uptake (cpm)
Figure 4. Effect of nuclear accumulation of PRAS40 on insulin action. A. Western blots of phosphorylated PRAS40-Thr246, PRAS40-Ser183, S6K-Thr389, PKB/Akt-Thr308, and PKB/Akt-Ser473 phosphorylation in A14 cells transfected with wild-type hPRAS40 (WT) or mutant L225A/L227A-hPRAS40 (L225A/L227A). 24 h after transfection, serum-starved cells were stimulated with insulin for 5 min at 100 nM (+, filled bars) or kept untreated (-, open bars). B-F. Quantification of Western blots of phosphorylated PRAS40-Ser183 (B), PRAS40-Thr246 (C), S6K-Thr389 (D), PKB/Akt-Thr308 (E), and PKB/Akt-Ser473 (F). G. Glucose uptake in L6 myotubes expressing wild-type or mutant L225A/L227A-hPRAS40. All data is expressed as mean ± SEM of three independent experiments. *: p<0.05 compared to untreated cells, #: p<0.05 compared to cells expressing wild-type hPRAS40.

Discussion

In the present study, we examined the subcellular localisation of PRAS40 in A14 fibroblasts and rat skeletal muscle. We found that the majority of PRAS40 is localized in the cytoplasm and that insulin stimulation results in phosphorylated PRAS40-Thr246 in the nucleus in cultured A14 cells and rat skeletal muscle. Insulin-mediated nuclear accumulation of phosphorylated PRAS40-Thr246 was not observed in skeletal muscle from insulin resistant HFD-fed rats. Finally, we identified a NES within PRAS40, and forced accumulation of ectopic PRAS40 in the nucleus may sensitize the insulin signalling pathway.

The predominant cytosolic localisation of PRAS40, which was observed in subcellular fractionation experiments on untransfected cells, as well as in immunofluorescence studies on cells transfected with hPRAS40, differs from previous immunofluorescence and immunohistochemistry studies using phospho-specific antibodies against PRAS40-Thr246 (20;26;27). Staining untransfected cells with phospho-specific antibodies against PRAS40-Ser183 also showed a predominant nuclear localisation of the endogenous phosphorylated PRAS40 (data not shown). The reason for discrepancy is not clear. However, since phosphorylated PRAS40 can interact with 14-3-3 proteins (13;16;28;29), we cannot exclude the possibility that the epitopes recognized by the phospho-specific PRAS40 antibodies are masked in the cytosol when the endogenous PRAS40 protein is bound to 14-3-3 chaperones.

The observation that PRAS40 shuttles between the cytoplasm and the nucleus is supported by the presence of a functional NES, and the original identification of PRAS40 as nuclear phosphoprotein in Hela cells (19). The presence of phosphorylated PRAS40 in the nucleus of insulin-stimulated cells further substantiates the occurrence of shuttling of the PRAS40 protein. However, we could not identify a nuclear localisation signal within the PRAS40 sequence. Proteins smaller than 60 kDa can freely move between the cytoplasm and the nucleus (14) and since PRAS40 is a 40 kDa protein, it should be able to shuttle independent of active transport between cytoplasm and nucleus. Nevertheless, expression of hPRAS40 in A14 cells resulted in a cytosolic localisation of the protein, while co-expression of GFP
resulted in both nuclear and cytosolic distribution of GFP indicating that an alternative mechanism may underlie the translocation of PRAS40 to the nucleus.

A possible mechanism for nuclear import could involve the interaction of phosphorylated PRAS40 with 14-3-3 proteins. Multiple studies have implicated 14-3-3 proteins in the control of nuclear-cytosolic shuttling (30;31). Using GST pull-down experiments, we confirmed previous reports showing that insulin promotes the binding of PRAS40 to 14-3-3 proteins. Interactions were observed between 14-3-3 isoforms beta, tau, eta, zeta and PRAS40 (data not shown) (13;16;28;29). The importance of the different phosphorylation sites as well as the contribution of 14-3-3 binding as chaperone for nuclear import, however, remains to be clarified.

In addition to PRAS40, other components of the mTORC1 signalling pathway, like mTOR, raptor, TSC2, S6K, and PKB/Akt have been found in the nucleus and/or contain a NES (18;32-35). The function of the nuclear accumulation of these proteins is not completely elucidated. In the present study, nuclear accumulation of PRAS40 resulting from expression of mutant L225A/L227A-hPRAS40 or following incubation with LMB is associated with increased insulin-mediated phosphorylation of PKB/Akt (13;36). Previous reports have shown that neither overexpression nor silencing of PRAS40 affects the phosphorylation of PKB/Akt. Conversely, one study demonstrated that silencing PRAS40 abrogates insulin-mediated phosphorylation of PKB/Akt through degradation of IRS1 (11). Although these contradicting findings do not exclude the possibility that PRAS40 may potentially contribute to the regulation of PKB/Akt, further studies are clearly required to address this issue.

Interestingly, we found that forced nuclear accumulation of PRAS40 is associated with increased insulin-mediated phosphorylation of S6K. Previous reports have suggested that PRAS40 can act as substrate inhibitor of mTORC1 (11;13-15). In line with these findings, we also found that expression of wild-type hPRAS40 in A14 cells blunts the activation of S6K by insulin (data not shown). Accordingly, expression of mutant L225A/L227A-hPRAS40, which accumulates in the nucleus, may augment the ability of mTORC1 to phosphorylate S6K by relieving the inhibitory constraint on mTORC1. A limitation of this study is that we cannot determine whether the amount of endogenous PRAS40 accumulated in the nucleus is sufficient to relieve the inhibitory constraint on mTORC1. We have estimated that approximately 3% of the total amount of Thr246-phosphorylated PRAS40 was found in the nucleus of following insulin stimulation of rat skeletal muscle and A14 fibroblasts (data not shown). Also, quantification of confocal images obtained from cell expressing wild-type hPRAS40 showed that insulin increased the amount of hPRAS40 in the nucleus from 0.7 to 3% (data not shown). Alternatively, PRAS40 may have a direct function in the nucleus. Recently, mTORC1 has been found to regulate expression of mitochondrial genes (37). PRAS40 as component and proposed regulator of mTORC1
PRAS40 contains a nuclear export signal might via this way directly influence mitochondrial gene transcription. Finally, in immunofluorescence studies, we observed staining of the midbody using phospho-specific antibodies against PRAS40-Thr246 and phospho-PRAS40-Ser183 (data not shown). Proper cell division is regulated through this protein complex (38). However, at this point we are unable to supply evidence that PRAS40 is indeed involved in the process of cell division.

In conclusion, we have analyzed the subcellular localisation of PRAS40. Phosphorylated PRAS40-Thr246 has a cytosolic localisation, but after insulin stimulation phosphorylated PRAS40-Thr246 is detected in the nucleus of the cell. Furthermore, we identified a nuclear export signal in PRAS40 and observed that forced nuclear accumulation of hPRAS40 sensitizes insulin-mediated activation of S6K. Further research and understanding of PRAS40 function will benefit disease knowledge linked to the PKB/Akt and mTORC1 signalling pathway like type 2 diabetes and cancer.

Acknowledgements
The authors would like to acknowledge Annelies van der Laan for assistance with the confocal microscope, Amina Teunisse for practical assistance with the GST pull down experiments, S. Lam for assistance with the LMB experiments, and A.G. Jochemsen for discussions and help with the GST pull down experiments. The financial support of the Dutch Diabetes Research Foundation (grant 2004.00.063), European Union COST Action BM0602 and Top Institute Pharma (grants T1-106 and T2-105) is greatly acknowledged.

References


Chapter 5