Identification and characterization of Nuclear Localization Signal of pRS1 protein

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SUMMARY

RS1, a gene product of *RSC1A1*, is critically involved in cell density-dependent transcriptional down-regulation of SGLT1 in LLC-PK_1 cells and in the post-transcriptional down-regulation of SGLT1 in small intestine. RS1 inhibits the release of SGLT1 containing vesicles from the *trans*-Golgi network and migrates into the nucleus where it inhibits transcription of SGLT1.

In the present work we identified a novel 21 amino acids-long nonconventional nuclear localization sequence (RS1 NLS) in porcine RS1 (pRS1) that is necessary and sufficient for nuclear targeting of pRS1. RS1 NLS is framed by two consensus sequences for phosphorylation which are responsible for confluence-dependent regulation of RS1 NLS: a casein kinase 2 (CK2) site in position 348 and a protein kinase C (PKC) site in position 370. Confluence-dependent nuclear targeting was observed with amino acids 342-374 (R-NLS-Reg). Mutation analysis suggested that nuclear targeting is blocked by phosphorylation of serine 370 (PKC) and that phosphorylation of serine 348 (CK2) prevents phosphorylation of serine 370. Because CK2 is down-regulated and PKC is up-regulated during confluence of LLC-PK_1 cells, our data suggest that nuclear localization coordinates cell density-dependent changes in transcriptional and post-transcriptional inhibition of SGLT1 expression.

ZUSAMMENFASSUNG

RS1, ein Genprodukt von RSC1A1, ist entscheidend an der zelldichteabhängigen transkriptionellen Herunterregulation von SGLT1 in LLC-PK_1 Zellen und an der post-transkriptionellen Herunterregulation von SGLT1 im Dünndarm beteiligt. RS1 hemmt die Freigabe von SGLT1 enthaltenden Vesikeln aus dem *trans*-Golgi Netzwerk und wandert in den Zellkern wo es die Transkription von SGLT1 inhibiert.

In der vorliegenden Arbeit identifizierten wir eine neuartige 21 Aminosäuren lange nicht-konventionelle Kernlokalisierungssequenz (RS1 NLS) in RS1 vom Schwein (pRS1), die für die Kernlokalisierung von pRS1 nötig und ausreichend ist. RS1 NLS ist von zwei Konsensussequenzen für Phosphorylierung umrahmt, welche für die konfluenzabhängige Regulierung von RS1 NLS verantwortlich sind: Eine Stelle für Casein Kinase 2 (CK2) in der Position 348 und eine Stelle für Protein Kinase C (PKC) in der Position 370.

Es wurde eine konfluenz-abhängige Kernlokalisierung mit den Aminosäuren 342-374 (R-NLS-Reg) beobachtet. Die Mutationsanalyse deutete darauf hin, dass Kernlokalisierung durch die Phosphorylierung von Serin 370 (PKC) geblockt wird, und dass die Phosphorylierung von Serin 348 (CK2) die Phosphorylierung von Serin 370 verhindert. Da während der Konfluenz CK2 herunterreguliert und PKC hochreguliert wird, deuten unsere Daten darauf hin, dass die Kernlokalisierung die zelldichteabhängigen Veränderungen in der transkriptionellen und posttranskriptionellen Hemmung von SGLT1 Expression koordiniert.

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INTRODUCTION

One of the most important processes of receiving energy by organisms is glucose absorption. Absorption of glucose takes place in small intestine and is highly regulated. In small intestine D-glucose is translocated across the epithelial cells by glucose transporters, which belong to two families: Na^+-D-glucose cotransporters of the SGLT family in the brush-border membrane, and Na^+-independent glucose transporters of the GLUT families in the basolateral membrane (Hediger and Rhoads, 1994, Bell et al., 1993). The regulation of glucose transport by transporters from both families is cell type-specific, and is brought about by various mechanisms (Hediger and Rhoads, 1994, Klip et al., 1994, Wright et al., 1997, Scheepers et al., 2004).

The facilitative GLUT transporters utilize the gradient of glucose across plasma membranes and exhibit different substrate specificity, kinetic characteristics, and expression profiles, thereby allowing a tissue-specific adaptation of glucose uptake through regulation of gene expression of GLUT (Scheepers et al., 2004, Wood and Trayhurn, 2003). SGLT transport glucose (and galactose) via a secondary active transport mechanism. They use a sodium gradient to transport glucose across the apical membrane against its concentration gradient. In human three members of the SGLT family – SGLT1, SGLT2, SGLT4 function as sugar transporters (Tazawa et al., 2005) and SGLT3 functions as a sensor (Diez-Sampedro et al., 2003). SGLT1 has a limited tissue expression and is found essentially on apical membranes of the small intestinal absorptive cells (enterocytes) and renal proximal strait tubules (S3 cells) (Hediger and Rhoads, 1994), small amount is found in brain, heart, testis and prostate (Wright et al., 2007).

Some years ago the RS1 protein, that changed the expression of SGLT1 in *Xenopus laevis* oocytes (Veyhl et al., 1993), was found. The RS1 protein has been shown to inhibit glucose transport by blocking transcription of SGLT1 in the nucleus (Korn et al., 2001) and to prevent the translocation of SGLT1 transporters from the *trans*-Golgi network to the plasma membrane (Kroiss et al. 2006).

Since the glucose transport has fundamental physiological and biomedical importance, the investigation of RS1 and its functions is important.

1. The RS1 protein

RS1 is a 67-68 kDa hydrophilic protein, that is broadly expressed in mammals. It was cloned from the four species, pig (Veyhl et al., 1993), rabbit (Reinhardt et al., 1999), mouse (Osswald et al., 2005) and human (Lambotte et al., 1996). The RS1 protein exhibits about 70 % identity on the amino acids level through all four species (Figure 1). It contains two conserved consensus sequences for phosphorylation by protein kinase C (PKC), three conserved consensus sequences for casein kinase II (CK2), and a conserved C-terminus of 42 amino acids that contains an ubiquitin-associated domain (UBA). RS1 is encoded by an intronless single copy gene that is located at chromosome 1p36.1 in human. This protein has wide tissue distribution. In the cell RS1 is localized within the nucleus, in the cytoplasm and associated with the plasma membrane (Valentin et al., 2000). It was shown that it is up-regulated in subconfluent poorly differentiated epithelial cells and down-regulated during confluence (Korn et al., 2001). The RS1 protein inhibits the expression of a group of transporters that includes the sodium-D-glucose cotransporter SGLT1, the sodium-*myo*inositol cotransporter SMIT, the organic cation transporter OCT2, the organic anion transporter OAT1 and the Na^+-cotransporter for serotonin SERT, but not the sodium-independent glucose transporter GLUT1 and the proton-peptide cotransporter PEPT1 (Veyhl et al., 1993, Lambotte et al., 1996, Reinhardt et al., 1999, Veyhl et al., 2003 and Jiang et al., 2005).

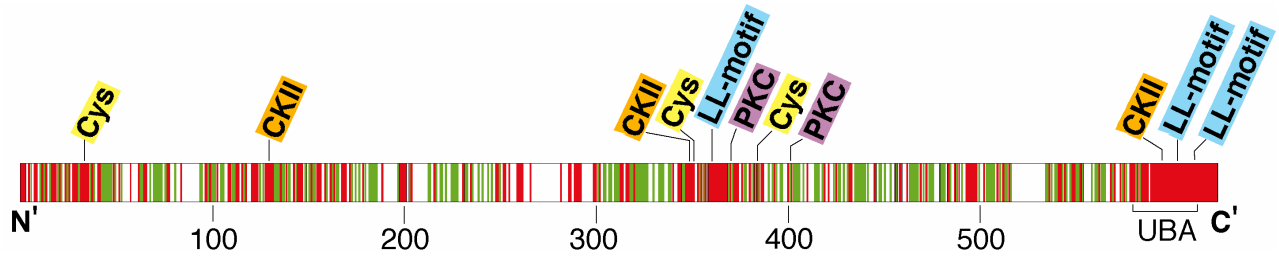


Figure 1. Comparison of homologous regions of RS1 protein from porcine, human, rabbit and mouse. *Red* – identical amino acid residues, *green* – similar amino acid residues. Predicted phosphorylated sites for casein kinase 2 (CK1), protein kinase C (PKC), LL-motifs, cystein residues (Cys) and ubiquitin-associated domain (UBA) are indicated

1.1. The localization of RS1

The RS1 protein was identified in many different cells and tissues, including S1, S2 and S3 segments of renal proximal tubules, small intestine epithelial and subepithelial cells, hepatocytes and neurons. Small amount of RS1 was found in lungs and spleen, but not in skeletal and heart muscle, colon or stomach (Veyhl et al., 1993, Reinhardt et al., 1999). In rabbit and pig RS1 was found in a 200,000×g supernatant of homogenized cells and a small amount of proteins was associated with the plasma membrane (Veyhl et al., 1993, Reinhardt et al., 1999). Similarly in porcine kidney RS1 protein was found in brush-border membrane fraction (Valentin et al., 2000). In small intestine, mRS1 was localized in epithelial and subepithelial cells, within the nucleus and below the plasma membrane (Osswald et al., 2005). Employing immunohistochemistry with specific antibodies and laser scanner microscopy, porcine RS1 (pRS1) protein was located at the intracellular side of the plasma membrane, at the entire *trans*-Golgi network (TGN) and within the nuclei of native subconfluent LLC-PK_1 cells (Kroiss et al., 2006).

1.2. The functions of RS1

RS1 was originally discovered as a protein what was able to modulate the activity of Na^+-glucose cotransporter SGLT1 in *Xenopus laevis* oocytes. According to this, RS1 was first proposed to be a regulatory subunit of SGLT1. But later the inhibitory effect of RS1 on other transporters was found. So, the regulatory function of RS1 was not specific for SGLT1, but for several other transporters.

RS1 is proved to be important for the regulation of glucose absorption in small intestine. After removal of the gene coding RS1 (Rsc1A1) in mice, SGLT1 activity was up-regulated and glucose absorption in small intestine was increased. In addition the animals developed an obese phenotype (Osswald et al., 2005).

It has been shown that RS1 regulates SGLT1 activity transcriptionally and post-transcriptionally.

Experiments using the LLC-PK_1 cells showed a relative correlation between the presence of pRS1 in plasma-membrane enriched (PME) fraction and transport activity of SGLT1. Thus, in pRS1-overexpressed cells, the phlorizin-inhibitable transport of an α-methylglucose (AMG) and the amounts of SGLT1 mRNA and SGLT1 protein in the PME fraction were much lower than in the native LLC-PK_1 cells. But in the RS1-antisense cells, where the expression of pRS1 protein was blocked by over-expressed antisense mRNA, the

amounts of mRNA and protein of SGLT1 were higher then in native cells. Also nuclear run-off assay performed with isolated nuclei of porcine LLC-PK_1 cells showed that the transcription of SGLT1 was increased when the expression of RS1 was reduced by antisense-strategy (Korn et al., 2001).

Post-transcriptional inhibition of SGLT1 by RS1 was demonstrated in *Xenopus laevis* oocytes. The experiments showed that the effect of RS1 on SGLT1 transport was dependent on the species. Thus, the co-expression of cRNA of human RS1 (hRS1) or rabbit RS1 (rbRS1) together with human cRNA-SGLT1 strongly inhibited the transport of glucose, but co-expression of cRNA of rabbit or porcine RS1 with cRNA-rbSGLT1 increased the glucose uptake in oocytes (Korn et al., 2001, Reinhardt et al., 1999). Additionally, co-expression of mRNAs showed inhibition of hOCT2 transport by hRS1 but no effect on hOCT2 transport by rbRS1. Recently it was shown that the injection of the hRS1 protein into oocytes, expressing hSGLT1 or hOCT2, also inhibits the glucose uptake (Veyhl et al., 2006). This supports the idea that RS1 inhibits the SGLT1 activity on post-transcriptional level.

Since (*i*) post-transcriptional inhibition of hSGLT1 was not altered by inhibitors of endocytosis but was prevented when exocytosis was blocked by botulinum toxin B, (*ii*) the down-regulation of hSGLT1 was dynamin-dependent and (*iii*) the *trans*-Golgi network (TGN) was modified by brefeldin A, it was concluded that RS1 inhibits the release of SGLT1 containing vesicles from the TGN to the plasma membrane. This interpretation was supported by immunohistochemical localization of RS1 to the TNG of subconfluent LLC-PK_1 cells and by showing the release of RS1 from TGN by brefeldin A (Kroiss et al., 2006).

RS1 is essentially involved in confluence dependent up-regulation of SGLT1 in the native LLC-PK_1 cells. During confluence the amount of intracellular RS1 and the amount of RS1 in nuclei were down-regulated (Kroiss et al., 2006), the concentration of the pRS1 protein in PME fraction was strongly decreased, whereas the amount of pRS1-mRNA was not changed (Korn et al., 2001). At the same time, in the dense cells the amount of SGLT1-mRNA, the concentration of SGLT1 protein in plasma-membrane reached (PME) fraction and the phlorizin-inhibitable transport of AMG were strongly increased. This inverse relationship between RS1 and SGLT1 gave rise the hypothesis that RS1 suppresses transcription of SGLT1 in subconfluent cells and that the up-regulation of SGLT1 after confluence is caused by a relief of this inhibition. The different nuclear location of RS1 before and after confluence suggests that the RS1 protein interacts with the transcriptional complex of SGLT1

In the RS1-antisense cells after proliferation, the SGLT1 protein concentration was 20-fold higher than in control cells expressing pRS1 and the amount of SGLT1 mRNA after

confluence was also 5-10-fold increased, although in the presence of native RS1 protein no changes of SGLT1 mRNA and protein during the proliferation was observed. In confluent RS1-antisense cells V_{max} of AMG transport was increased without changes of K_m value, due to the increasing of the number of SGLT1 transporters in PME fraction by releasing from TGN vesicles, but not due to the change of capacitance. Consequently the confluence dependent inhibition of SGLT1 is regulated by pRS1 protein in two ways. First, confluence dependent inhibition of nuclear migration of RS1 releases the transcriptional complex of SGLT1 following the increasing of the SGLT1-mRNA amounts. Second, the decreased amount of pRS1 protein allows the SGLT1 protein release from TGN vesicles to the plasma membrane.

Recently interaction between RS1 and ischemia/reperfusion inducible protein (IRIP), the other regulator of plasma membrane-transporter, was found. The 28 kDa IRIP is induced after renal ischemia and reperfusion (Jiang et al., 2005). It is widely expressed in the tissues, mostly in the testis, secretory and endocrine organs. The IRIP protein could inhibit the expression of a group of transporters that include SGLT1, OCT2, OAT1, GAT and SERT but not GLUT1 and PEPT1. In addition, down-regulation of endogenous expression of IRIP increased transporter uptake activities, supporting the inhibitory function of IRIP protein. The interaction of IRIP and RS1 was shown with yeast two-hybrid screening and proved by co-immunnoprecipitation assays. Interestingly, the inhibitory effect of RS1 on OCT2 could be blocked by a dominant negative mutant of IRIP. Moreover, the levels of RS1 and IRIP mRNAs were drastically increased in mouse kidney after ischemia/reperfusion, whereas mRNA levels of SGLT1, OCT1 and OCT3 were decreased. Both RS1 and IRIP significantly decreased V_{max} of OCT2-mediated uptake but did not changed its K_m, suggesting that they do not affect the catalytic activities of transporters, but decrease the amount of transporters on the plasma membrane. Both proteins, RS1 and IRIP, seem to be a part of common regulatory mechanism controlling transporter activities.

2. The nuclear migration of proteins

2.1. The nuclear pore complex

In eukaryotic cells the double-membrane nuclear envelope (NE) spatially separates the cytoplasm from the nucleus. Nuclear pore complex (NPC) embedded in NE allows the ions and small molecules diffuse across the NE and facilitates the receptor-mediated bidirectional transport of proteins, RNAs and ribonucleoproteins particles. The NPC in mammal cells contains approximately 50 different proteins components and its mass is estimated to be 125 MDa. It has three parts: a central framework, surrounded by cytoplasmic filaments and a nuclear basket. The central framework is composed of eight multidomain spokes, which consist of two halves that are placed back-to-back in the midplane of the NE. These membrane-spanning domains are

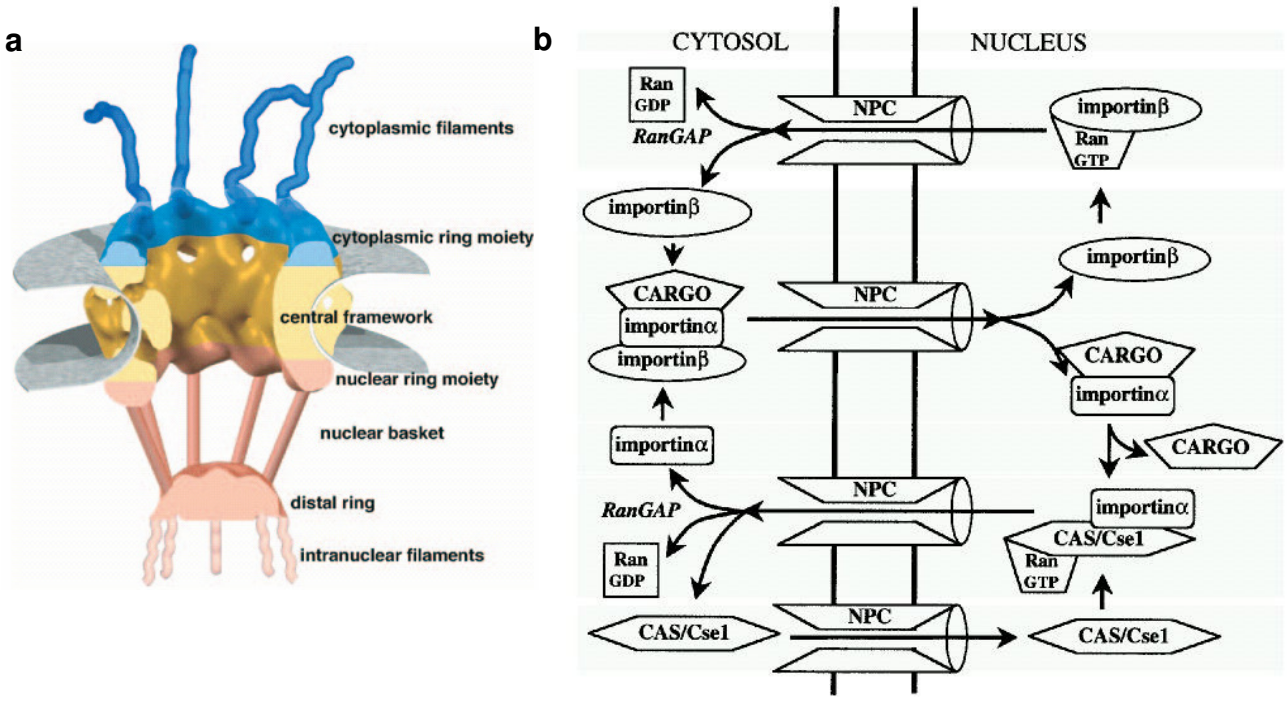


Figure 2. a, the structure of nuclear pore complex (Fahrenkrog, 2006), **b**, mechanism of cargo import by the importin-α/importin-β pathway. The cargo binds to an adaptor, importin-α rather then directly to importin-β. The complex is dissociated in the nucleus by RanGTP. Importin-α requires carrier for export, called CAS (Cse1 in budding yeast). Importin-β-RanGTP and importin-α-CAS-RanGTP complex return to the cytosol, where they are dissociated by hydrolysis of the GTP (Macara, 2001).

continuous with eight short filaments towards the cytoplasm and with a nuclear ring moiety to the nucleus. Eight filaments extend from the nuclear ring and are attached to a distal ring forming the nuclear basket (Allen et al., 2000) (Figure 2, a). Nucleoporins (nuclear pore complex proteins) on the both sides of the NPC and in the central channel contain small phenylalanine-glycine (FG) repeats, which are very flexible due to their irregular structure. They collect cargo complexes close to the transport channel and move them into the channel.

2.2. Nuclear import and export of proteins

Although small molecules may diffuse between the cytosol and the nucleus, macromolecules greater then ~39 nm diameter (Pante and Kann, 2002) are transported actively through the NPC along the central axis. This active transport requires special signals: a nuclear localization signal (NLS) to import the proteins into the nucleus, and a nuclear export signal (NES) for exporting them to the cytoplasm. There are different nuclear trafficking pathways, but generally, proteins containing NLS do not interact directly with the NPC, but first they are bound to soluble carriers of the importin-β/karyopherin-β family, directly or via adapter importin-α (Figure 2, b). Following translocation, the complex is dissociated by nuclear RanGTP releasing the cargo. After binding of the importin-β to RanGTP, it is recycled back to the cytoplasm, whereas importin-α makes a trimeric complex with CAS (importin-β-related molecule) and RanGTP to migrate to the cytoplasm. In the cytoplasm the RanGAP promotes RanGTP hydrolysis and thus dissociates the complex (Figure 2, b) (Steward et al., 2001, Gasiorowski et al., 2003, Johnson et al., 2004).

Analogous pathways were determined for protein and RNA export from the nucleus. Proteins containing leucine-rich NES build a complex with the export receptor CRM1 and RanGTP in the nucleus. Following translocation through the NPC, complex is localized in the cytoplasm, where the trimeric complex is dissociated by converting of RanGTP to GDP-form by GAP, a RanGTPase activation protein. The RanGDP binds to a small NTF2 protein, what transports it back to the nucleus. The Ran guanidine nucleotide exchange factor (GEF) RCC1, associated with chromatin, stimulates the release of nucleotides bound to Ran. Since GEF distributes in the nucleus and GAP in the cytosol, it leads to distribution of primary GDP-bound Ran in the cytoplasm and GTP-bound Ran in the nucleus. GTP-hydrolysis seems not to be involved in the nuclear transport.

The mechanism of translocation of protein complexes has not been clarified. It is known that nucleoporins containing small phenylalanine-glycine (FG) repeats are directly

involved in the nuclear traffic. FG sequence repeats are based on highly conserved cores that contain one or two phenyl residues framed by different sequences that are rich in charge and polar residues (Doye and Hurt, 1997, Rout et al., 2000, Ryan and Wente, 2000). The two most common cores are FxFG and GLFG. The FG repeats-containing regions of nucleoporins have a very flexible structure. FG nucleoporins are located on both sides of the NPC and in the central channel. They are involved in collecting cargo complexes close to the transport channel and hopping the material into the channel.

2.3. Nuclear Localization Signal

The nucleocytoplasmic trafficking of proteins, which exceed the passive diffusion limit of ~40-50 kDa, occurs through the nuclear pore complex and is mediated by active and selective mechanisms that are controlled by saturable transport receptors and special transport signals. The Nuclear Localization Signal (NLS) is a sequence of amino acids that is recognized by importin family proteins for trafficking into the nucleus.

One special signal, the M9 sequence of the human ribonucleoprotein (hnRNP) A1 protein, functions both as nuclear import and nuclear export signal (NES) (Siomi et al., 1995).

The best characterized NLSs comprise one or two short stretches of amino acids. These basic, generally lysine-rich signals, have been typified by the simian virus 40 large T-antigen NLS (PKKKRKV) (Kalderon et al., 1984) and the cellular nucleoplasmin protein NLS (KRPAATKKAGQAKKKK) (Robbins et al., 1991), often called as classical NLS. Other examples are human single-minded proteins SIM1 and SIM2 (Yamaki et al., 2004), kanadaptin (Hübner et al., 2002) and many others. These sequences are recognized in cytoplasm by importin-α bound to importin-β or by importin-β alone. The formed protein complexes are translocated into the nucleus.

More recently, a variety of nonclassical NLS that are not rich in lysine residues have been identified. Some of them have been characterized as nonconvential importin- α interacting motifs, for example, the influenza virus NP protein (Wang et al., 1997), the cellular transcription factor Stat1 (McBride et al., 2002; Melen et al., 2001) or the NLS of UL84 protein of human cytomegalovirus containing 282 amino acids residues, which all are necessary for binding to the importin-α proteins (Lischka et al., 2003). Within the human immunodeficiency virus type 1 Rev and Tat proteins, an arginine-rich NLS has been identified, that binds directly to importin-β receptor (Truant et al., 1999). The hnRNP A1

protein contains NLS of 38 amino acids residues, which is rich in glycine and aromatic residues. This import sequence is recognized and carried into the nucleus by transportin, one of the importin-like molecules (McBride et al., 2002; Pollard et al., 1996). Nowadays many proteins migrating to the nucleus have been described to contain different non-classical nuclear localization signals.

2.4. The other pathways of nuclear import

Besides the classical mechanism, other pathways of nuclear traffic have been identified. There are transport factors, which are unrelated to importin family and proteins that can translocate through the NPC in the absence of other soluble factors. For example, there is Ran-independent dissociation of the importin-cargo complex (cyclin B1-cdc2) (Takizawa et al., 1999). MNS5, beta-transportin family protein, could work as importin with the DNA-damage response protein and as exportin with trans-factor Pho4 (Kaffman et al., 1998). Various proteins that are not related to the importin family are able to transport cargo proteins. ER-resident protein calreticulin protein exports the glucocorticoid receptor (Holaska and Paschal, 1998). Transportin-1, a member of the transportins family (Rebane et al., 2004) recognizes the nuclear location signal M9 of hnRNP A1 mRNA-binding protein and translocates it into the nucleus. Proteins such as hnRNPK and β-catenin probably interact directly with NPCs without any cytoplasmic receptors (Fagotto et al., 1998, Michael et al., 1997, Yokoya et al., 1999).

2.5. Regulation of nuclear migration

Masking and exposing of NLS by binding proteins are the common regulatory pathways of the nuclear migration. These processes are mediated by phosphorylation or dephosphorylation, acetylation, ubiquitination or sumoylation.

Thus, the phosphorylation of serines, which lie directly within cytoplasmic retention signal (CRS) in cyclin B1 protein, mediates nuclear translocation of the protein, whereas nonphosphorylated cyclin B1 is localized in the cytoplasm (Li et al., 1997, Yang et al., 2001). The aryl hydrocarbon receptor contains two sites for PKC one amino acid upstream to both segments of NLS (Ikuta et al., 2004, *BBRC*). Mimicking of phosphoserine by aspartate inhibits the nuclear localization of the protein. The nonclassical nuclear localization signal of human heat shock cognate protein 70 is up-regulated by heat shock and is able to mask NES of this protein (Tsukahara and Maru, 2004). In *Saccharomyces cerevisiae* transcriptional

factor Swi6p, phosphorylation of sites adjacent to an NLS decreases the affinity for importin-α and nuclear accumulation (Harreman et al., 2004). Furthermore phosphorylation of a PKA site adjacent a NLS of *Drosophila* transcriptional factors (Tfs) modulates NLS recognition by importins 58/97 complex (Briggs et al., 1998).

Another regulation mechanism is releasing anchored protein from the membrane. For example, the membrane-bound transcriptional factor SRE-binding protein (sterol response element) induces the transcription of sterol-regulated genes. It consists of a hydrophobic segment of the protein anchored in the membrane, the transcriptional factor and helix-loop-helix leucine zipper. By two proteolytic cleavages the transcription factor was released and located into the nucleus (Brown and Goldstein, 1997, Horton et al., 2002).

Some proteins have an alternative nuclear localization signal except of classical NLS. For example, the yeast hsp70 S194p protein normally is translocated into the nucleus by importin-α /importin-β pathway, but after ethanol shock was transferred into the nucleus by importin- family receptor Nmd5p, that binds to another NLS within the protein (Quan et al., 2004).

3. The aims of the study

The RS1 protein is the widespread protein that was found at the intracellular side of the plasma membrane, at vesicles below the plasma membrane, at the *trans*-Golgi network (TGN) and within the nucleus. This protein was shown to regulate the activity of the sodium-D-glucose cotransporter SGLT1, the organic cation transporter OCT2, the organic anion transporter OAT and the Na^+-cotransporter for serotonin SERT post-transcriptionally as well as on expression level.

The aim of the study was to identify the nuclear localization signal (NLS) of pRS1 protein and to determine the ways of its regulation during confluence. Because the nuclear localization was previously demonstrated on transiently transfected preconfluent LLC-PK_1 cells using a GFP-pRS1 fusion construct, the same method was used in the beginning of this study. For detailed identification of NLS, pRS1 fragments were inserted in β-Gal-GFP constructs and these constructs were transiently transfected into preconfluent LLC-PK_1 cells. The GFP fluorescence was detected by light microscopy, and cells containing fluorescence within nuclei were calculated. We also studied the regulation of RS1 localization and its dependence on cell density.

Besides nuclear localization, the ways of degradation of the pRS1 protein were investigated. For degradation experiments, transiently transfected by pRcCMV-pRS1 vector HEK 293 cells were used. The protein content was detected by Western blot applying polyclonal anti-pRS1 antibody.

MATERIALS AND METHODS

1. MATERIALS

1.1. Chemicals

In the work we used chemicals mostly from Serva (Heidelberg, Germany), Sigma-Aldrich (Taufkirchen, Germany), AppliChem (Darmstadt, Germany), Roth (Karlsruhe, Germany) and Merck (Darmstadt, Germany).

1.2. Enzymes and kits

Restriction endonucleases were obtained from New England Biolabs (Frankfurt, Germany) and MBI Fermentas (St. Leon-Rot, Germany). Pfu DNA and T4 DNA ligase were purchased from MBI Fermentas (St. Leon-Rot, Germany). Midi and Maxiprep kits for DNA isolation were obtained from Qiagen (Hilden, Germany), DNA purification kit "Easy Pure" – from Biozym Scientific GmbH (Oldendorf, Germany).

1.3. Equipment

Camera Polaroid MP4 – Polaroid Corporation (USA); Thermostat IPP-400 – Memmert GmbH (Germany); UV transilluminator TM-20 – UVP Inc. (USA); Axioplot 2 microscope – Carl Zeiss (Jena, Germany); Horizontal Blot-apparat – Novablot, Pharmacia (Freiburg, Germany); PCR amplificator – "Omnigene TR3 SM5" Hybaid Limited (United Kingdom).

1.4. Oligonucleotides

All oligonucleotides were synthesized by MWG-BIOTECH AG (Ebersberg, Germany) and Biomers.net GmbH (Ulm, Germany).

Table 1. The list of oligonucleotides, used as primers in PCR (restriction sites are underlined).

Oligos	**Position on pRS1 (hRS1) sequence (see attachment)**	**5´-Sequence-3´**	**Restriction Sites**
FR1-for	1104-1121	GCGCTAGCCTTCATGAGCTTTTGGTC	Nhe I, BspHI
FR2-rev	1190-1172	GCACCGCGGCTCAGTTACTATCTCTGAC	SacII
FR3-rev	1292-1275	GCACCGCGGGGCCTGATAGAAGGAGAC	SacII

Table 1 (continue). The list of oligonucleotides, used as primers in PCR (restriction sites are underlined).

FR4-for	858-880	GCGCTAGCTCTGATCTCACCGTAGATAATCC	NheI
FR5-rev	110-1121	GCACCGCGGGACCAAAAGCTCATGAAGTTCC	SacII, BspHI
FR6-for	1062-1078	GCGCTAGCGCAGAGGAATCTTGCTC	NheI
FR7-for	1035-1053	GCGCTAGCGGCACTTGTCAGCCCTCTG	NheI
FR8-for	942-964	GCGCTAGCTCCACTCAGAATTTACAGCTTCC	NheI
FR9-for	1041-1058	GCGCTAGCTGTCAGCCCTCTGTAGAG	NheI
FR10-for	1145-1129	GCACCGCGGCGCTGGTTTAC	NheI
FR11-rev	1232-1218	GCACCGCGGTTGGGTCCATCTTTC	SacII
FR12-for	(1180-1196)	GCGCTAGCGCAGAAGAATCTTGCCC	SacII
DEL1-rev	1428-1413	GCAGGGCCCCCCTAGACCTGGGGAC	ApaI
DEL2-rev	1290-1270	GCAGGGCCCTGATAGAAGGAGACTTGTG	ApaI
DEL3-rev	1188-1168	GCAGGGCCCAGTTACTATCTCTGACCGAC	ApaI
DEL4-rev	1139-1129	GCAGGGCCCTTCTAACGCTGGTTTACTAC	ApaI
DEL5-rev	1247-1218	GCAGGGCCCTGTAAGATGCTCACTTTGGG	ApaI
DEL-NLS-F	1044-1061/1140-1155	CAGCCCTCTGTAGAGTCA-TTAGAAAATACATCTG	
DEL-NLS-R	1155-1140/1061-1044	CAGATGTATTTTCTAA-TGACTCTACAGAGGGCTG	
F591L-R	1797-1777	GCAAGGTAAGACCGGCCCGAAG	
F591L-F	1779-1799	CGGGCCGGTCTTACCTTGCAG	
PrsG590P-R	1794-1184	CCTGCAAGGTAAAGGGAGCCCGAAGAATCCGG	
PrsG590P-F	1772-1189	GATTCTTCGGGCTCCCTTTACCTTGCAGGAAGC	
LL614,615AA-R	1845-1878	GCACTTCTTGTTGCTGCAGCAAAGAACATTGTAG	
LL614,615AA-F	1875-1841	CAATGTTCTTTGCTGCAGCAACAAGAAGTGCAAGG	
S348A-R	1072-1047	GATTCCTCTGCAGCCTCTACAGAGGG	
S348A-F	1049-1076	CTCTGTAGAGGCTGCAGAGGAATCTTGC	
S348E-R	1072-1047	GATTCCTCTGCCTCCTCTACAGAGGG	
S348E-F	1049-1076	CTCTGTAGAGGAGGCAGAGGAATCTTGC	
S370A-R	1111-1140	TTTGGTCATTGCTAGCAAACCAGCGTTAGA	NheI
S370A-F	1115-1144	ACGCTGGTTTGCTAGCAATGACCAAAAGCT	NheI
S370A-R(2)	1110-1139	CGCTGGTTTACTCGCGATGACCAAAAGCTC	
S370A-F(2)	1110-1139	GAGCTTTTGGTCATCGCGAGTAAACCAGCG	
S370E-R	1135-1110	GGTTTACTCTCAATGACCAAAAGCTC	
S370E-F	1116-1139	TTGGTCATTGAGAGTAAACCAGCG	
S400A-R	1229-1209	GGTCCATCTTTCGGCCAGGTCCTTAACATC	EaeI
S400A-F	1203-1230	GTTAAGGACCTGGCCGAAAGATGGACCC	EaeI
S400E-2R	1227-1193	TCCATCTTTCCTCAAGATCTTTAACATCTGTTTGG	BglII
S400E-2F	1198-1230	CAGATGTTAAAGATCTTGAGAAAGATGGACCC	BglII
S400D-R	1227-1193	TCCATCTTTCGTCAAGATCTTTAACATCTGTTTGG	BglII
S400D-F	1198-1227	CAGATGTTAAAGATCTTGACGAAAGATGGA	BglII
RS1-rev	1897-1881	TTCCATGGTTATGTAGG	
RS2-rev	1589-1573	GAAGCTGGATGACAAGG	
RS3-for	810-836	CTTAATTCAGCAGGCGG	
RS4-for	1112-1129	GCTTTTGGTCATTAGTAG	
RS5-for	1427-1444	GGAATCAGTAAACGAGAG	
RS6-for	1262-1279	GCAGTGTTCACAAGTCTCC	
RS7-for	453-472	CCACAGAGGCTTATAGGTG	
BETA-Gal1		CAGTATCGGCGGAATTCC	
829-rev		GCCATCCAGTTCCACGAG	

1.5. Plasmids

The plasmids pRcCMV (Invitrogen, The Netherland), pEGFP(C1) (Clontech, Heidelberg) and pBluescript II SK-pRS1 (Veyhl et al., 1993, Valentin, 1998) were used for most of the experiments. pHM829 and pHM830 plasmids were kindly provided by Dr. S. Hübner (Hübner et al., 2002). Plasmid pRS1/pRcCMV, GFP-pRS1 fusion protein with the N-terminus pRS1 linked to the C-terminus of GFP as well as truncations of GFP-pRS1 Δ1-281 and Δ365-623 were made in our laboratory earlier (Baumgarten, 1999, Kühlkamp, 2000).

As selective agents the following antibiotics were used:
100 µg/ml ampicillin – for constructs on the basis of pRcCMV, pHM829 and pHM830 plasmids
30 µg/ml kanamycin – for constructs on the basis of pEGFP(C1) plasmid

1.6. Bacteria

The bacterial *E.coli* strain *DH10B* (Grant et al., 1990) was used for selection and amplification of plasmids.

1.7. Antibodies

Polyclonal primary antibodies against recombinant porcine protein RS1 were raised in rabbit against a peptide of pRS1, identical to amino acids 462-477 of pRS1 (Valentin et al., 2000). Mouse monoclonal antibodies against GFP, secondary goat anti-rabbit IgG and goat anti-mouse IgM coupled to HRP were purchased from Sigma (Taufkirchen, Germany).

1.8. Cell lines

The transfection experiments were carried out with HEK 293 and LLC-PK_1 cell lines. HEK 293 is a human embryonic kidney cell line what expresses a small amount of native RS1 protein (Kroiss et al., 2006). The cells were used for over-expressing the pRS1 protein and its mutant forms. LLC-PK_1 cells were derived from porcine kidney proximal tubule cells (renal epithelial cells), which express SGLT1 and RS1 proteins. After confluence they were differentiated form tight junctions and build brush-border enzymes. Both cell lines were transiently transfected with plasmids, encoding either GFP-pRS1 or pRS1-truncated variants or fusionproteins comprising β-Gal, pRS1 fragments and GFP.

2. METHODS

2.1. Buffers and Medias

All buffers and media were prepared, diluted using distilled water and autoclaved.

Table 2. Contents of buffers and medias.

A-Blockbuffer	1 x TBST, 0.5 % BSA
A-Elution buffer	0.1 M citric acid, 500 mM NaCl, 0.05 % (v/v) Tween-20, pH 2.5
A-Shift buffer	50 % A-Block buffer, 50 % Tris/HCl pH 8.8
Blocking buffer	1 x TBST, 5 % milk powder
Blotting buffer	25 mM Tris, 192 mM glycine, 10 % (w/v) methanol pH 8.3
Buffer H, hypotonic	20 mM HEPES, pH 7.5, 10 mM KCl, 15 mM $(CH_3CO_2)_2Mg$, 1 x protease Inhibitor mix, 10 µM MG-132
Coat buffer	15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.5
Detaching buffer	Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffered saline (DPBS), 28 mM $NaCHO_3$, 0.5 mM EDTA, 10 mM HEPES pH 7.4.
Laemmli buffer	0.001 % bromphenolblue, 10 % glycerole (v/v), 0.5 M β-mercaptoethanol, 2 % SDS (w/v), 0.1 M Na_2HPO_4 pH 6.8
LB-agar	LB-media, 1.5 % agar (w/v)
LB-media	2 % bacto-tryptone (w/v), 0.5% yeast extract (w/v), 170 mM NaCl, pH 7.0
Loading buffer (x 5)	30 % glycerine, 0.25 % (w/v) bromphenol blue, 0.25 % (w/v) xylencyanol in TAE buffer
Lysation buffer	25 mM Tris-HCl pH 7.5, 100mM NaCl, 1 % Igepal, 1 x PI mix
NM	Dulbecco's Modified Eagle's Medium (DMEM), 10 % (v/v) fetal bovine serum, 4 mM L-glutamine, 0.1 mg/ml streptomycin sulfate and 100 U/ml penicillin G
ORI buffer	5 mM MOPS pH 7.4, 100 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$
PAO	4 ml poly-α-olefine, 0.9 ml Tween-81, 0.3 ml Tween-80
PBS buffer	137 mM NaCl, 20 mM, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4 x $2H_2O$ pH 7.14
Protease Inhibitor mix (100 x PI mix)	1 mM benzamidin, 5 µg/ml aprotinin (Fluka, Bucks, Switzerland), 5 µg/ml leupeptin hemisulfate (Fluka, Bucks, Switzerland), PMSF
Running buffer	8 % PAA, 0.1 % SDS (w/v), 375 mM Tris/HCl pH 8.8
SOB-media	2 % bacto-tryptone (w/v), 0.5% yeast extract (w/v), 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ pH 7.0
SOC-media	SOB-media, 10 mM $MgSO_4$, 20 mM glucose

Table 2 (continue). Contents of buffers and medias.

Stocking buffer	5 % PAA, 0.1 % SDS (w/v), 125 mM Tris/HCl pH 6.8
T-Blockbuffer	1 x PBS (pH 7.4), 200 mM NaCl, 0.5 % BSA, 0.1 % natriumazidin
T-Substrate buffer	100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris/HCl pH 9.8, 1 mg/ml para-nitrophenylphosphate (104 substrate, Sigma)
T-Washbuffer	1 x PBS (pH 7.4), 200 mM NaCl, 0.05 % (v/v) Tween-20
TAE buffer	40 mM Tris-acetate pH 8.0, 1 mM EDTA
TBST buffer	137 mM NaCl, 50 mM Tris/HCl, 2.7 mM KCl, 0.05 % (v/v) Tween 20 pH 8.0
TBST-washing buffer	1 x TBST, 1% milk powder
TE buffer	10 mM Tris-HCl pH 8.0, 1 mM EDTA

2.2. Methods of molecular cloning

The desired fragments of pRS1 were duplicated by polymerase chain reaction (PCR) (2.2.2.), cut with restrictases for 3 hours in required conditions (2.2.4.), gel-purified in 1% agarose gel using DNA purification kit "Easy Pure" (Biozym Scientific GmbH, Oldendorf, Germany) (2.2.5.) and ligated with restricted vectors by T4 DNA ligase in ligation buffer (2.2.6.). After ligation for 16 h at +14°C the mix was desalted by NH_4Cl and polyacrylamide carrier (2.2.7.) and transformed by electroporation method in Cs-treated *E.Coli* cells (2.2.8.). Then the *E.coli* cells were grown on agarose plates containing either 100 µg/ml ampicillin or 25 µg/ml kanamycin as selective antibiotics.

Several clones were amplified by miniprep (2.2.9.1.) and were controlled by restrictases, which cut construct with required mutation in a different way as they cut native plasmids. The sequences of selected clones of all generated truncations and mutations were confirmed by DNA sequencing.

DNA of approved constructs was amplified by endotoxin-free midi or maxiprep (Qiagen, Hilden, Germany) in *E.Coli* cells (2.2.9.2.). Concentration of DNA was measured (2.2.10.). DNA was diluted till 1 µg/ml concentration and used for further experiments.

2.2.1. Construction of RS1-expression plasmid

For identification of Nuclear Localization Signal (NLS) of pRS1 protein, following constructs were made.

2.2.1.1. pRcCMV-pRS1 and pEGFP(C1)-pRS1 constructs

Deletion / truncation constructs – constructs expressing green fluorescent protein (GFP)-pRS1 truncations were prepared as followed:

Δ219-581: the pEGFP(C1)-pRS1 plasmid was cut with *PstI*, the internal *PstI* fragment was removed, and the plasmid was religated.

Δ375-623, Δ391-623, Δ411-623, Δ425-623 and Δ475-623: fragments of pRS1 were amplified by polymerase chain reaction (PCR) using forward primer RS7-for corresponding to C-terminus of pEGFP(C1) and reverse primer, containing *ApaI* site and covering the end of the required region. PCR products were digested by *PshAI* and *ApaI*, and the regions of pRS1 (amino acids 1-374, 1-390, 1-410, 1-424 and 1-474) were cloned into the pEGFP(C1) plasmid cut with the same enzymes. In that way we have got GFP-pRS1 fusion proteins lacking of C-terminal parts.

constructs	Reverse primer (5')
Δ375-623	DEL4-rev
Δ391-623	DEL3-rev
Δ411-623	DEL5-rev
425-623	DEL2-rev
Δ475-623	DEL1-rev

Δ524-623: the pEGFP(C1)-pRS1 plasmid was digested with *XcmI* and *SmaI* restrictases, the C-terminal 551 bp-fragment of pRS1 was removed and the plasmid was religated.

Δ583-623: a C-terminal exonuclease *Bal3l*-truncation variant of pRS1 in pBluescriptII SK plasmid (made in our laboratory by Katharina Baumgarten) was digested with *SacI* and *KpnI* and recloned into the pEGFP(C1) vector.

Point mutations – Point mutations of pRS1 and GFP-pRS1 fusion proteins were generated by polymerase chain reaction (PCR) using the overlap extension method (Ho et al, 1989). In the case of substitutions in the ubiquitin-associated domain UBA (G590P, F591L and L614A/L614A mutants) the primer RS6-for and a primer corresponding the vector region downstream of pRS1 were used and the amplified products were digested by *BbsI* and *ApaI* restrictases and cloned into the previously restricted pEGFP(C1)-pRS1 or pRcCMV-pRS1 vectors. For point mutations of phosphorylation sites (S348A, S348E, S370A, S370E, S400A and S400E) primers S348A-rev – A400D-for were used and amplified products were cut with *PshAI* and *BbsI* and cloned into the pEGFP(C1)-pRS1 plasmid cut with the same enzymes.

Double point mutation S370A/S400A – Second mutation S400A was inserted by the overlap extension method (Ho et al, 1989) into the GFP-pRS1/S370A construct. The amplified product was digested by *PshAI* and *BbsI* and cloned into the previously cut pEGFP(C1)-pRS1 vector.

GFP-pRS1 ΔNLS (Δ349-374) – This plasmid was constructed by two PCR-amplification steps using pEGFP(C1)-pRS1 plasmid as a matrix. In the first step the 5'- and 3'-flanking regions of the NLS fragment was separately amplified. For amplification of the 5'-flanking part the primers RS7-for and DEL-NLS-R and for amplification of the 3'-flanking part the primers DEL-NLS-F and RS2-rev were used. In the second step both flanking regions were combined into one DNA fragment by additional PCR using primers RS7-for and RS2-rev. The PCR product lacking the NLS sequence was digested with *PshAI* and *BbsI*, gel purified and cloned into the GFP-pRS1 plasmid, what was previously cut with the same enzymes.

2.2.1.2. β-Gal-rs fragments-GFP constructs

For investigation of the short fragments of pRS1 protein, following constructs were utilized. Plasmids pHM829 containing C-terminal β-Galactosidase (β-Gal) and N-terminal GFP, and pHM830 containing N-terminal β-Galactosidase and C-terminal GFP (Sorg and Stamminger, 1999) were used. The fragments of pRS1 were cloned in multiple cloning site between GFP and β-Gal in pHM829 or pHM830 plasmids.

The fragments (amino acids 281-368, 281-391, 309-391, 340-391, 342-368, 342-374, 342-406, 349-368, 349-374, 349-391, 349-406, 363-425, 363-425) were amplified from the pEGFP(C1)-pRS1 vector using primers containing *NheI* and *SacII* restriction sites (reverse primer 1 and forward primer 2, respectively), gel-purified and cloned into the *NheI* and *SacII*-sites of the pHM829 plasmid. pHM829 without (β-Gal-GFP) or with the nuclear localization sequence of nucleoplasmin (NP-NLS, KRPAATKKAGQAKKKK) (β-Gal-NP-NLS-GFP) were used as negative and positive controls, respectively (Hübner et al., 2002).

constructs, a. a. of hRS1 in β-Gal-GFP	**Primer 1**	**Primer 2**
345-370	FR12-for	FR10-rev

constructs, a. a. of pRS1 in β-Gal-GFP	Primer 1	Primer 2
281-368	FR4-for	FR5-rev
281-391	FR4-for	FR2-rev
309-391	FR8-for	FR2-rev
340-391	FR7-for	FR2-rev
342-368	FR9-for	FR5-rev
342-374	FR9-for	FR10-rev
342-406	FR9-for	FR11-rev
349-368	FR6-for	FR5-rev
349-374	FR6-for	FR10-rev
349-391	FR6-for	FR2-rev
349-406	FR6-for	FR11-rev
363-425	FR1-for	FR3-rev
363-425	FR1-for	FR3-rev

Point mutations – point mutations (S348A, S348E, S370A, S370E, S400A and S400E) in 342-406 and 349-406 pRS1-fragments were performed by PCR from the pEGFP(C1)-pRS1 vector using the overlapping extension method (Ho et al, 1989), with flanking primers BETA-Gal and 829-rev. The amplified products were digested by *NheI* and *SacII* restrictases and cloned into the pHM829 vector.

Double point mutations – second mutation (S400A or S400E) was inserted into β-Gal-[349-406]-GFP /S370A or /S370E plasmids by PCR using the overlap extension method (Ho et al, 1989). The amplified products were digested by *NheI* and *SacII* and cloned into the pHM829 vector. In that way four double mutations (S370A/S400A, S370A/S400E, S370E/S400A and S370E/S400E) in β-Gal-[349-406]-GFP construct were performed.

2.2.1.3. Triple mutations in β-Gal-[342-406]-GFP construct

To investigate the effect of phosphorylation on nuclear migration, the special constructs with three mutated serines 348, 370 and 400 were performed. Serines in β-Gal-[342-406]-GFP construct were substituted to alanine and glutamate resulting in eight triple mutants: AAA, AAE, AEA, EAA, EEA, EAE, AEE and EEE.

For that two PCR-amplification steps using pRS1-GFP constructs carrying the point mutations S400A or S400E as matrix were performed. First, one substitution was inserted using the overlap extension method (Ho et al, 1989), and then the PCR was repeated for generation of the second point mutation using the mix from the first PCR as a matrix. The amplified products were digested by *NheI* and *SacII* and cloned into the pHM829 vector.

S348	S370	S400	Matrix, pRS1-GFP/	Primers for PCR 1	Primers for PCR 2
A	A	A	/S400A	S348A-R, -F	S370A-R(2), -F(2)
A	A	E	/S400E	S348A-R, -F	S370A-R(2), -F(2)
A	E	A	/S400A	S348A-R, -F	S370E-R, -F
E	A	A	/S400A	S348E-R, -F	S370A-R(2), -F(2)
E	E	A	/S400A	S348E-R, -F	S370E-R, -F
E	A	E	/S400E	S348E-R, -F	S370A-R(2), -F(2)
A	E	E	/S400E	S348A-R, -F	S370E-R, -F
E	E	E	/S400E	S348E-R, -F	S370E-R, -F

2.2.2. Polymerase chain reaction (PCR)

The PCR was used to amplify the plasmid constructs encoding mutated RS1 protein. The reaction was carried out in a 50 µl volume in 0.5 ml Eppendorf cups and was covered by oil. The reaction mix contained 1 µM of each primer, 10 ng of DNA matrix, 200 µM of each nucleotide and buffer for Pfu DNA polymerase. The 2.5 units Pfu DNA polymerase was added after heating. The reaction mix was covered with 50 µl paraffin oil. For most applications the standard program 1 was used:

1 cycle – denaturation (94°C, 30 sec);

25 cycles – denaturation (94°C, 30 sec), annealing (50-55°C, 30 sec), elongation (72°C, 30 sec);

1 cycle – elongation (72°C, 5 min).

The amplified DNA fragment of expected size was obtained. To get deletion constructs the fragment was purified, digested and subcloned into the vector.

For point mutations two fragments from the both sites of the mutation were amplified separately (A reaction mix and B reaction mix), then mixed and amplified together, so the fusion fragments with the point mutation in the middle were prepared. The new C reaction mix contained 1-3 µl of the A reaction mix, 1-3 µl of the B reaction mix, 200 µM of each

nucleotide and buffer for Pfu DNA polymerase. The 2.5 units Pfu DNA polymerase was added after heating. The standard program 2 was used:

1 cycle – denaturation (94°C, 3 min);

10 cycles – denaturation (94°C, 30 sec), annealing (55°C, 30 sec), elongation (72°C, 30 sec);

1 cycle – elongation (72°C, 5 min).

Then 1 µM of each flanking primer and new portion of Pfu DNA polymerase were added and next PCR was made (program 3):

1 cycle – denaturation (94°C, 3 min);

25 cycles – denaturation (94°C, 30 sec), annealing (55°C, 30 sec), elongation (72°C, 30 sec)

1 cycle – elongation (72°C, 5 min).

After the PCR 3-5 µl of the mixture were analyzed on an agarose gel.

2.2.3. Chloroform extraction

To remove paraffin oil and proteins and precipitate DNA after PCR the chloroform extraction was performed. First the PCR solution was mixed with the same volume of chloroform-isoamylalcohol (24:1). After centrifugation for 7 min at 14,000×g the upper phase was transferred to a new tube. DNA was precipitated with 2.5V of 100% ethanol in the presence of 0.3 M Na-acetate, pH 5.0 for 30 min at -20°C and spun down at 14,000×g for 15 min. The pellet was washed with 70% ethanol, air-dried and dissolved in 20 µl TE buffer.

2.2.4. DNA digestion with restriction enzymes

The procedure was performed to cut DNA fragments and then to insert them into cloning sites of the desired plasmid. 1µg DNA was mixed with 5-15 units enzyme in appropriate buffer. The enzymatic reaction was performed for 3 hours at +37°C. Enzymatic activity was stopped by heat inactivation or addition of 1 x Loading buffer. The following restriction enzymes (New England Biolabs and MBI Fermentas) were used in different applications: *ApaI, BbsI, BglII, BspHI, KnpI, NheI, PshAI, PstI, SacI, SacII, SmaI, XcmI.*

2.2.5. Preparative agarose gel electrophoresis

For isolation of DNA fragments after restriction we performed the preparative agarose gel electrophoresis. The digesting mix was combined with loading buffer and applied to a big slot of agarose gel. After electrophoresis (2.2.11.) the gel was observed under UV light and a part of the gel corresponding to a desired fragment was cut out. The DNA was isolated from the agarose gel using the DNA purification kit "Easy Pure" (Biozym Scientific GmbH, Hess.

Oldendorf, Germany)

2.2.6. Ligation

T4 DNA ligase joins blunt and cohesive ends by catalyzing the formation of phosphodiester bonds between 5`-phosphate and 3´-hydroxyl terminus in duplex DNA. It was used for cloning the DNA fragments into the plasmids. For most ligation reactions, a molar ratio 4:1 of fragments to vector was used. T4 DNA ligation buffer and 5 units T4 DNA ligase were added to the mixture and incubated overnight at +14°C.

2.2.7. Transformation of bacteria and clone selection

20 µl electrocompetent *E.coli* cells were mixed with 1 µl of desalted ligation mix and pipetted to ice-cold electroporation cuvette. After electrical pulse (1.6 kV, 5 msec, Biojet MJ) bacteria were suspended with 1 ml of SOC buffer and incubated for 1 h at 37°C. 250-500 µl of the bacterial suspension were spread on agar plate, containing appropriate antibiotic, ampicillin or kanamycin. In 16 h at +37°C transformed bacteria grew in single colonies. For further selection several colonies were transferred to a new agar plate and into tubes with 2.5 ml LB medium as well. The agar plates or LB tubes with selected bacterial clones were incubated for 16 h at +37°C as usual.

2.2.8. Plasmid isolation

Plasmid extraction was performed by endotoxin-free midi or maxiprep kits (Qiagen, Hilden, Germany).

2.2.8.1. Analytical purification of plasmid DNA (miniprep)

Plasmid DNA purification is based on alkaline lysis of bacterial cells followed by selective precipitation of genomic bacterial DNA and proteins by lowering the pH.

Bacterial clones were cultured overnight in a 2,5 ml LB medium containing the specific antibiotic (ampicillin or kanamycin). The 1,5 ml of the night culture was spun for 15 min. in a microcentrifuge at 14,000×g. The supernatant was discarded and the pellet was resuspended in 300 µl P1 buffer. After addition of 300 µl P2 buffer tubes were gently mixed by inversion for 6 times and set on ice. Immediately 300 µl P3 buffer was added and the samples were incubated for 20 min. on ice. The cell debris and chromosomal DNA were precipitated with sodium dodecyl sulfate (SDS) in the P2 buffer. After pelleting the debris the plasmid DNA was precipitated from the 800 µl supernatant with 680 µl isopropanol and spun

for 15 min in the microcentrifuge at 14,000×g. The pellet was washed with cold 70% ethanol, air-dried and resuspended in 20 µl TE buffer.

The DNA was controlled by restriction analysis and subjected to sequencing.

P1 buffer 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg/ml RNase A

P2 buffer 200 mM NaOH, 1% SDS

P3 buffer 10 mM Tris-HCl pH 8.0, 1 mM EDTA

2.2.8.2. Preparative purification of plasmid DNA (midiprep or maxiprep)

50 or 250 ml overnight bacterial culture inLB medium were taken for plasmid purification. The Midi or Maxi plasmid isolation kits (Qiagen, Hilden, Germany) were used. The DNA concentration was determined, adjasted to 1 µg/ml concentration and DNA was stored at -20 C.

2.2.9. Determination of DNA concentration

The concentration of DNA was determined by absorbance at 260 nm. For determination of the purity of DNA sample the absorbance was measured at 280 nm as well. A ratio between A_{260} and A_{280} should be approximately 1.8 - 2.0 for the pure preparation of DNA.

2.2.10. Analytical agarose gel electrophoresis of DNA

DNA fragments were separated and analyzed in 1 % agarose gel running in TAE buffer with 0.1 µg/ml ethidium bromide (Sigma-Aldrich). The electrophoresis was performed in TAE buffer at a voltage of 10 V/cm. 1kb and 10 kb Ladder (MBI Fermentas) were used as a molecular size marker.

2.3. Protein analysis methods

2.3.1. Isolation of proteins

The LLC-PK_1 or the HEK 293 cells were transiently transfected (3.4.3.), and after 24-48 h they were washed three times with PBS buffer and harvested in lysation buffer. The cells were rotated for 30 min at +4°C for denaturation of the cell membranes. The membranes were sedimented by centrifugation at 1,200×g for 10 min. All buffers were previously cooled at +4°C and all experiments were performed on ice. To avoid the degradation of the proteins all buffers contained 100-fold diluted Protease Inhibitor Mix and 10 µM of the proteosome inhibitor MG-132 (Sigma-Aldrich, Taufkirchen, Germany).

Lysation buffer	25 mM Tris-HCl pH 7.5, 100mM NaCl, 1 % Igepal, 1 x PI mix
PBS	137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4
Protease Inhibitor Mix (100 x PI mix)	5µg/ml aprotinin (Fluka, Bucks, Switzerland), 5µg/ml leupeptin, 1 mM benzamidin

2.3.2. Subcellular fractionation

It is known that the LLC-PK_1 cells contain native pRS1 protein. To determine localization of the native protein within the cell subcellular fractionation was performed.

Subcellular fractionation of pRS1 in LLC-PK_1 cells was done as described by Kühlkamp (2000). For some experiments LLC-PK_1 cells were pre-incubated with 10 µM MG-132, to inhibit proteosomal degradation. 4 hours after addition of MG-132 cells were washed 3 times with PBS and mechanically detached from culture plates. Then they were suspended for 20 min in 2 volumes of ice-cold hypotonic buffer H, frozen in liquid nitrogen and thawed 3 times, and finally homogenized by 10 strokes in a tight-fitting glass homogenizer.

The nuclei were sedimented by 5 min centrifugation at 1,200×g. Then pellet was suspended for 5 min in hypotonic buffer H with 0.5% (w/v) Igepal CA-630 (Sigma-Aldrich, Taufkirchen, Germany) and washed with H buffer containing 0.1 % Igepal CA-630.

For isolation of plasma membrane fraction, the supernatant was centrifuged 1 h (+4°C) at 40,000×g. The associated cytosolic proteins were removed by washing the pellet two times with H buffer.

A cytosolic fraction of proteins was obtained as supernatant after centrifugation of the 40,000×g-supernatant at 200,000×g for 2 h.

2.3.3. Determination of protein degradation

Degradation of the pRS1 protein was investigated by using different inhibitors for lysosomal and proteosomal enzymes.

HEK 293 cells were transiently transfected by plasmid, encoding RS1 protein. After 48 h the cells were washed three times with PBS and harvested in lysis buffer. The cells were rotated for 30 min at +4°C for denaturation of the cell membranes. The membranes were sedimented by centrifugation at 1,200×g for 10 min, and the supernatant, containing total proteins, was analyzed by 10% SDS-PAGE and Western blot as usual. Enrichment of different fractions by pRS1 protein was detected by measuring space scanned films.

When proteosome or lysosome inhibitors were used, HEK 293 cells were pre-incubated for 4 h before harvesting with 10µM MG-132 (inhibitor of proteosome degradation, Sigma-Aldrich, Taufkirchen, Germany), 10mM methylamine, 10µM 3-methyladenine, or 10µM NH_4Cl (inhibitor of lysosomal degradation).

2.3.4. Determination of protein concentration

The protein content of the samples was determined according to Bradford (Bradford, 1976). The method is based on the reaction between Coomassie brilliant blue G-250 and aromatic and basic amino acids of proteins.

5-10 µl protein solution was diluted in 95-90 µl H_2O and 1 ml Bradford reagent (Biorad, München, Germany) and incubated for 5 min at RT. The extinction of the samples was measured at 595 nm and correlated with the extinction of water. The extinction of 1 µg bovine serum albumin (BSA) was taken as standard.

2.3.5. SDS-polyacrylamide gel electrophoresis

(Laemmli, 1970)

Protein samples were prepared by boiling for 5 min at +95°C in 50 mM Laemmli buffer containing 2-mercaptoethanol, which is a mild reducing agent for cleaving disulfide bonds to thriols, and SDS, a denaturant of native proteins. 20 µg of the protein samples were loaded onto polyacrylamide gel and separated in the Running buffer at 10 V/cm in the stacking gel and at 15 V/cm in resolving gel.

Polymerization of the gels was started by addition of 10% ammonium-persulfate

(PSA) and tetramethylethylendiamine (TEMED). The prestained protein ladder for SDS-polyacrylamide gel electrophoresis (BenchMark™, Life Technologies, Inc.) was used as a marker.

Stacking gel	4 % PAA, 0.1 % (w/v) SDS, 125 mM Tris-HCl pH 6.8
Resolving gel	10% PAA, 0.1 % (w/v) SDS, 375 mM Tris-HCl pH 8.0
Running buffer	24.8 mM Tris-HCl, 192 mM glycin, 0.1% (w/v) SDS pH 8.3

2.3.6. Western blot and immunodetection

(Kyhse-Anderson, 1984)

After electrophoresis the proteins were transferred onto nitrocellulose membrane using a semi-dry system and detected by polyclonal or monoclonal antibodies.

For semi-dry transfer of proteins the horizontal Blot-apparatus (Novablot, Pharmacia, Freiburg) with two graphite plates was used. Before transfer Whatmann filter papers, the gel and the membrane were incubated in the Blotting buffer for 10 min, and additionally the nitrocellulose membrane was pre-soaked in methanol for 1 min and washed in water for 5 min. The transfer was performed in Blotting buffer at 1.5 mA/cm^2 for 2 hours.

cathode

3 x Whatmann 3MM filter paper

SDS-PAGE gel

nitrocellulose membrane

3 x Whatmann 3MM filter paper

anode

After blotting the membrane presence of proteins on the nitrocellulose membrane was demonstrated using Ponceau dye (Salinovich and Montelaro, 1986). The membrane was incubated for 40 sec in the dye and washed thoroughly with water. During the next washing step with TBST buffer the Ponceau strips were washed away.

For detection of pRS1 protein the previously characterized affinity-purified pRS1 antibodies (pRS1-ab), raised in rabbit against recombinant RS1 (1:2500), were used (Valentin et al., 2000). As secondary antibody, peroxidase-conjugated goat anti-rabbit IgG antiserum from Sigma (1:5000) was used. The immunodetection of GFP was performed with polyclonal antibodies against GFP (Clontech Laboratories, Heidelberg) in a dilution of 1:1000 in TBST-

washing buffer.

During the incubation the membrane was shaken on the rotator. First, the all binding sites on the membrane were blocked by incubation with 5 % milk powder in TBST buffer for 1 h at room temperature. Then the membrane was incubated with the primary specific antibodies for 1 h at RT or overnight at +4°C, following washing step (3 x 15 min with TBST-washing buffer). The secondary antibodies carrying horseradish peroxidase (HRP) were bound to the primary antibodies during incubation for 1 h at RT. Unbound antibodies were rinsed away with the next washing step. The presence of protein was detected using ECL PlusTM Detection Kit (Amersham Pharmacia).

Blotting buffer	25 mM Tris, 192 mM glycine, 10 % methanol (w/v) pH 8.3
TBST buffer	137 mM NaCl, 2.7 mM KCl, 0.05 % (v/v) Tween-20, 50 mM Tris/HCl pH 8.0
TBST-washing buffer	TBST, 1 % milk powder

2.3.7. Antibodies

(Catty et al., 1989)

For identification of the RS1 protein the polyclonal antibodies were raised in rabbit, isolated and affinity purified on ELISA plates, which have been coated with the affinity purified rRS1 protein (Valentin et al., 2000).

2.3.7.1. Immunization

6 month-old rabbits have been immunized with recombinant pRS1 protein (rRS1), what was isolated from *E. coli* (Valentin et al., 2000). For immunization, the protein was mixed with PBS and suspended with PAO (4 ml poly-a-olefine, 0.9 ml Tween-81, 0.3 ml Tween-80) in proportion of 1 to 4, additionally 100 µg of adjuvant peptide (muramyldipeptide, Sigma) was added. For the first immunization 500 µg of antigen were injected. Then 3 times 250 µg of the rRS1 protein were injected every 21 days. 7-10 days after the last immunization 10-20 ml of blood from the ear artery were collected. After incubation at 3 hours at RT and 16 hours at +4°C the blood was centrifugated (10,000 ×g, 10 min at +4°C) and supernatant was isolated. The serum was divided onto 0.5-1 ml fractions and stored at -70°C.

2.3.7.2. Titer identification

First, purified recombinant pRS1 was coated onto the plate. 100 µl of Coat buffer containing 1 µg RS1 protein were loaded into 96-well plate (Nunc-Immuno™ Plate, MaxiSorp™ Surface, Denmark) and incubated for 16 h at +4°C. Then the non-immobilized places on the plate were blocked by incubation with T-Blockbuffer, containing 0.5 % BSA for 2 h at RT and washed three times with T-Washbuffer. The antiserum was diluted in T-Blockbuffer (dilutions from 1 : 400 to 1 : 204,800) and 100 µl from each dilution were added to the wells. After 16 h incubation at the +4°C the plate was washed again three times with T-Washbuffer. Each well was filled with 100 µl of diluted goat anti-rabbit secondary antibodies, coupled with F(ab) alkaline phosphate (1 : 250 dilution in T-Blockbuffer) and incubated for 4 h at RT. The secondary antibodies were washed away (3 x T-Washbuffer) and 100 µl of Equilibration buffer were placed in each well for 5 min at RT. 100 µl of T-Substrate buffer were added and the colour reaction was observed and blocked by 100 µl of EDTA (100 mM, pH 8.0). In ELISA-reader (Multiskan Plus, Titerek) the extinction at 405 nm was measured and the extinction at 450 nm (background) was subtracted to give final results.

Coat buffer	15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.5
Equilibration buffer	
T-Blockbuffer	1 x PBS (pH 7.4), 200 mM NaCl, 0.5 % BSA, 0.1 % natriumazidin
T-Substrate buffer	100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris/HCl pH 9.8, 1 mg/ml para-nitrophenylphosphate (104 substrate, Sigma)
T-Washbuffer	1 x PBS (pH 7.4), 200 mM NaCl, 0.05 % (v/v) Tween-20

2.3.7.3. Antibody purification

For affinity purification of anti-RS1 antibodies, the recombinant pRS1 protein was immobilized on polystyrene plates and coated with serum containing the antibodies against pRS1. Bound anti pRS1-ab were eluted from the plate and stored.

The recombinant pRS1 was diluted to a concentration of 100 µg/ml Coat buffer and 200 µl were added into 96-well plate and incubated for 16 h at +4°C. Non-immobilized places on the polystyrene plate were blocked by 1 h incubation with A-Blockbuffer at RT and washed four-times with TBST buffer. 200 µl of serum (diluted to a ratio 1 : 20 in A-Blockbuffer) were added and incubated for 2 h at RT and the plate was washed as before. For elution, the wells were covered with 200 µl of A-Elution buffer for 3 min and the samples

were quickly transferred into wells containing 200 µl of A-Shiftbuffer for pH-neutralization. The fractions were conserved with 0.01 % thimerozal, 2 % skimmed milk powder was added and the antibodies were stored at +4°C.

A-Blockbuffer	1 x TBST, 0.5 % BSA
A-Elution buffer	0.1 M citric acid, 500 mM NaCl, 0.05 % (v/v) Tween-20, pH 2.5
A-Shiftbuffer	50 % A-Block buffer, 50 % Tris/HCl pH 8.8
Coat buffer	15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.5
TBST buffer	137 mM NaCl, 50 mM Tris/HCl, 2.7 mM KCl, 0.05 % (v/v) Tween-20 pH 8.0

2.4. Cell culture

Native and transfected cells of the human embryonal kidney (HEK 293) and the porcine renal epithelial (LLC-PK_1) cell lines were maintained in Normal Medium (NM). Cells were grown at +37°C on Petri dishes or 200 ml flasks in the presence of 5% (v/v) CO_2, and the medium was replaced every 2 to 3 days.

2.4.1. Passage

For passage, the HEK 293 cells were detached mechanically whereas the LLC-PK_1 cells were detached by incubation for 20 min at +37°C in the Detaching buffer. The aspirated cells were pelleted by 5 min centrifugation at 250×g and resuspended in NM. 10 % of the cells were transferred into new flask, containing NM media.

NM	Dulbecco's Modified Eagle's Medium (DMEM, Sigma), 10 % (v/v) fetal bovine serum, 4 mM L-glutamine, 0.1 mg/ml streptomycin sulfate and 100 U/ml penicillin G
Detaching buffer	Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffered saline (DPBS, Sigma), 28 mM $NaHCO_3$, 0.5 mM EDTA, 10 mM HEPES pH 7.4

2.4.2. Cryoculture

Detached and sedimented cells were resuspended in Cryomedium at concentration of 10^6 cells per ml and immediately placed at -20°C. On the next day the cells were transferred at -70°C, then stored in liquid nitrogen.

To bring the frozen cells again into culture, we thawed them in waterbath at +37°C, then resuspended in 5 volumes NM and immediately sedimented for 10 min at 250×g to remove DMSO. The cell pellet was again resuspended in NM and transferred into a flask. After 16 h the culture medium was replaced and the cells were grown as usual.

Cryomedium DMEM, 20% FCS, 19% (v/v) dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Taufkirchen, Germany)

2.4.3. Transient transfection of mammalian cells

Although LLC-PK_1 and HEK 293 cell lines contain small amount of native RS1 protein, for number of our experiments, higher amounts of protein were needed. Therefore we have used over-expressed pRS1 protein, where the plasmid encoding pRS1 or GFP-pRS1 fusion protein was transiently transfected in the cells.

For the transient transfection of pRS1 and GFP-fusion proteins in HEK 293 and LLC-PK_1 cells, the FuGENE6 transfecting reagent (Boehringer-Mannheim Biochemica, Mannheim, Germany) was used. Usually 1 x 10^5 cells were transfected with 1 µg DNA using 2.4 µl transfected reagent per cover slip following the instruction of the manufacturer. After cultivating the cells for 24-48 hours until 50-70% confluence, cells were fixed for microscopic observation or harvested. In some experiments preincubation with 4,5,6,7-tetrabromobenzotriazole (TBB, Biaffin GmbH & Co KG, Kassel, Germany), 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), tamoxyfen, calphostine C or s*n*-1,2-dioctanoyl-glycerol (DOG) (Calbiochem, Darmstadt, Germany) before fixation or harvesting was performed.

To compare nuclear localization in subconfluent and confluent cells, LLC-PK_1 cells were seeded onto polyester membrane at a density of 10^4 x cm^2. One day later subconfluent cells were transfected as described above. Other cells were grown for 3-4 days until confluence and then transfected. 24 h after transfection the cells were fixed.

2.5. Fluorescent microscopy of pRS1-GFP fusion proteins

LLC-PK_1 cells were transiently transfected with plasmids encoding GFP, GFP-pRS1 fusion protein or β-Gal-rs fragments-GFP fusion protein. For 48 h the cells were grown on glass slides or polyester membrane (Costar Korning) till about 50-70% confluence. They were washed 3 times with ORI buffer and fixed in 4% paraformaldehyde in ORI buffer (20 min at RT). After three washing steps for 5 min cells were embedded in Fluorescent-Mounting Medium DAKO (Diagnostika GmbH, Hamburg, Germany).

For detection of GFP-fluorescence, fluorescent microscopy was performed using the Axioplot 2 microscope (Carl Zeiss) and laser scanning microscopy (LSM) using the confocal microscope LSM 510 (Carl Zeiss). A standard filter set was used for fluorescent microscopy of GFP. Laser excitation was performed at 488 nm and emission was registered at 505 nm using a long pass filter. On the light microscope strong, weak or no fluorescence at nuclei and cytoplasm were observed. LSM indicated that cells with strong fluorescence at the nuclei in the light microscopy contained GFP in the nuclei, whereas weak fluorescence at the nuclei in the light microscopy was due to GFP in the cytosol above or below the nuclei. Additionally nuclei were counterstained with 4,6-diamindino-2-phenylindole (DAPI, Molecular Probes, Eugene, USA).

Cells were scored according to the cellular localization of GFP-pRS1 fusions as predominantly nuclear (with strong GFP fluorescence within nuclei) or cytoplasmic (weak or no nuclear GFP fluorescence). For each determination, 80-160 cells were scored from 3 to 6 independent transfections.

ORI buffer 5 mM MOPS pH 7.4, 100 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$

RESULTS

1. Identification of Nuclear Localization Signal of pRS1 protein

1.1. GFP-pRS1 fusion protein

Green fluorescent protein (GFP) is a small protein of ca. 27 kDa that can passively diffuse through the nuclear pore complex (NPC) into the nucleus (Figure 2). GFP-fusion protein with molecular weight >50 kDa are too big for passive translocation. The GFP-pRS1 fusion protein could be used to study nuclear localization of pRS1. Only fusion proteins >50 kDa containing nuclear localization signal (NLS) can be transported through the NPC. In the fluorescent experiments GFP alone was uniformly distributed throughout the cytosol and the nucleus, whereas GFP-pRS1 fusion protein was enriched within the nucleus except for the nucleoli (Figure 3). This strongly supports the assumption that pRS1 has some region, which is responsible for nuclear migration.

Because GFP-pRS1 fusion protein was found within the nucleus, although it does not contain any known nuclear localization sequence, we expected that pRS1 contains a new nonconventional nuclear localization signal.

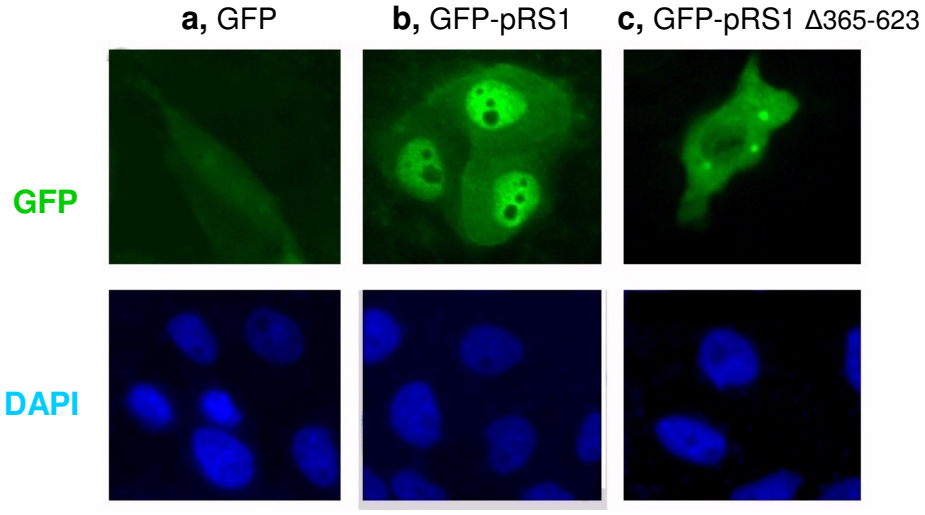


Figure 3. Localization of GFP-fluorescence. 48 h following transfection of LLC-PK$_1$ cells, the fluorescence of GFP was observed by light microscopy. GFP, green fluorescence of GFP indicates localization of GFP fusion proteins; DAPI, nuclei were visualized by immunofluorescence. **a**, cells were transfected by GFP vector . **b**, cells were transfected by GFP-pRS1 vector, fixed and observed as usual. GFP-pRS1 fusion protein showed predominantly nuclear localization of GFP-fluorescence. **c**, cells were transfected by GFP-pRS1 Δ365-623, fixed and observed as usual. GFP-pRS1 Δ365-623 construct could not migrate to the nucleus.

1.2. GFP-pRS1 constructs with N- and C-terminal truncations

To identify the domain of pRS1 that is required for nuclear targeting we analyzed fusion proteins between the C-terminus of GFP and N-termini of truncated pRS1-proteins for nuclear localization.

In Figure 4 some truncated constructs are presented. The structure, name of constructs and the percentage of cells having nuclear localization of GFP with respect to all number of GFP-containing cells (nuclear fractions) are shown. Thus, GFP-pRS1 wild type (wt) demonstrated 85.8 ± 2.4 % of nuclear localization of GFP fluorescence. Nuclear fraction was significantly ($P < 0.5$) reduced to 73.0 ± 2.7 %, when 281 N-terminal amino acids (Δ1-281) were removed (Figure 4, a). But in the construct lacking about 260 C-terminal amino acids (Δ365-623), nuclear localization was completely abolished (2.4 ± 1.4 %). Thus we suggest that the C-terminal part of pRS1 protein contains the nuclear localization region.

1.3. Function of the UBA domain for nuclear migration of pRS1 protein

The 46 C-terminal amino acids of the RS1 protein (583-623 a. a.) are highly conserved among all four species and contain a consensus sequence for an ubiquitin-associated domain (UBA). The UBA domain is able to bind mono-ubiquitin and multi-ubiquitin (Wilkinson et al., 2001). Ubiquitin-associated (UBA) domains are found in a large number of proteins with diverse functions involved in ubiquitination, DNA repair, and signalling pathways. Like the UBA domains of HHR23A, RAD23 and other proteins (Dieckmann et al., 1998, Watkins et al., 1993), the UBA domain of pRS1 reveals the typical three-helical fold and two hydrophobic surface patches called epitope 1 and 2 (Thomas Müller, University Würzburg, unpublished). Because the point mutations in the epitope 1 of some proteins have been shown to abolish the protein binding (Withers-Ward et al., 2000, Layfield et al., 2004), we decides to introduce into pRS1 the mutations that should inactivate the epitope 1.

The substitution of glycine 590 by proline (G590P) on epitope 1 of RS1 UBA decreased the nuclear migration to 56.3 ± 6.8 % in comparison with the GFP-pRS1 protein fusion (Figure 5). The further mutations on the epitope 1, F591L and L614A/L615A, also showed reduced the nuclear localization (72.7 ± 3.0 % for F591L and 64.9 ± 1.8 % for L614A/L615A). But none of the UBA domain mutants was able to abolish the nuclear migration.

Then we prepared two truncated constructs, one without the UBA domain (Δ583-623)

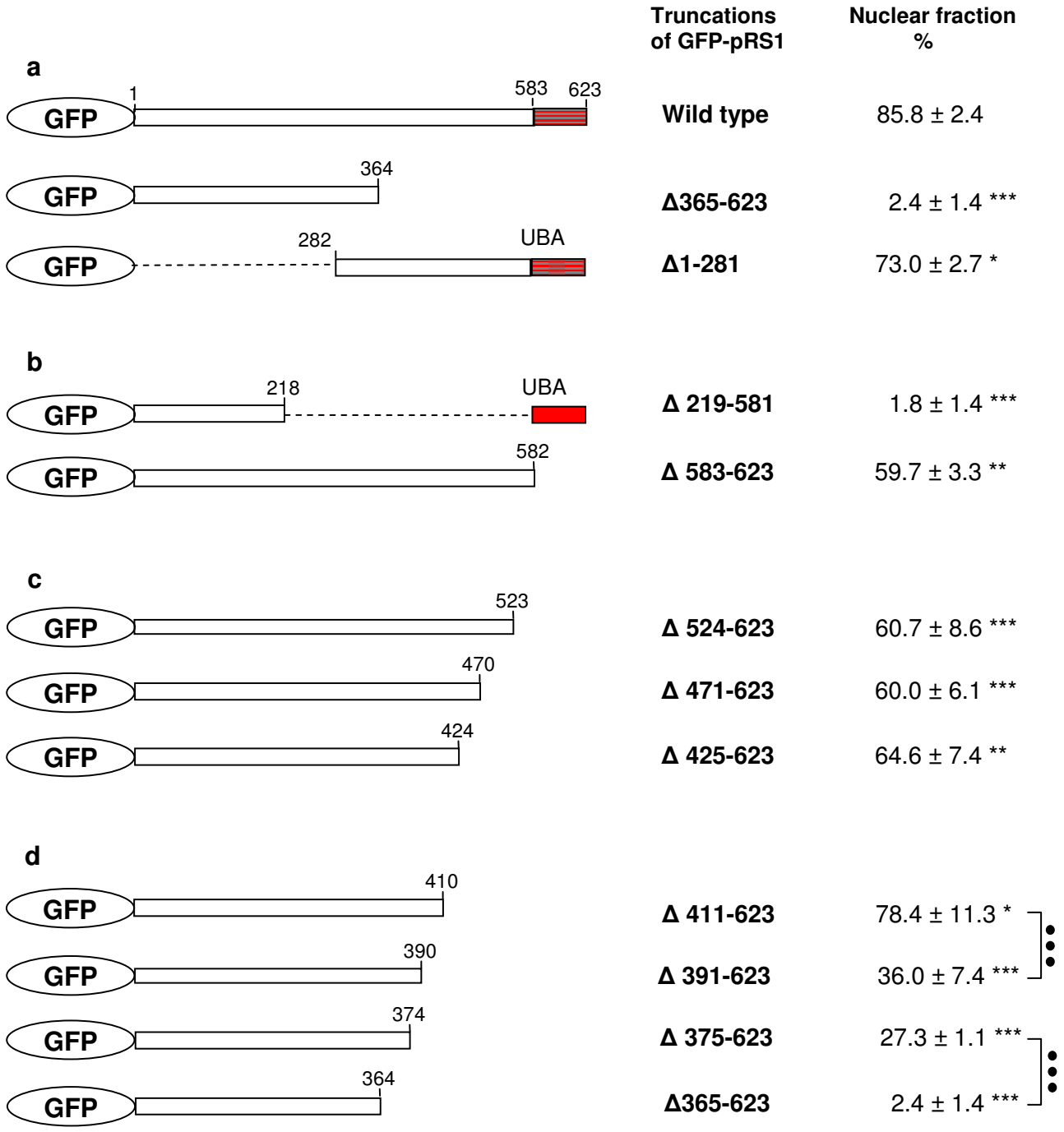


Figure 4. Identification of pRS1 region that is involved in nuclear targeting. The LLC-PK$_1$ cells were transiently transfected with pEGFP-RS1-vectors, expressing GFP-pRS1 or the indicated GFP-pRS1 truncations. Cells were grown on the glass slides and GFP-fluorescence was evaluated for nuclear localization. Mean values ± SD of 3 to 6 independent experiments are indicated. Significant differences in comparison with intact GFP-pRS1 (* $P < 0.05$; ** P 0.01; *** $P < 0.001$) and between selected construct (••• $P < 0.001$) are indicated. **a**, wild type, C-terminal truncation (Δ365-623) and N-terminal truncation (Δ 1-281). **b**, the role of UBA domain in nuclear migration: Δ 219-583 without the middle part but with the UBA domain, Δ583-623 lacks the UBA domain. **c**, Δ524-623, Δ471-623 and Δ425-623 decrease nuclear localization but do not block it. **d**, successive truncations of the C-terminal part of pRS1 with consecutive reduction of nuclear localization (Δ411-623, Δ391-623, Δ375-623 and Δ365-623).

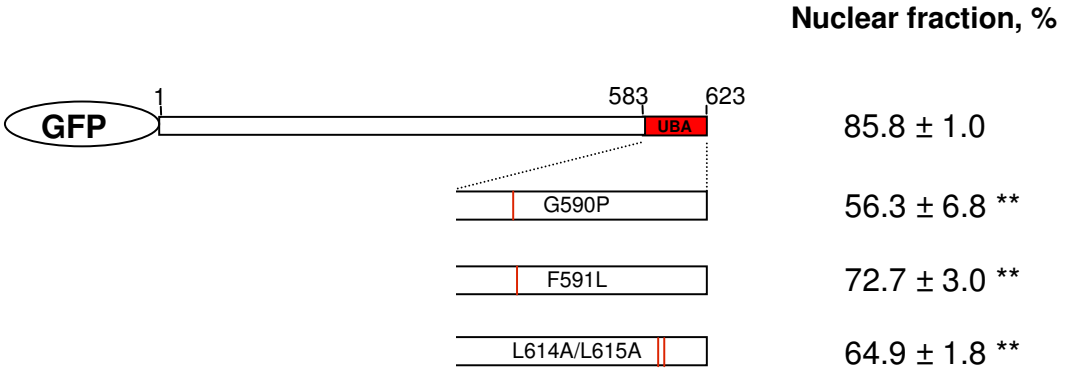


Figure 5. The effect of point mutations on the 1 epitope of the UBA domain in GFP-pRS1 construct on nuclear localization. The LLC-PK$_1$ cells were transiently transfected with GFP-pRS1 vectors. Mean values ± SD of 3 to 6 independent experiments are indicated. Significant difference in comparison to GFP-pRS1 construct is indicated: ** $P < 0.01$.

and another one containing the UBA domain but lacking the middle part of pRS1 protein (Δ219-581) (Figure 4, b). The truncation of the middle part leaving the UBA domain intact (Δ219-581) showed nearly no nuclear localization (1.8 ± 1.4 %, $P < 0.001$), whereas the nuclear fraction of GFP-pRS1 lacking only the UBA domain (Δ583-623) was reduced to the level of that for single mutations in the epitope 1 (59.7 ± 3.3 %).

Since there was no nuclear localization (1.8 ± 1.4 %) of GFP-pRS1 Δ219-581 construct (Figure 4, b), where the middle part was removed leaving the UBA domain intact, we may conclude that RS1 UBA is not involved in nuclear targeting but may support the nuclear localization of pRS1 by binding of RS1 UBA with (poly)ubiquitin in the nucleus.

1.4. Successive truncations from C-terminus of GFP-pRS1 fusion constructs

For identification of NLS on pRS1 several additional truncation variants of pRS1 protein were constructed. Successive truncations of C-terminus (Δ524-623, Δ471-623 and Δ425-623) showed some reduction of nuclear localization in comparison with GFP-pRS1 wt (Figure 4, c), but could not completely block it. But the further truncations showed continuous decreasing of the nuclear localization (Figure 4, d): 78.4 ± 11.3 %, 36.0 ± 7.4 % and 27.3 ± 1.1 % for Δ411-623, Δ391-623 and Δ375-623 constructs, respectively. Noteworthy, the nuclei were almost free of GFP-pRS1, if the C-terminus was truncated by 11 further amino acids (2.4 ± 1.4 % for Δ365-623, Figure 4, d). This suggests that a motif between 365 and 374 amino acids is essential for nuclear targeting.

Although data show the importance of 365-374 amino acids, only nuclear accumulation

of Δ411-623 construct was comparable to wild type, consequently amino acids in front of the amino acid 425 also play a role in nuclear localization.

1.5. β-Galactosidase-rs fragments-GFP fusion constructs

Since GFP-pRS1 Δ1-281 truncation was still able to migrate into the nucleus we conclude that NLS of pRS1 protein is located between 281 and 425 amino acids. To analyze this region we needed another plasmid system, because GFP-pRS1 truncations became too short resulting in a fusion protein being able to enter the nucleus passively through the nuclear pore complex. So, we cloned different small fragments of pRS1 (281-425) between β-Galactosidase (β-Gal) and GFP, the plasmids were transiently transfected into the LLC-PK_1 cells and cellular distribution of GFP fluorescence was analyzed as described above (Figure 6). We used β-Gal-GFP fusion as a negative control, and the nuclear localization sequence of nucleoplasmin (KRPAATKKAGQAKKKK), inserted between β-Gal and GFP (β-Gal-NP-NLS-GFP), as positive control (Hübner et al., 2002).

Using this approach we found that β-Gal-[363-425]-GFP construct (with the C-terminal part of the 281-425 region) did not localized in the nuclei (2.1 ± 2.4 %, Figure 7), whereas the construct containing the N-terminal part of the 281-425 region (β-Gal-[281-368]-GFP construct) showed 71.1 ± 6.8 % of nuclear localization. β-Gal-[309-391]-GFP construct, containing middle part of this region, was also able to transfer fusion protein into the nucleus

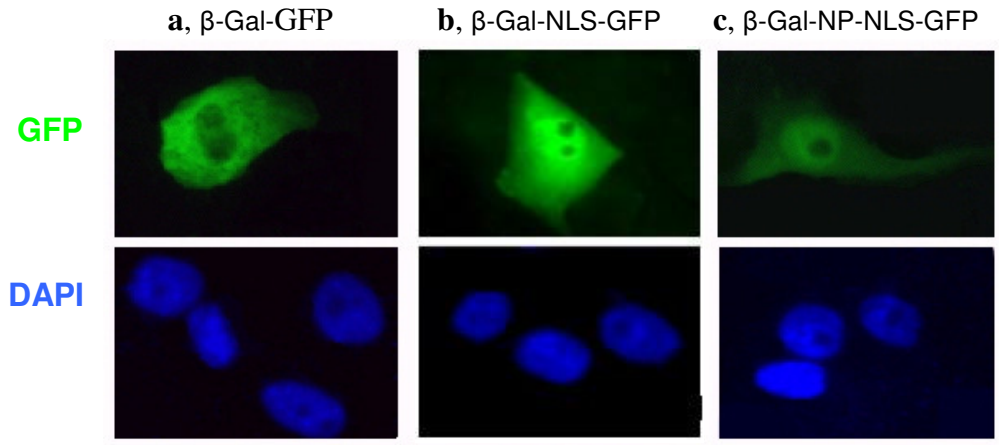
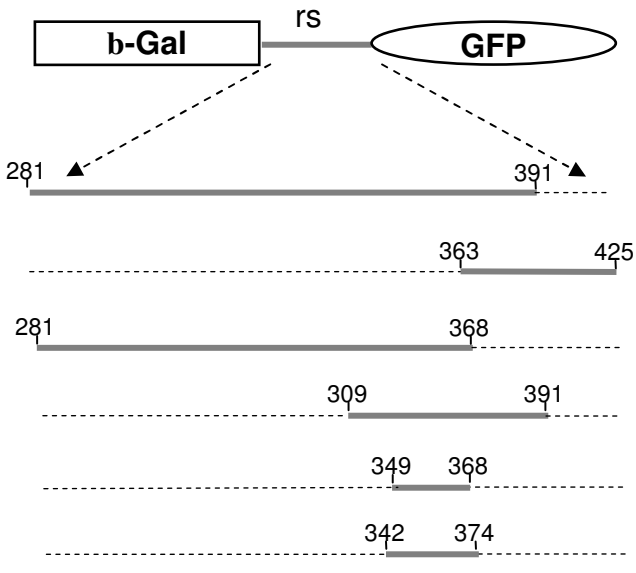


Figure 6. Localization of β-Gal-GFP constructs. 48 h following transient transfection of LLC-PK_1 cells, the fluorescence of GFP was observed by light microscopy. *GFP*, green fluorescence of GFP indicates localization of β-Gal-GFP fusion proteins; *DAPI*, nuclei were visualized by immunofluorescence. **a**, negative control, cells were transfected by β-Gal-GFP alone. **b**, β-Gal-NLS-GFP construct containing 349-368 a. a. of pRS1 migrated to the nucleus. **c**, positive control, β-Gal-NP-NLS-GFP construct showed nuclear localization of GFP-fluorescence.



Fragment of pRS1, a. a.	Nuclear fraction %
281-391	88.0 ± 5.4
363-425	2.1 ± 2.4 ***
281-368	71.1 ± 6.8
309-391	67.2 ± 9.0
349-368	53.7 ± 2.5 **
342-374	78.0 ± 6.4

Figure 7. Final localization of NLS on the pRS1 sequence by using the β-Galactosidase-rs fragments-GFP fusion constructs. Significant difference in comparison with intact GFP-pRS1 construct is indicated: ** $P < 0.01$; *** $P < 0.001$.

(67.2 ± 9.0 %). Since the other middle part [342-374], shorten from both sites, could target the fusion construct to the nucleus (78.0 ± 6.4 %, Figure 7) as well, we conclude that the nuclear localization signal is located within this region.

1.6. Presumed secondary structure of RS1 NLS

The nuclear localization sequence of RS1 protein (RS1 NLS) is highly conserved through the species showing the one amino acid exchange in rabbit and the two exchanges in human. Using the programs compiled in NPS@Network Protein Sequence Analysis, we analyzed the predicted structure of central part of pRS1 (309-391 amino acids). The used programs proposed an α-helix in the region between 349 and 365 amino acids residues (Figure 8, b), where the RS1 NLS is localized. Moreover, the α-helix of RS1 NLS is amphiphilic and contains five charged residues on one side and a patch of hydrophobic amino acids on the other side.

A search for proteins containing amino acids stretches with homologous to RS1 NLS revealed proteins that have been shown to exhibit functional activities within the nucleus (Figure 8, a): DNA repair protein RAD5 from *Saccharomyces cerevisiae* (Johnson et al, 1992 and 1994), DNA-dependent RNA polymerase III of *Giardia lamblia* (Lanzerdörfer et al., 1992), deoxyribonucleoside kinase *Dm*-dNK of *Drosophila melanogaster* (Zheng et al., 2000, Munch-Petersen et al, 2000), human oxysterol binding protein related ORP7 (Lehto et al.,

2001, Johansson et al, 2003), human ubiquitin specific protease USP36 (Quesada et al., 2004) and ubiquitin activating enzyme E1 from *Plasmodium falciparum* (Finley and Chau, 1991). Remarkably, the seven residues on the hydrophobic side are conserved between the RS1 protein and the homologous domains of these six proteins.

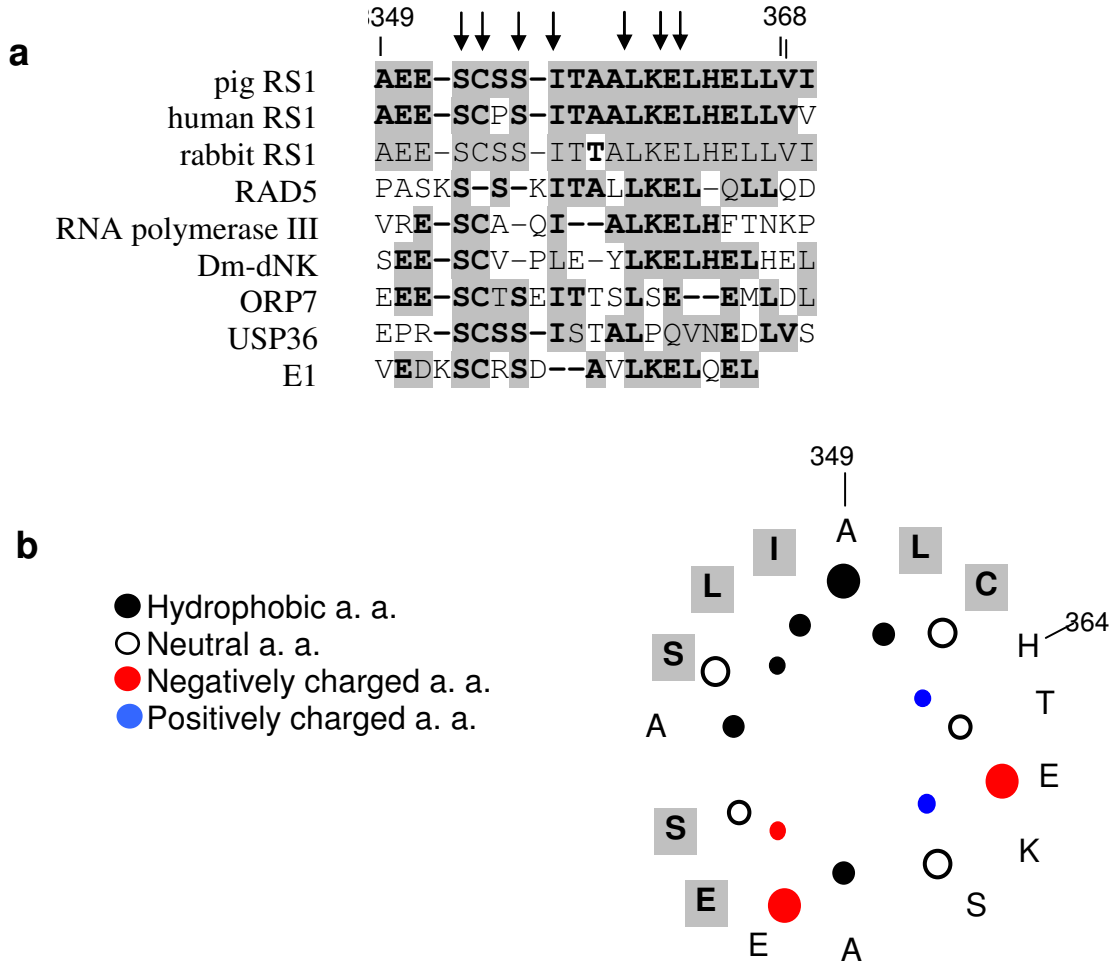


Figure 8. Alignment of pRS1-NLS with homologous domains of other proteins and α-helical wheel presentation of RS1 NLS. Identical and similar amino acids are shadowed, identical amino acids are showed in bold face. **a**, alignment of NLS (a. a. 349-368) including some adjacent amino acids on each side to humanRS1, rabbit RS1, and to homologous domains of other proteins (see in text). **b**, α-helical wheel presentation of RS1 NLS. α-helix is shown from up to down, charged and hydrophobic amino acids are indicated.

We tested whether the amino acids of human RS1 (345-370) corresponding to the identified NLS of the porcine RS1 (NLS-R, detailed description of different pRS1-NLS variants see in the Figure 11) mediate nuclear migration of β-Gal-GFP fusion protein in subconfluent LLC-PK$_1$ cells. Indeed, when amino acids 345-370 of hRS1 were inserted between β-Gal and GFP, 88.2 ± 13.6 % of transfected cells showed GFP fluorescence in the nucleus. This value was similar to that of the corresponding fragment of pRS1 (Figure 9, a).

The part of RAD5 protein, corresponding to R-NLS of pRS1 protein that was inserted between β-Gal and GFP, was not sufficient for nuclear migration (Figure 9, a). Perhaps, the region in RAD5 corresponding to α-helix of pRS1 is involved in other processes in the nucleus, while the identified classical NLS is responsible for the nuclear migration.

1.7. Deletion of RS1 NLS from the GFP-pRS1 fusion

To prove that the region between 349 and 368 amino acids is important for the nuclear migration of the pRS1 protein, the construct GFP-pRS1 lacking 349-374 amino acids was made. Indeed, without this region the construct Δ349-374 GFP-pRS1 was not able to migrate into the nucleus (1.8 ± 0.2 %, Figure 9, b). Data suggest that pRS1 protein contains only one nuclear localization signal between 348 and 374 amino acids.

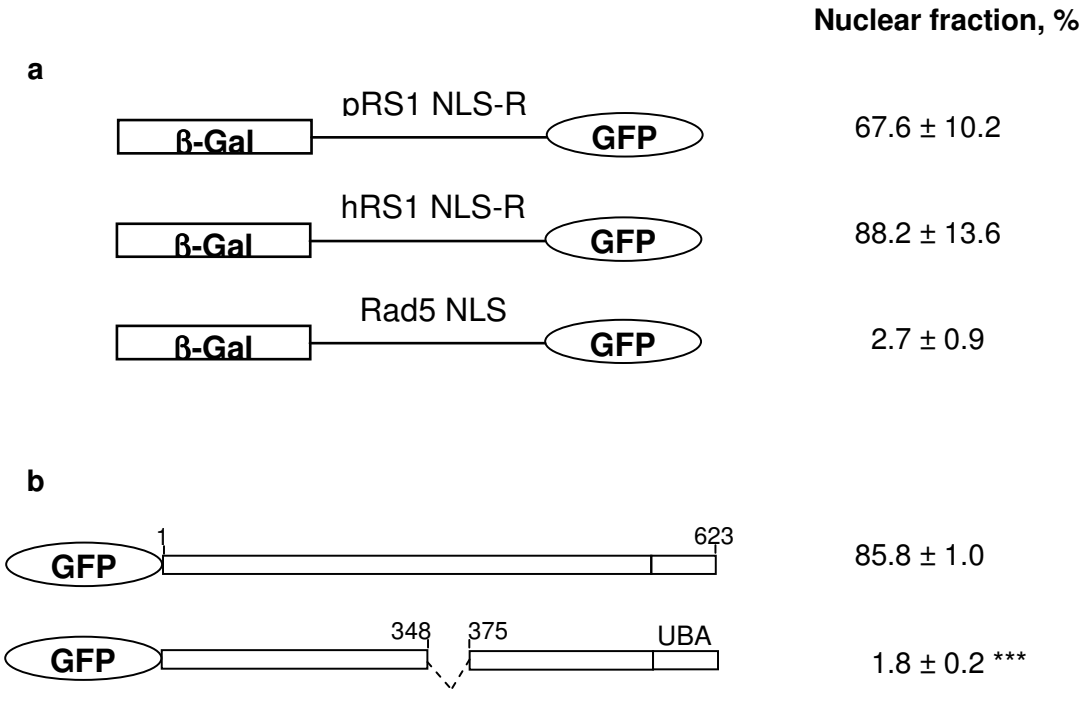


Figure 9. Effect of pRS1-NLS and NLS-homologous region of hRS1 and Rad5 on nuclear migration. **a**, homologous NLS domains of pRS1 (349-374 a. a), hRS1 (345-370 a. a.) and RAD5 protein in β-Gal-GFP construct transiently transfected in LLC-PK_1 cells; **b**, total GFP-pRS1 and construct Δ349-374 GFP-pRS1 lacking the NLS-R fragment. Significant difference in comparison to GFP-pRS1 construct is indicated (*** $P < 0.001$).

2. Regulation of RS1 NLS by phosphorylation

2.1. The influence of serines 348, 370 or 400 on the nuclear migration of β-Gal-rs fragments-GFP fusion proteins

RS1 protein contains several putative sites for phosphorylation, which were predicted around RS1 NLS. They are conserved among all four species. There are one site for casein kinase 2 (CK2) serine 348 (S348), and two sites for protein kinase C (PKC): S370 and S400 (Figure 10). It is known that nuclear migration of proteins can be regulated by phosphorylation or dephosphorylation of amino acids near NLS. To investigate the impact of serines flanking the RS1 NLS several experiments were performed.

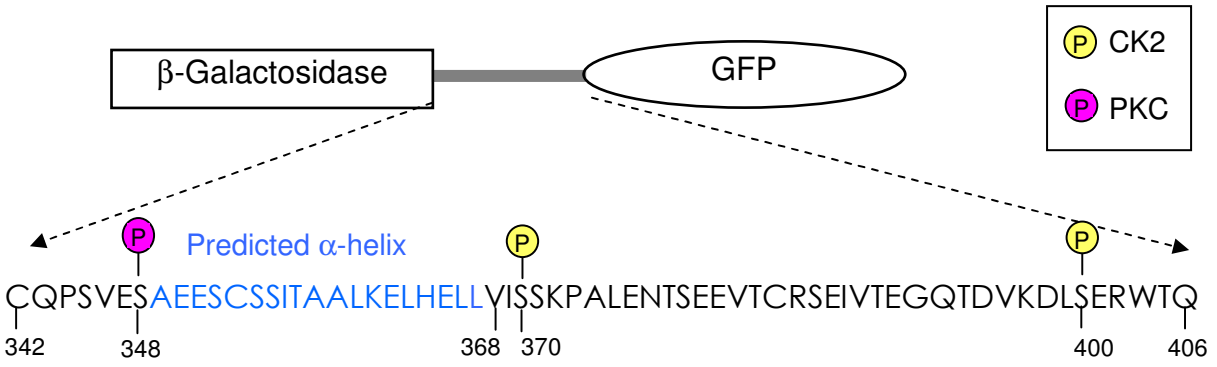
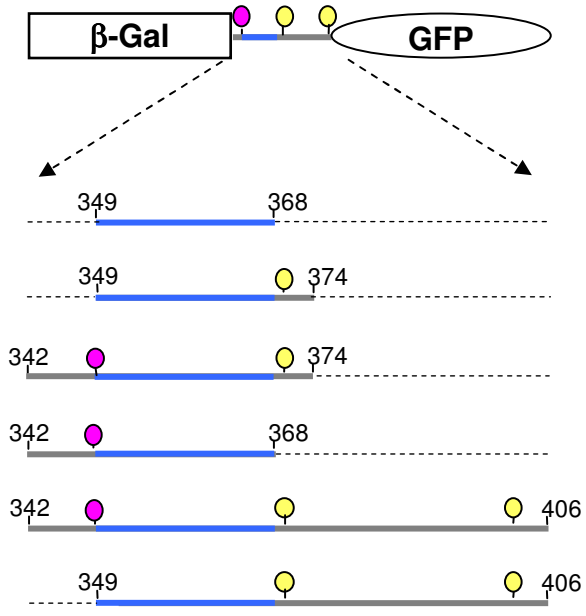


Figure 10. Sequences of RS1 (R-NLS-Reg) harbouring NLS with putative phosphorylation sites for CK2 and PKC.

We have investigated the β-Gal-rs fragments-GFP fusion constructs, containing pRS1 fragments of different lengths, with or without the serines 348, 370 and 400 around RS1 NLS. We observed that the phosphorylation sites had strong effect on the nuclear migration of the constructs (Figure 11). The presence of the serine 348 in construct R-NLS (342-368 a. a.) or 370 in construct NLS-R (349-374) did not change the nuclear fraction of the fusion constructs (44.1 ± 14.6 % and 67.6 ± 10.2 %, respectively, in comparison with 53.7 ± 2.5 % for NLS construct). The presence of both serines 348 and 370 together in R-NLS-R construct (342-374 a. a.) significantly increased the nuclear fraction to 78.0 ± 6.4 % ($P<0.5$). But the nuclear fraction was decreased in comparison with R-NLS-Reg to 61.8 ± 8.7 %, when further 30 amino acids were added (R-NLS-Reg construct, 342-406 a. a.). The construct NLS-Reg (349-406 a. a.), containing both sites for PKC but not S348 for CK2 phosphorylation, showed no

further change in the nuclear migration (59.0 ± 23.3 %). We can conclude that at least two phosphorylation sites around RS1 NLS, serine 348 for CK2 and serine 370 for PKC, are involved in the regulation of the nuclear migration.



Name of constructs	Fragments of pRS1, aa	Nuclear fraction, %
NLS	**349-368**	53.7 ± 2.5
NLS-R	**349-374**	67.6 ± 10.2
R-NLS-R	**342-374**	78.0 ± 6.4 *
R-NLS	**342-368**	44.1 ± 14.6
R-NLS-Reg	**342-406**	61.8 ± 8.7
NLS-Reg	**349-406**	59.0 ± 23.3

Figure 11. Presence and absence of sites for phosphorylation around NLS change nuclear migration. The LLC-PK_1 cells were transiently transfected with β-Gal-rs fragments-GFP vectors. Significant difference in comparison with RS1 NLS (349-368 a. a.) is indicated (* $P < 0.5$).

2.2. Substitution of serines 348, 370 or 400 by alanine or glutamate

Phosphorylation occurs on hydroxyl group of serine, resulting in the change of size and charge of residue. In turn it could alter the tertial structure of the protein around phosphorylation site, so that the NLS domain of RS1 could be opened for interactions or hidden inside the protein.

By the method of site-directed mutagenesis, single serine residues were substituted by alanine or glutamate. The glutamate residue has similar size and charge as phosphorylated serine and the alanine residue mimics non-phosphorylated serine. Thus we imitated the conditions, where conformational changes could be invented due to phosphorylation reaction (glutamate) or could not occur at all (alanine).

Both total pRS1 fused to GFP and β-Gal-GFP construct, containing short fragments of pRS1 with RS1 NLS and amino acids around it, carried the point mutations of required serines.

Interestingly, some substitutions changed the migration of the proteins into the nucleus (Figure 12, 13). Thus, both mutations of S348, to alanine or glutamate, strongly inhibited the nuclear migration of GFP-pRS1 fusion (Figure 13, 34.6 ± 5.6 % for ASS; 22.7 ± 5.4 % for ESS) as well as of R-NLS-Reg construct (Figure 12, a, 19.7 ± 1.8 % for ASS; 10.1 ± 0.8 % for ESS). Besides, the nuclear fraction of the alanine mutants in both constructs was significantly higher ($P < 0.5$) than of the mutants containing glutamine.

In all constructs the substitution of serine in position 370 by alanine showed higher nuclear fraction than glutamate substitution (Figures 12, 13). For the GFP-pRS1 fusion protein the nuclear fraction of the S370-alanine substitution was significantly higher then glutamate substitution S370E (SAS: 52.9 ± 4.4 % compared to SES: 20.4 ± 3.4), although it did not reach the level of GFP-pRS1 (85.8 ± 2.4 %, Figure 13).

In the R-NLS-Reg construct (Figure 12, a) the difference between S370A (SAS) and S370E (SES) substitutions was large (73.4 ± 2.4 % and 7.6 ± 2.6 %, respectively, $P < 0.001$). Interestingly, the nuclear fraction of S370A was significantly higher than of wt NLS-Reg (73.4 ± 2.4 % for SAS compared to 63.6 ± 3.6 % for SSS, $P < 0.5$).

The construct NLS-R (349-374 a. a.), containing only one phosphorylation site S370, showed similar nuclear location (Figure 12, b). The mutant S370A, mimicking non-phosphorylated serine, had significantly higher nuclear fraction (A: 90.1 ± 3.2 %) than wt (S: 67.6 ± 3.5 %, $P < 0.5$) or phosphorylated S370E (E: 58.3 ± 4.6 %, $P < 0.01$).

For other construct NLS-Reg (349-406 a. a.), lacking S348, effects were the same: S370A (91.4 ± 9.5 %) showed higher nuclear localization than wt (59.0 ± 9.5 %), and S370E substitution significantly inhibited nuclear migration to 39.2 ± 6.4 % (Figure 12, b).

When double mutations of serine residues 370 and 400 were done in the NLS-Reg construct (349-406 a. a.), the effects on the nuclear migration were similar. The S370A substitution increased the nuclear fraction (AA: 82.5 ± 13.2 % and AE: 97.8 ± 1.1 % in comparison with SS: 74.2 ± 11.4%). The substitution of serine by the glutamate in 370 position blocked or significantly decreased the nuclear fraction (EA: 0 % and EE: 29.3 ± 5.9 %). Interestingly, the mutation of serine 400 to glutamate supported the nuclear migration. Thus it increased the nuclear fraction from 82.5 ± 13.2 % to 97.8 ± 1.1 % (AA and AE, respectively), when added to the S370E substitution, it recovered the nuclear fraction from 0 % to 29.3 ± 5.9 % (EA and EE, respectively).

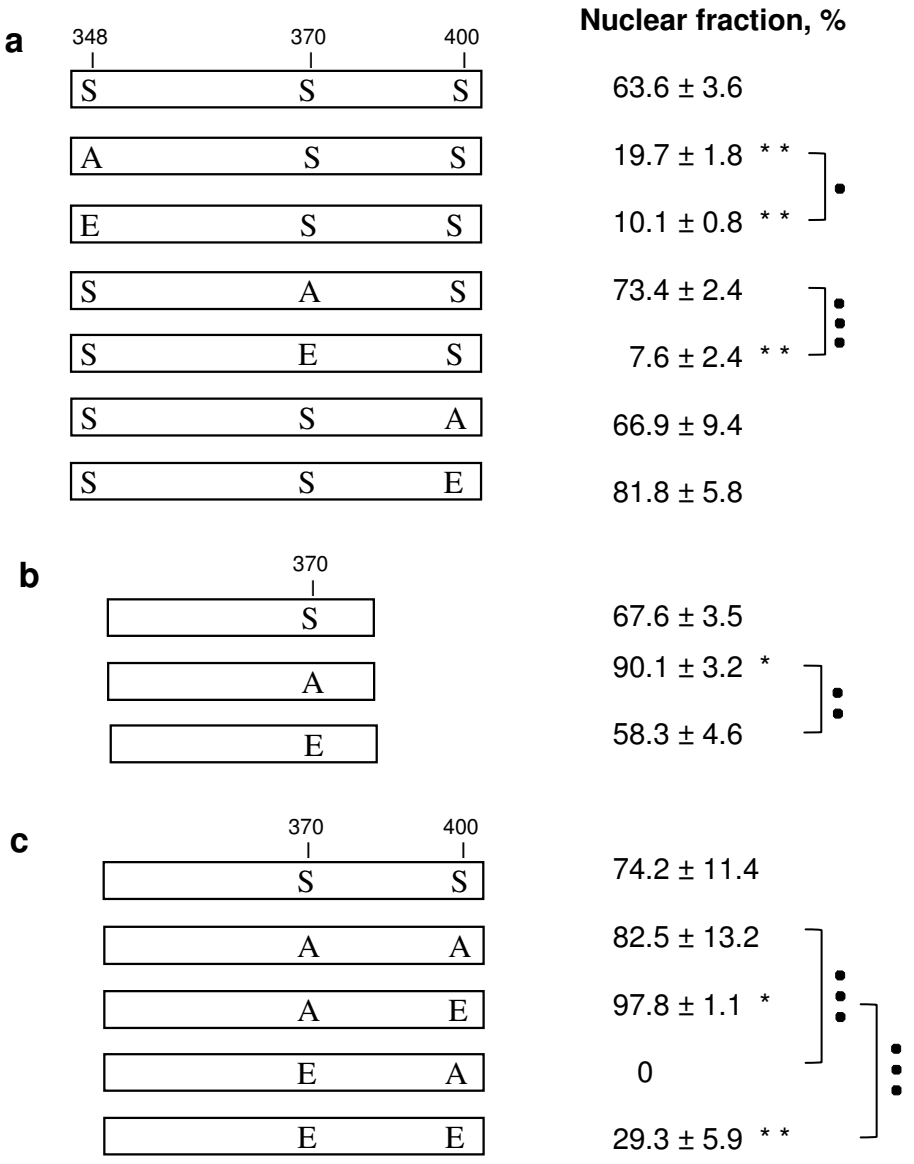


Figure 12. The effect of substitutions of serine 348, 370 or 400 to alanine (A) or glutamate (E) on the nuclear migration of β-Gal-rs-fragments-GFP constructs. Significant differences in comparison with control (* P < 0.5; ** P <0.01) and between individuals mutants (•• P < 0.01; ••• P < 0.001) are indicated. **a**, single substitutions of S348, S370 or S400 in R-NLS-Reg (342-406 a. a.) construct; **b**, single substitutions of S370 in NLS-R (349-374 a. a.) construct; **c**, double substitutions of S370 and S400 in NLS-Reg (349-406 a. a.) construct.

The same effect of the supported nuclear migration was observed in the R-NLS-Reg construct. The substitution by alanine S400A did not change the nuclear fraction (SSA: 66.9 ± 9.4 % compared to SSS: 63.6 ± 3.6 %), whereas the glutamate substitution S400E increased the nuclear fraction to 81.8 ± 5.8 % (SSE, in comparison with SSS: 63.6 ± 3.6 %, P < 0.1).

Our data suggest that in LLC-PK$_1$ cells serine 370 is partly phosphorylated and that

phosphorylation partially prevents nuclear localization of the protein. Phosphorylation of S400 probably supports but is not sufficient for the nuclear migration. The serine 348 appears to be involved in the regulation of the nuclear migration.

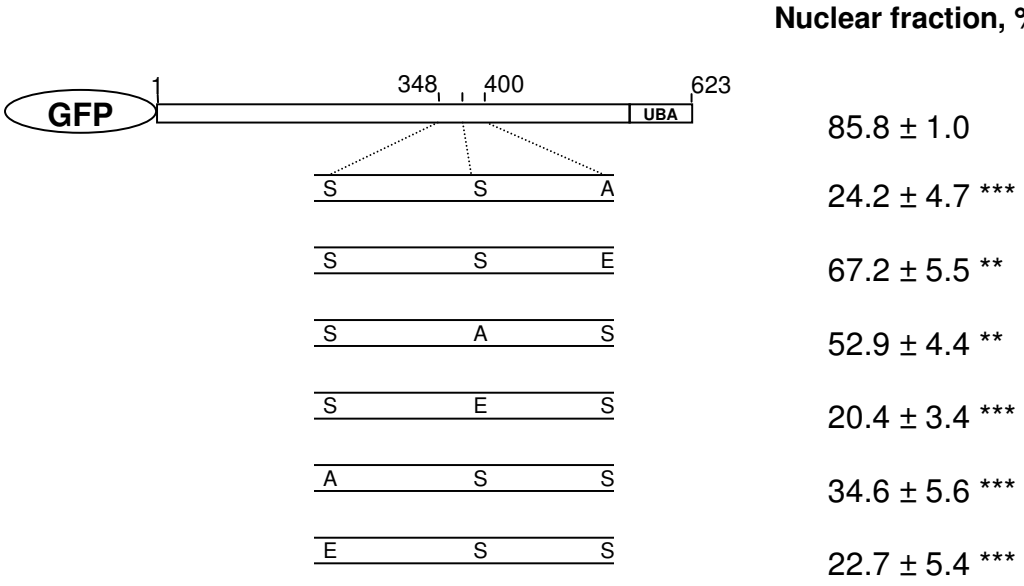


Figure 13. The effect of the point mutations of the phosphorylation sites around the NLS in GFP-pRS1 construct on nuclear localization. Significant difference in comparison to GFP-pRS1 construct is indicated: ** $P < 0.01$, *** $P < 0.001$.

2.3. Triple substitution of serines 348, 370 and 400

Reasoning that mutations of individual serine residues in R-NLS-Reg (β-Gal-[342-406]-GFP) construct may alter nuclear targeting indirectly by changing the accessibility of other serines for phosphorylation, we performed the additional experiments in which all three serine residues in the construct were exchanged by alanine or glutamate in all possible combinations (Figure 14).

When all three serines were substituted to alanines (AAA construct), 78.8 ± 9.3 % of transfected cells contained fluorescence in the nucleus. This indicates that non-phosphorylated R-NLS-Reg construct is able to mediate the nuclear migration. AAE mutant showed no significant effect on nuclear localization (77.5 ± 10.0 %) in comparison with AAA (78.8 ± 9.3 %). Interestingly, for the mutant EAA the nuclear location was reduced to 65.0 ± 8.3 % ($P < 0.01$ for difference with AAA, 78.8 ± 9.3 %). Moreover the reduction of the nuclear localization was decreasing to 15.0 ± 8.4 % for EAE construct with additional S400E

substitution. This suggests that phosphorylation of serine 348 mediates an inhibitory effect on the nuclear migration of R-NLS-Reg construct that is supported by phosphorylation of serine 400.

When serine 370 was substituted to glutamate, the nuclear migration was abolished independently whether other serines where mutated to alanine or glutamate (compare mutant AEA, 2.3 ± 1.2 % with AAA: 78.8 ± 9.3 %; or AEE: 2.7 ± 2.0 % with AAE: 77.5 ± 10.0 %; or EEA: 1.2 ± 1.5 % with EAA: 65.0 ± 8.3 %; or EEE: 2.0 ± 2.4 % with EAE: 15.0 ± 8.4 %). Mutation of serine 370 to glutamate (S370E) alone in R-NLS-Reg inhibited the nuclear localization as well (7.6 ± 2.4 %, Figure 12, a). Data strongly suggest that the phosphorylation of serine 370 prevents the nuclear localization.

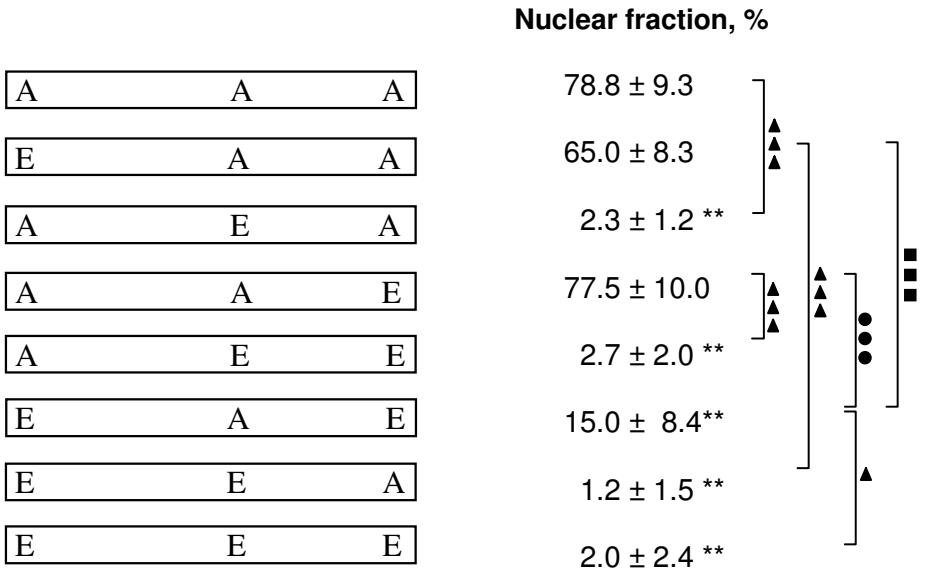


Figure 14. Triple substitutions of serines in β-Gal-GFP containing R-NLS-Reg (342-406 a. a.). The preconfluent LLC-PK_1 cells were transiently transfected with constructs. Serine residues for casein kinase 2 (S348 of pRS1) and for PKC (S370 and S400 in pRS1) were mutated together. Significant differences in comparison with AAA mutant (* P<0.5; ** P<0.01), between A348 and E348 (●●● P<0.001), between A370 and E370 (▲ P<0.05; ▲▲▲ P<0.001) and between A400 and E400 (■■■ P<0.001) are indicated.

2.4. Stimulation and inhibition of PKC and CK2

There are three predicted sites for phosphorylation around nuclear localization signal: S348 for casein kinase 2, S370 and S400 for protein kinase C. Using substitution of the serines by alanines or glutamates it was shown that phosphorylation / dephosphorylation of

the serines around RS1 NSL influenced the nuclear migration. For further investigation of the effects of phosphorylation / dephosphorylation, an activator of PKC (DOG), inhibitors of PKC (tamoxyfen, calphostin) and/or inhibitors of CK2 (DRB, TBB) were used and the nuclear location of GFP-RS1 and β-Gal-[342-406]-GFP constructs in subconfluent LLC-PK_1 cells was observed.

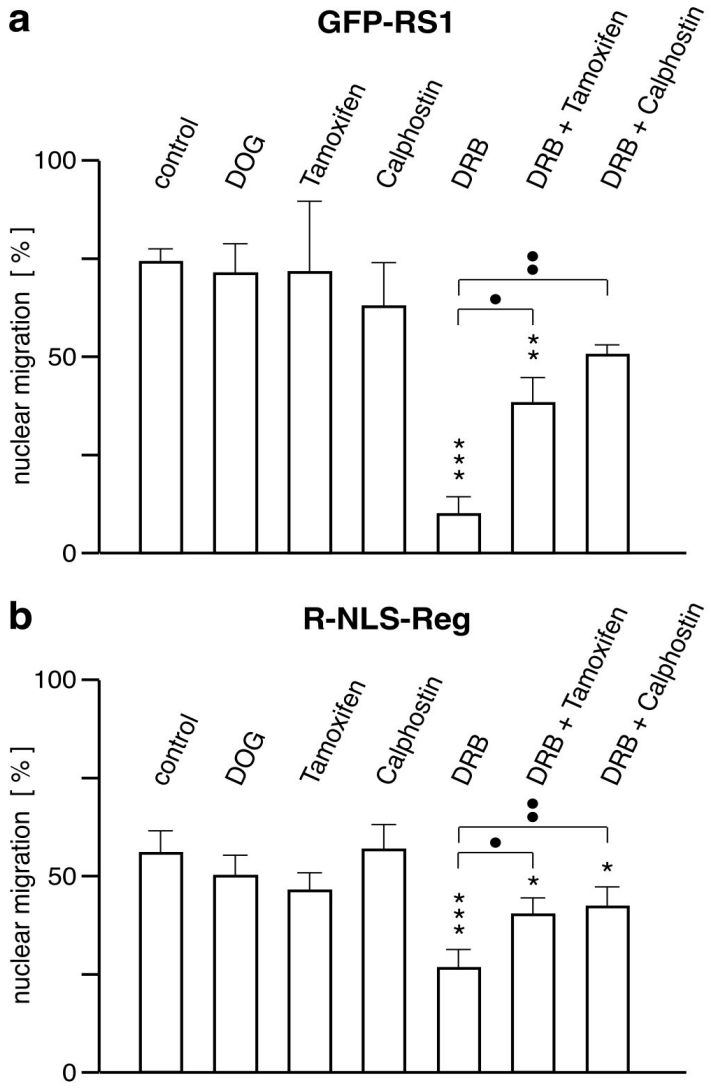


Figure 15. The effect of Inhibitors and activators of PKC and CK2 on the nuclear migration of GFP-pRS1 and R-NLS-Reg (β-Gal-[342-374 a. a. of pRS1]-GFP) constructs. Cell were incubated 24 h before fixation with PKC inhibitor 100 nM calphostin, CK2 inhibitor 100μM DRB, or 1 h before fixation with PKC activator 5μM DOG or PKC inhibitors 20μM tamoxyfen. Control cells were not incubated. Significant differences in comparison with control (* $P < 0.5$, ** $P < 0.01$, *** $P < 0.001$) or between transfected cells treated with DRB or DRB plus tamoxyfen/calphostin are indicated (• $P < 0.05$, •• $P < 0.01$). **a**, GFP-pRS1. **b**, R-NLS-Reg.

When subconfluent cells were incubated for 1 h with 5 μM PKC stimulator DOG, for

1 h with 20 µM PKC inhibitor tamoxyfen or for 24 h with 0.1 µM PKC inhibitor calphostin C, no significant effect on nuclear localization of GFP-pRS1 and β-Gal-[342-406]-GFP (R-NLS-Reg) were observed (Figure 15). This suggests that in subconfluent cells the serine 370 was not phosphorylated before or after activation of PKC. Otherwise, when the subconfluent cells were incubated with 100 µM CK2 inhibitor DRB, the nuclear location was drastically decreased from 71.2 ± 5.8 % to 9.9 ± 3.6 % for GFP-pRS1 and from 62.9 ± 5.5 % to 26.4 ± 4.0 % for R-NLS-Reg. But this inhibitory effect of DRB was partly reduced by addition of PKC inhibitor tamoxyfen (with GFP-pRS1 from 9.9 ± 3.6 % to 38.1 ± 5.8 %, with R-NLS-Reg from 26.4 ± 4.0 % to 39.4 ± 3.7 %) or calphostin (with GFP-pRS1 from 9.9 ± 3.6 % to 50.4 ± 2.3 %, with R-NLS-Reg from 26.4 ± 4.0 % to 44.0 ± 3.4 %). Probably the dephosphorylation of S348 activated the phosphorylation of S370 and thereby inhibited the nuclear migration. So when PKC inhibitors were added they prevented the phosphorylation of S370 and the constructs could migrate into the nucleus.

To test the hypothesis that S348 of pRS1 but not other protein was required for the inhibitory effect of DRB on the nuclear localization, the construct without the serine 348 was used (NLS-Reg, β-Gal-[349-406 a. a.]-GFP). Indeed, the incubation with DRB did not decrease the nuclear location of the construct (78.7 ± 4.4 % compared to 70.3 ± 11.8 % without treatment). Data suggest that serine 348 in phosphorylated stage prevents phosphorylation of serine 370 and therefore retains the nuclear migration.

3. Confluence dependence of the pRS1 nuclear localization

It was shown that the nuclear localization of pRS1 is dependent on cell confluence (Kroiss et al., 2006). To support these data we performed the further investigation of confluence dependence on nuclear accumulation of the pRS1 protein.

Cells were seeded at a density of $10^4/cm^3$ on polyester membranes (Trans-Clear Polyester Membrane, Corning Incorporated, Corning, USA) and were grown on supplemented DMEM medium as described in “Materials and Methods”. On the next day (subconfluent cells) or 3-4 days after seeding (confluent cells) cells were transiently transfected by GFP-pRS1, by NLS-Reg (349-406), by R-NLS-R (342-374), by RS1 NLS without regulatory part (349-368), by R-NLS-Reg (342-406) or by R-NLS-Reg with substitution of serines 348, 370 or 400 by alanine. After 24 h cells were fixed and GFP-fusions distribution was observed through light microscopy (Figure 16).

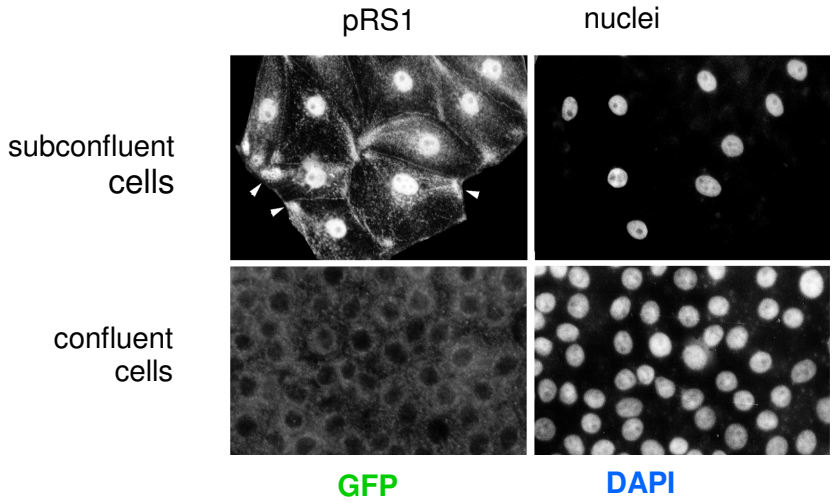


Figure 16. Nuclear localization of GFP-pRS1 construct before and after confluence of LLC-PK$_1$ cells. LLC-PK$_1$ cells were seeded on polyester membranes and grown for different time intervals. They were transiently transfected with GFP-pRS1 48 h before fixation and observation through light microscope, and the nuclei were counter-stained with DAPI (Kroiss, et. al., 2006).

After confluence the localization of the pRS1 protein changed (Table 3). In confluent cells the nuclear fraction (25.5 ± 8.0 %) of GFP-pRS1 fusion protein was reduced by more than half of subconfluent stage (84.2 ± 2.1%). The regulated R-NLS-Reg construct also showed the drastic decrease of the nuclear localization after confluence (47.7 ± 10.0 % for subconfluent compared to 16.0 ± 1.3 % for confluent, P< 0.01 for difference), whereas RS1 NLS alone (349-368) showed no difference between subconfluent and confluent stage (55.8 ± 7.7 % compared to 68.9 ± 12.7 %). The data indicate that the RS1 NLS domain, comprising amino acids 342-406, mediates confluence dependent nuclear targeting.

To investigate the role of phosphorylation / dephosphorylation of the serine residues 348, 370 and 400 on nuclear migration during confluence, in R-NLS-Reg construct serines were mutated to alanines and nuclear location after confluence was observed. In the mutant S400A nuclear location in the confluent cells was reduced to 18.9 ± 5.6 % (compared to 51.1 ± 2.3 % in subconfluent cells, P<0.01, Table 3). So phosphorylation of S400 does not affect an inhibition of the nuclear migration after confluence. On the contrary, the substitution S370A decreased the inhibition of the nuclear localization after confluence (50.4 ± 4.7 % before confluence compared to 59.5 ±1.0 % after confluence). Mutant S348A also showed no inhibitory effect on the nuclear location after confluence (15.8 ± 4.5 % in subconfluent cells and 20.0 ± 0.8 % in confluent cells). With NLS-Reg construct, which contains serines 370 and 400 but not serine 348, we observed no significant difference between fluorescence in the nucleus in subconfluent and confluent cells (45.5 ± 1.7 % for subconfluent cells and 40.3 ± 4.4 % for confluent cells). And the nuclear fraction of R-NLS-R construct, containing serine

348 for CK2 and serine 370 for PKC, was inhibited after confluence (60 ± 5.7 % for subconfluent cells and 16 ± 6.3 % for confluent cells).

Data suggest that RS1 NLS without the regulatory part can migrate into the nucleus after confluence as well as in subconfluent cells; whereas the nuclear migration of the R-NLS-Reg construct is down-regulated after confluence. The confluence dependent nuclear migration of RS1 NLS is mediated by the regulatory part around NLS. The phosphorylation of serine 370 inhibits the nuclear targeting and serine 348 is somehow involved in the regulation of the R-NLS-Reg construct nuclear localization.

So we conclude that the nuclear migration of GFP-pRS1 and of R-NLS-Reg, containing RS1 NLS with additional amino residues around it (342-374 a. a.), is confluence dependent. It is regulated by phosphorylation and dephosphorylation of serines 348 and 370 around RS1 NLS.

Table 3. The effect of the confluence on the nuclear localization of pRS1 mutants in LLC-PK_1 cells.

Constructs	Subconfluent cells	Confluent cells
GFP-pRS1	84.1 ± 2.1	25.5 ± 8.0 ***
RS1 NLS (349-368 a. a.)	55.8 ± 7.7	68.9 ± 12.7
R-NLS-Reg (342-406 a. a.)	47.7 ± 10.0	16.0 ± 1.3 **
R-NLS-Reg / S348A	15.8 ± 4.5	20.0 ± 0.8
R-NLS-Reg / S370A	50.4 ± 4.7	59.5 ± 1.0
R-NLS-Reg / S400A	51.1 ± 2.3	18.9 ± 5.6 **
R-NLS-R (342-374 a. a.)	62.0 ± 5.7	16.0 ± 6.3 ***
NLS-Reg (349-406 a. a.)	45.5 ± 1.7	40.3 ± 4.4

Data represent the mean values (nuclear fraction, %)± SD of 3 to 6 independent experiments. Significant differences between subconfluent and confluent cells (* P < 0.5; ** P <0.01; *** P < 0.001) and R-NLS-Reg (342-406 a. a.) and NLS-Reg (349-406 a. a.) in confluent cells (••• P < 0.001) are indicated.

4. Degradation of the pRS1 protein

During this study we observed that pRS1 protein is very unstable in cells and undergoes the degradation yielding several smaller fragments detectable by Western blot.

In order to find ways how the pRS1 protein degrades, the specific inhibitors of proteosomes and lysosomes were tested.

HEK 293 cells were transiently transfected with the pRcCMV-pRS1 vector, expressing pRS1 protein, and pre-incubated for 16 h before harvesting with 10µM MG-132 (inhibitor of proteosomal degradation), 10mM methylamine, 10µM 3-methyladenine, or 10µM NH_4Cl (inhibitors of lysosomal degradation).

The cells were harvested as described in "Materials and Methods", the SDS-PAGE was performed as usual and the proteins were detected by Wester blot. Densitometric quantification of immunostained proteins in Western blots was performed as described earlier.

The incubation with the lysosomal inhibitors did not drastically change the protein content, while the incubation with the proteosomal inhibitor MG-132 led to two-fold increase of the protein content (Figure 17). We can conclude that RS1 protein is degraded mostly by the proteosomal degradation way.

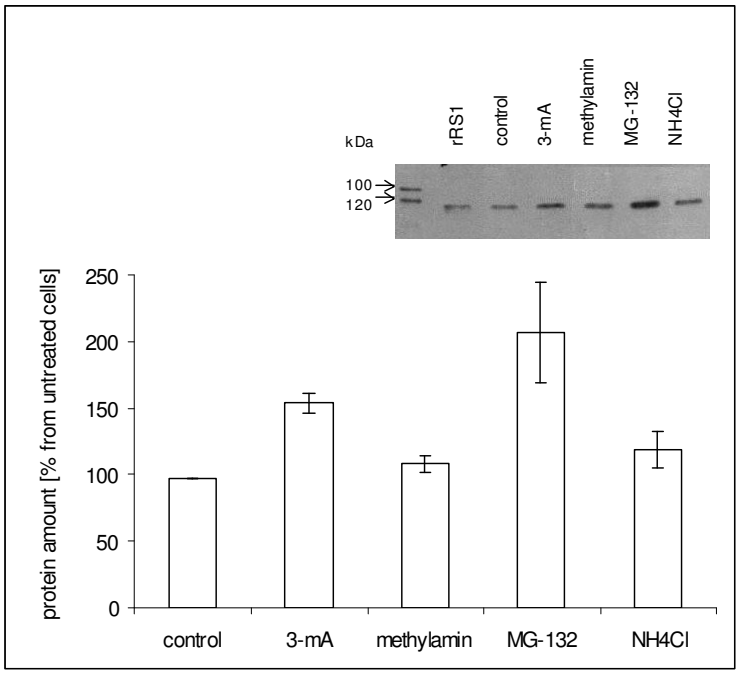


Figure 17. Degradation of RS1 protein. The HEK 293 cells were transiently transfected with pRcCMV-pRS1-vector, cells were grown on culture plates until 50-70% confluence and harvested without preincubation (control) or after 16h preincubation with inhibitors (10µM MG-132, inhibitor of proteosomal degradation; 10mM methylamine, 10µM 3-methyladenine, or 10µM NH_4Cl, inhibitors of lysosomal degradation). 20µg of protein were loaded on the PAA-gel. Mean values ± SD of 3 independent experiments and significant differences between untreated and treated cells are indicated: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. rRS1 – purified recombinant RS1 protein expressed in *E.coli*.

DISCUSSION

The main aim of this study was to identify the nuclear localization sequence of the pRS1 protein. For this purpose the fusion constructs between pRS1 and GFP were considered. First, we narrowed down the region of 92 amino acids in the middle part of the protein required for nuclear localization. Then by inserting the fragments of pRS1 between GFP and β-Galactosidase the exact nonconventional NLS of 20 amino acids residues was identified. Although the new identified NLS (349-368 amino acids) was able to transfer the β-Gal-GFP construct into the nucleus, the nuclear fraction was 53.7 % and did not reach the level of GFP-pRS1 nuclear localization (85.8 %). Perhaps the full-length protein contains some other regions that enhance the nuclear migration. Since the pRS1 protein contains no other nuclear localization signal, the identified NLS alone is sufficient for nuclear migration of RS1 protein.

Our next objective was to predict the structure of the nuclear localization sequence. Therefore several secondary structure simulated computer programs were used. They showed the α-helical structure between 349 and 365 amino acids (Figure 10). The α-helix is amphiphilic and contains hydrophobic amino acids on the one side, and some charged amino acids on the other side of the helix (Figure 8, b). RS1 NLS has no similarity with any of the previously identified classical and nonclassical NLS, but we discovered a high amino acid identity with domains of DNA repair proteins RAD5 from *Saccharomyces cerevisiae* (Johnson et al., 1992 and 1994), of DNA-dependent RNA polymerase III of *Giardia lamblia* (Lanzerdörfer et al., 1992), of deoxyribonucleoside kinase *Dm*-dNK of *Drosophila melanogaster* (Zheng et al., 2000, Munch-Petersen et al., 2000), of human oxysterol binding protein related ORP7 (Lehto et al., 2001, Johansson et al., 2003), of human ubiquitin specific protease USP36 (Quesada et al., 2004) and of ubiquitin activating enzyme E1 from *Plasmodium falciparum* (Finley and Chau, 1991). In the *Dm*-dNK and in the RAD5 proteins classical NLSs have been identified in addition to RS1 NLS homologous domains. By inserting the RAD5 homologous to RS1 NLS domain between β-Galactosidase and GFP no nuclear localization was found. So, we suggest that the homologous to RS1 NLS domain of Rad5 cannot alone target proteins to the nucleus. Either it functions together with other parts of Rad5 or plays another role in the nucleus.

The RS1 NLS is surrounded by three phosphorylation sites which are conserved through all four species: pig, rabbit, mouse and human. It is known that often phosphorylation or dephosphorylation near NLS regulates nuclear migration of proteins. For example, the

phosphorylation of a PKA site adjacent a NLS of *Drosophila* transcriptional factors (Tfs) modulates NLS recognition by importins 58/97 complex (Briggs et al., 1998). So, we suggest that the phosphorylation / dephosphorylation of serines (348, 370 or 400) regulates the nuclear migration. Our data showed that mimicking the phosphorylation form of serine 370 by glutamate strongly inhibited the nuclear location in all constructs we used, whereas the substitution by alanine, mimicking non-phosphorylated serine residue 370, recreates the nuclear migration. Also the S348 is involved in the regulation of the nuclear migration. The low nuclear fraction of both substitutions of S348, S348A and S348E, could be caused by the charge of the protein conformation which hides the NLS inside the protein. In addition all substitution and deletion experiments suggest that the serine 400 is not essential for the regulation of the nuclear location, but supports it. Taken together our data suggest that the serines 348 and 370 are crucial for the regulation of nuclear migration.

There is a strong correlation between the cell confluence and the amount and the distribution of the pRS1 protein in the cell. Thus, in LLC-PK_1 cells after confluence the native pRS1 protein leaves the nucleus and its total amount decreases (Korn et al, 2001, Kroiss et al, 2006). We investigated whether two conserved consensus sequences for the PKC C-terminal from RS1 NLS and one conserved consensus sequence for the CK2 N-terminal from RS1 NLS are involved in the confluence dependent regulation of the nuclear migration.

Cells, transiently transfected with fusion constructs GFP-pRS1 and R-RS1-Reg (β-Gal-[342-374 a. a. of pRS1]-GFP, containing the NLS and the regulation part around it), showed strong inhibition of the nuclear location after confluence, while RS1 NLS construct (349-368 a. a.) without the regulation part did not change the nuclear location in the dense cells (Figure 16, Table 3). Since the part around the RS1 NLS was essential for confluence dependent nuclear migration, we speculate that the conserved consensus sequence for CK2 dependent phosphorylation N-terminal of NLS (S348) and the consensus sequences for PKC C-terminal to NLS (S370) are involved. Notice, proteins with homologous sequences to RS1 NLS do not contain these phosphorylation sequences. Using point mutation analysis and experiments with kinase inhibitors we have demonstrated that serine 348 and serine 370 are required for the confluence dependent regulation of the nuclear migration, whereas other consensus sequence for PKC (serine 400) is not required.

Modulation of phosphorylation and dephosphorylation of amino acids near NLS and Nuclear Export Signal (NES) is known to regulate cellular distribution of different proteins (Rihs et al., 1991, Kann et al., 1999, Yang et al., 2001, Lee et al., 2002, Yuan et al., 2002). Also the confluence dependent nuclear/cytoplasmic location has been described for aryl

hydrocarbon receptor (Ikuta et al., 2004, *JBC*), for the adenomatous polyposis coli protein (Fagman et al., 2003) and for the cytosolic phospholipase A(2) (Grewal et al., 2002).

Since phosphorylation / dephosphorylation of proteins may affect on the nuclear migration and during the cell confluence the RS1 constructs showed different nuclear location, kinase activities of CK2 and PKC before and after confluence in LLC-PK_1 cells were measured (these experiments were performed by PhD student Alina Filatova from our lab). The measurements showed the strong 5-fold activation of PKC and 75 % down-regulation of CK2 in confluent cells (Figure 18). This correlates to our data, which showed that non-phosphorylated serine 370 is essential for nuclear targeting before confluence, and after confluence RS1 distributes mostly in the cytoplasm. This can be a result of phosphorylation of the serine 370 by up-regulated PKC as well as of dephosphorylation of the serine 348 by down-regulated CK2.

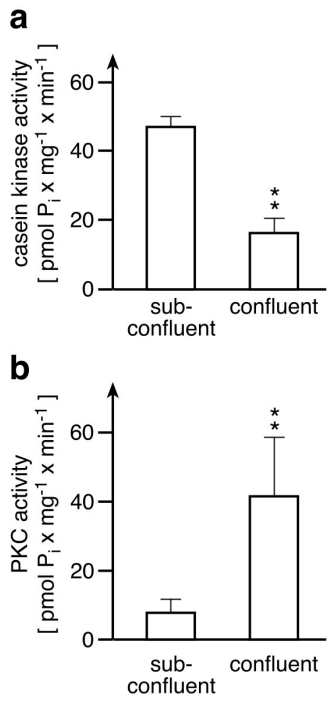


Figure 18. Activity of casein kinase 2 (CK2, **a**) and protein kinase C (PKC, **b**) in subconfluent and confluent LLC-PK_1 cells. Cells were grown on culture plates until 50% confluence or until two days after confluence. Cells were harvested and CK2 activity or PKC activity was measured. Mean values ± SD of 3 independent experiments and significant differences between activities in subconfluent and confluent cells are indicated: ** P <0.01.

It has been reported in the literature that in many cell lines amount of PKC protein but not mRNA was regulated during confluence. For example in Caco-2 (Abraham et al., 1998) and in rat osteogenic sarcoma cells (Geng et al, 2001) the amount of PKC protein was up-regulated. In LLC-PK_1 cells PKC was found in cytosol before confluence and shifted to the membrane fraction after confluence (Dawson and Cook, 1987). There are numerous reports in the literature that actively proliferating cells contain increased levels of CK2 activity (Allende

and Allende, 1995, Faust et al., 2000), which is necessary for the cell division (Pepperkok et al., 1994).

On the basis of the data, (*i*) measuring the effect of point mutations in serines 348, 370 and 400 of pRS1 that prevent or mimic phosphorylation on the nuclear localization, (*ii*) measuring the activity of CK2 and PKC before and after confluence, and (*iii*) measuring the effect of activators and inhibitors of CK2 and PKC on the nuclear migration, we propose a mechanistic model how phosphorylation / dephosphorylation of the CK2 and PKC dependent phosphorylation sites may regulate the confluence dependent nuclear migration of RS1 protein (Figure 19).

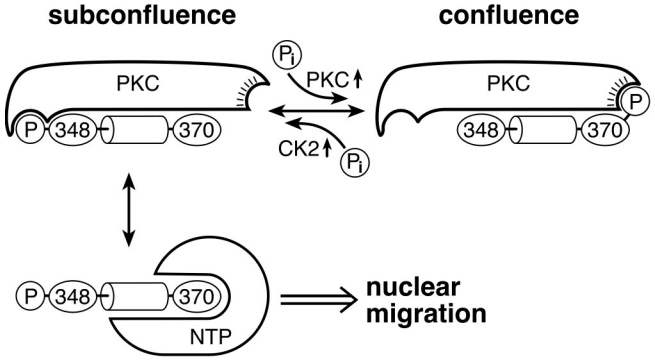


Figure 19. Schematic model of the regulation of pRS1 nuclear migration by differential phosphorylation of pRS1 by CK2 and PKC. The cells density regulates nuclear localization signal of pRS1 (R-NLS-R construct) comprised of 342-374 amino acids.

In subconfluent cells PKC activity is low whereas CK2 is active and perhaps it phosphorylates the serine 348, which prevents the phosphorylation of the serine 370. RS1 NLS binds to unknown nuclear transfer protein (NTP) that is required for nuclear migration. After confluence it is the other way round: CK2 is down-regulated leading to dephosphorylation of serine 348 that permits phosphorylation of the serine 370 by up-regulated PKC during confluence. The phosphorylation of serine 370 completely blocks the nuclear migration of the R-NLS-R construct and significantly reduces the amount of the pRS1 protein in the nucleus. We assume that phosphorylated serine 370 permits binding of NTP to RS1 NLS and thus inhibits the nuclear migration of RS1 after confluence.

Joint action of PKC and CK2 to the same protein was recently considered in p53, the tumor suppressor protein that plays a key role in cell cycle regulation and differentiation (Pospíšilová et al., 2004). Protein p53 protein is phosphorylated by different kinases, which are involved in protein stabilization and activation in response to cellular stress. Interestingly, phosphorylation by PKC occurs in undamaged cells, whereas ckc2 and CK2 are enhanced in

DNA-damaged cells. The authors showed *in vitro* that binding of one kinase to the protein does not block binding of other kinase to the same protein. But the phosphorylation of the p53 by CK2 prevents the phosphorylation of p53 by PKC. At the same time the phosphorylation of p53 by PKC does not block the phosphorylation of the p53 protein by CK2 bound to the protein.

Another example of the combined action of different protein kinases is adenomatous polyposis coli protein (APC), a multifunctional tumor suppressor. Phosphorylation at the CK2 site near one of two classical NLS on the C-terminus of APC (NLS2) increases, and phosphorylation at a PKA site near NLS2 decreases the nuclear localization of the protein. This regulation is cell density-mediated with predominantly nuclear localization in subconfluent MDCK and epithelial cells, and both cytoplasmic and nuclear localization after confluence (Zhang et al., 2001). The p38 mitogen-activated protein kinase (MAPK) is involved in both activation of CK2 and inhibition of PKA (Hildesheim et al., 2005). However in 6/6 thyroid and colorectal carcinoma cell lines this process is cell cycle-dependent, and a mutant, lacking NLS1 and NLS2, has similar cell distribution as wild type APC (Fagman et al., 2003). The authors suggest that perhaps the N-terminal part of APC contains some additional nonclassical NLS, which may be regulated in the same confluent-dependent way and increase nuclear import of APC.

Trying to understand how the phosphorylation of serine 348 by CK2 prevents the phosphorylation of the serine 370 by PKC, a model between R-NLS-R pRS1 and the PKC was made by Dr. Thomas Müller (University Würzburg). The model (Figure 20) used the crystal structure of the complex between the PKA and the inhibitory peptide fragment PK1 (4-25) (Bossemeyer et al., 1993) as well as the crystal structure of the catalytic sites of PKCθ (Xu et al., 2004). Based on (*i*) the similarity of the tertial structure of PKC and PKA catalytic sites, (*ii*) the same length of the predicted α-helix of RS1 NLS and an α-helix of PK1 (4-25), and that (*iii*) the C-terminal alanine in PK1 (4-25) is located on the same distance from α-helix as S370 from NLS α-helix in pRS1, we suggested that the interaction of R-NLS-R and the catalytic site of PKC is similar to the interaction of PK1 (4-25) and the catalytic site of PKA. According to this model, the phosphorylation of serine 348 disturbs the interaction of serine 370 with the catalytic site of PKC.

Using over-expression of the RS1 protein in the HEK cells it was found that the pRS1 protein is degraded mostly by proteosomes. It correlates with the fluorescent experiments on the native LLC-PK_1 cells, where the native pRS1 protein was degraded mostly by

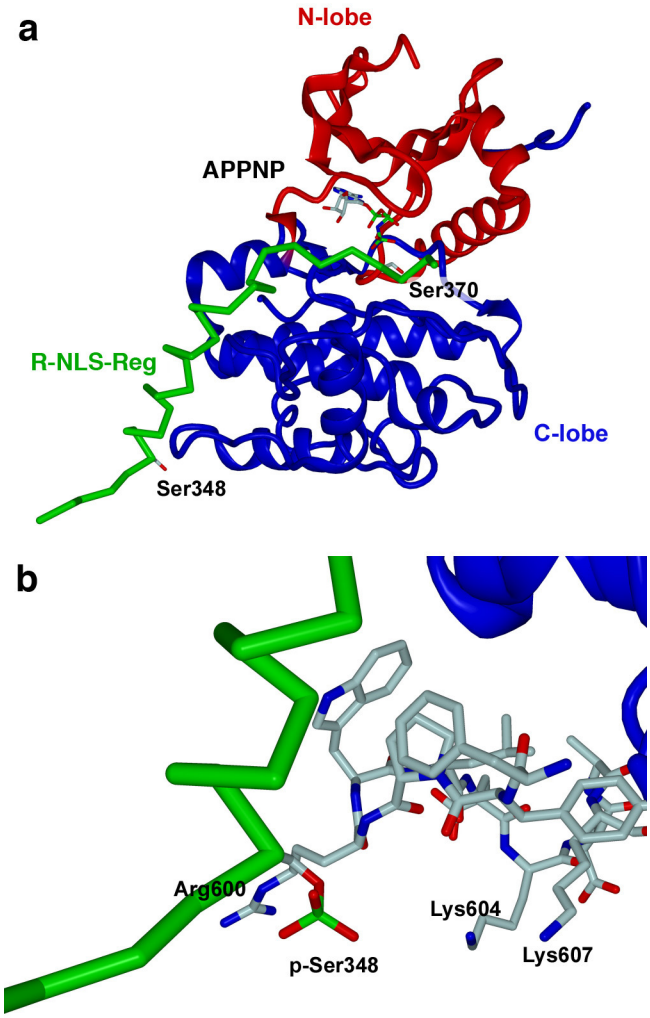


Figure 20. a, structural model of the interaction of R-NLS-Reg with PKC. PKC is shown as a ribbon representation with the N-lobe colored in red and the C-lobe marked in blue. The ATP analogue APPNP is indicated as sticks. R-NLS-Reg (residues Gln343 – Pro373 of pRS1) is shown as an α-trace with the two non-phosphorylated phosphorylation sites Ser348 and Ser370 shown as sticks. Serine 370 is located in the active site and may be phosphorylated when serine 348 is not phosphorylated. **b**, magnification of the area around Ser348. Phosphorylation of Ser348 is modeled showing the steric clash between the side chain of p-Ser348 and residues of PKC loop (residues 598 to 607). The resulting displacement of the helix of R-NLS-RS1 from the binding pocket of PKC would likely result in a weakening of the protein-protein interaction. Additionally the negative charge introduced by the phosphorylation of Ser348 might also lead to a displacement of the helix of RS1 by strong ionic interaction with a positively charged cluster formed by the PKC residues Arg600, Lys604 and Lys607.

proteosomes, and the intracellular concentration of pRS1 protein in the confluent LLC-PK_1 cells was drastically increased after incubation with the proteosomal inhibitor MG-132 (Kroiss et al., 2006). So the different cell localization of the pRS1 protein in the dense cells was not only the result of the inhibited nuclear migration, but it was supported by degradation of the protein in the nucleus.

To evaluate the potential physiological importance of RS1, we summarize all data about RS1. Subconfluent LLC-PK_1 cells have small amount of the RS1 protein that localizes at the plasma membrane, at the TGN and within the nucleus. In the subconfluent cells RS1 down-regulates SGLT1 transcriptionally and post-transcriptionally: it blocks the release of the SGLT1-containing vesicles from the TGN (Kroiss et al., 2006), and inhibits the transcription of SGLT1 (Korn et al., 2001; Veyhl et al., 2006). During confluence down-regulation of SGLT1 by RS1 occurs by at least two mechanisms. First, the RS1 protein is degraded more rapidly by proteosomes in the confluent cell (Kroiss et al., 2006). Second, the dephosphorylation of S348 after confluence allows the interaction of S370 with the catalytic site of PKC, and S370 is phosphorylated; the phospho-serine 370 disturbs the binding of NTP to RS1 NLS and the nuclear targeting of RS1 is inhibited. Further experiments are required to elucidate the following mechanism: how the cell confluence leads to the kinase-activity changes and whether the up-regulation of PKC and the down-regulation of CK2 during the confluence of the LLC-PK_1 cells influence the degradation of RS1 and post-transcriptional inhibition of SGLT1 by RS1.

In addition to the confluence dependent nuclear localization signal NLS in the central part of the protein, RS1 contains the ubiquitin-associated binding (UBA) domain at the C-terminus (a potential site for binding ubiquitin or ubiquitinylated proteins) and two short protein domains that are responsible for the post-transcriptional down-regulation of SGLT1 by RS1 (Vernaleken, A., Gorboulev, V., Veyhl, M., Kotta, G., Palm, D., Burckhardt, B.G., and Koepsell, H., unpublished data).

The IRIP and RS1 proteins are two components of the new regulatory pathway that is involved in the down-regulation of transporters during regeneration of renal tissue after ischemia and reperfusion (Jiang et al., 2005). One way to unravel the new regulatory pathway may be the identification of functional domains in RS1 and IRIP and to identify the proteins that interact with such domains. Many questions concerning the interaction of RS1 with regulatory pathways of transporters during differentiation remain to be solved including specificity, molecular mechanisms and its physiological significance.

LIST OF ABBREVIATIONS

a. a. – amino acids

AMG – α-methylglucose

APC – adenomatous polyposis coli protein

β-Gal – beta-galactosidase

bp – base pairs

BSA – bovine serum albumin

Cdc2 – cyclin-dependent kinase 2

CK2 – casein kinase

DAPI – 4,6-diamindino-2-phenylindole

DMEM – Dulbecco's Modyfied Eagle's Medium

DOG – *sn*-1,2-dioctanoyl glycerol

DPBS – Dulbecco's phosphate buffered saline

DRB – 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole

DTT – dithiothreitol

EDTA – ethynelediaminetetraacetic acid

GFP – green fluorescent protein

GLUT – sodium-independent glucose transporter

Ig – immunoglobulin

IRIP – ischemia/reperfusion inducible protein

MAPK – mitogen-activated protein kinase

NE – nuclear envelope

NES – nuclear export signal

NLS – nuclear localization signals

NLS-R – β-Gal-[349-374 a. a. from pRS1]-GFP construct

NLS-Reg – β-Gal-[349-406 a. a. from pRS1]-GFP construct

NM – normal medium

NP – nucleoplasmin, KRPAATKKAGQAKKKK

NPC – nuclear pore complex

PAA – polyacrylamide

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PKC – protein kinase C

PMSF – phenylmethylsulfonylfluoride

PSA – ammonium persulfate

RNase – ribonuclease

R-NLS – β-Gal-[342-368 a. a. from pRS1]-GFP construct

R-NLS-R – β-Gal-[342-374 a. a. from pRS1]-GFP construct

R-NLS-Reg – β-Gal-[342-406 a. a. from pRS1]-GFP construct

RS1 NLS – nuclear localization signal of pRS1 protein, β-Gal-[349-368 a. a. from pRS1]-GFP construct

RT – room temperature

SDS – sodium dodecyl sulfate

SGLT1 – Na^+-dependent D-glucose co-transporter

TBB – 4,5,6,7-tetrabromobenzotriazole

TGN – *trans*-Golgi network

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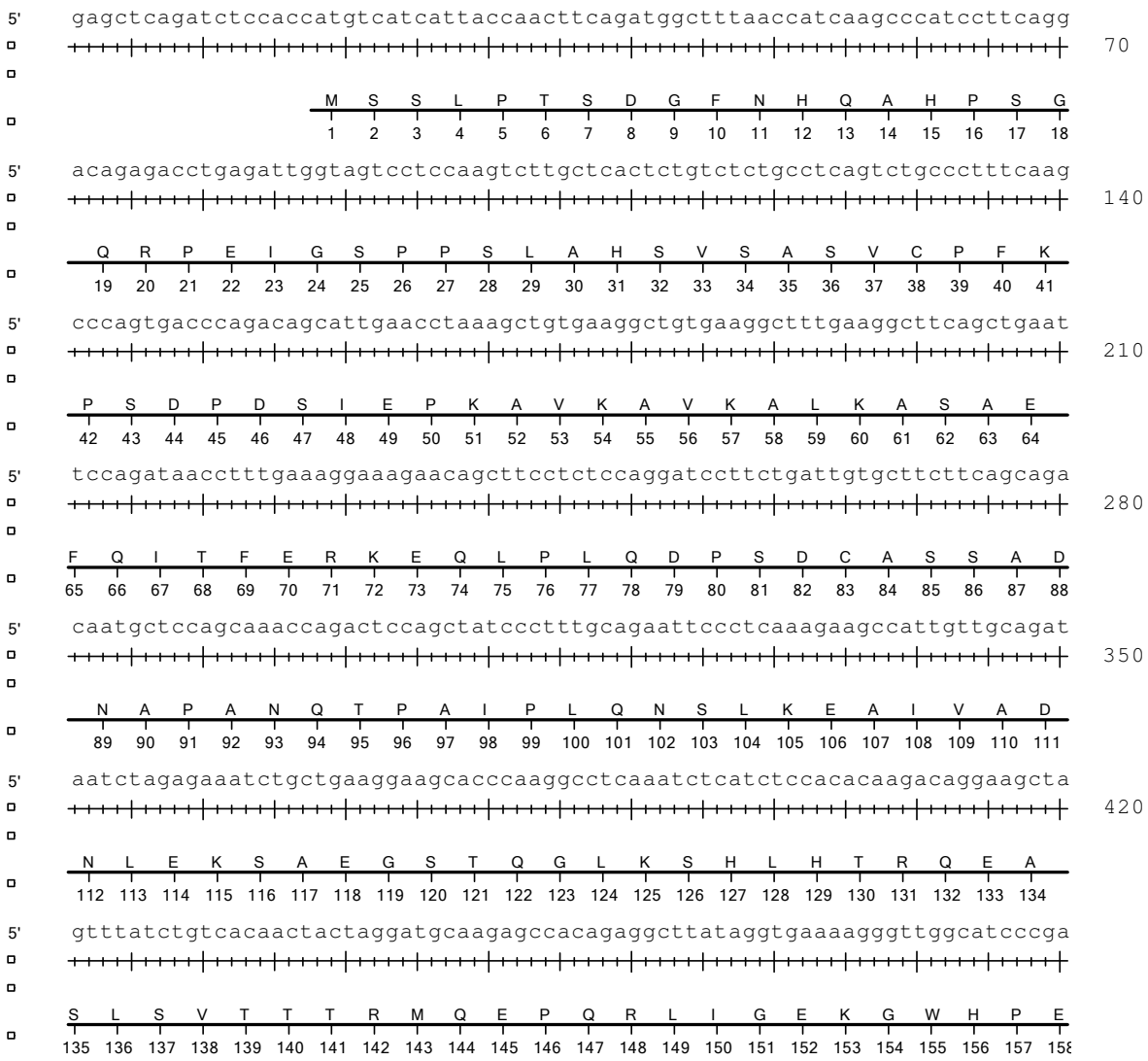
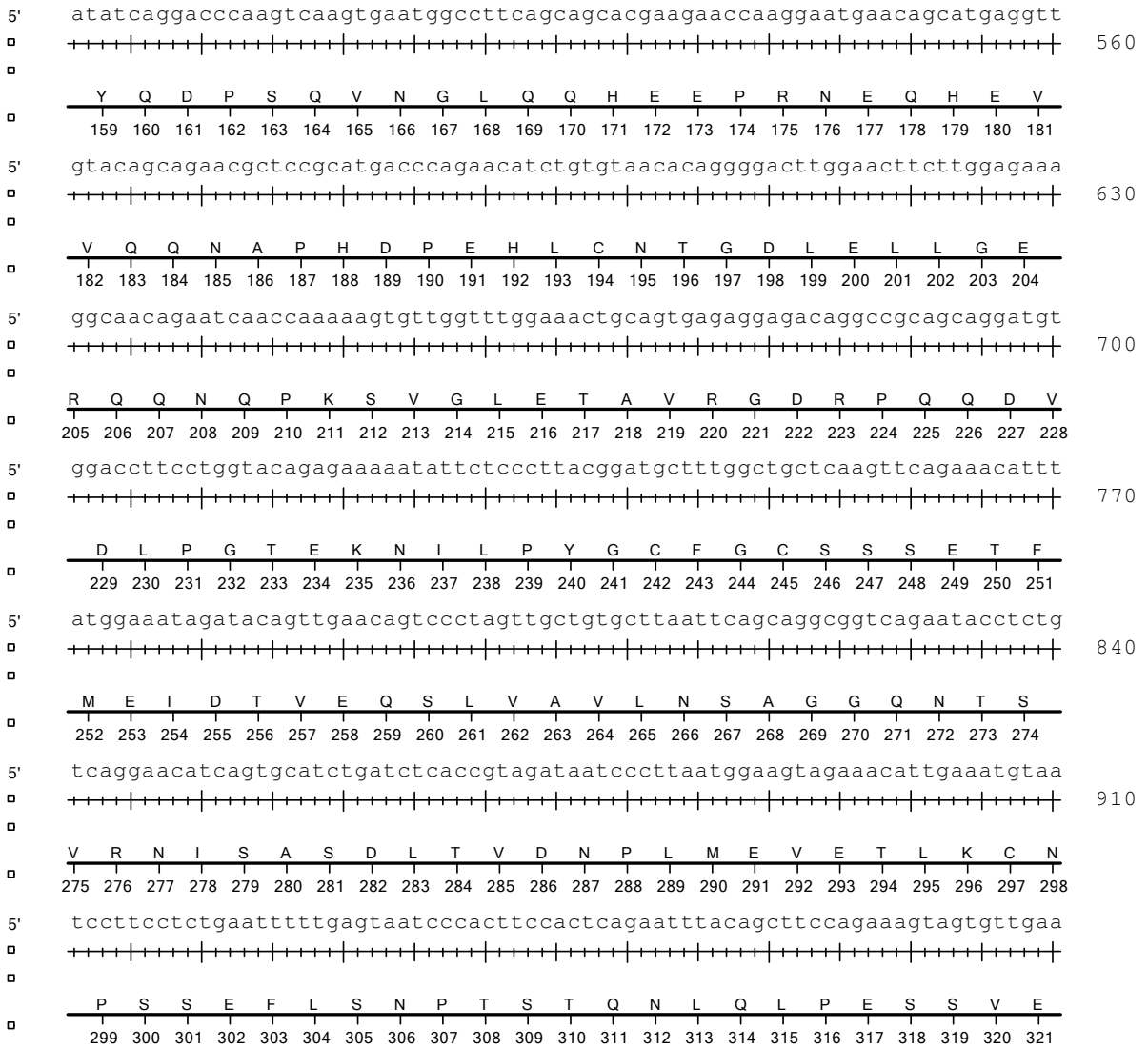
ATTACHMENT

DNA and amino residues sequences of pRS1 protein.

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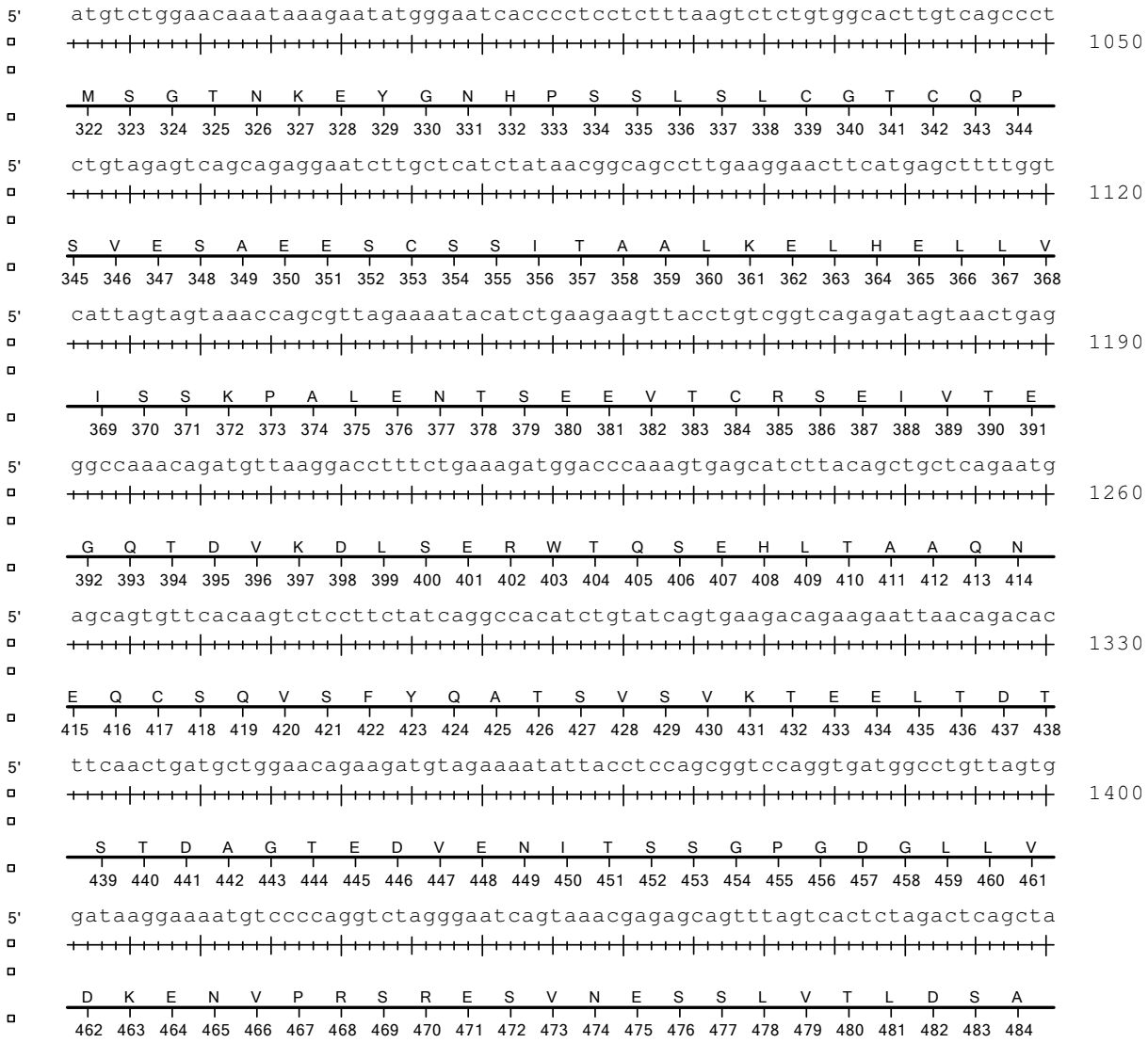
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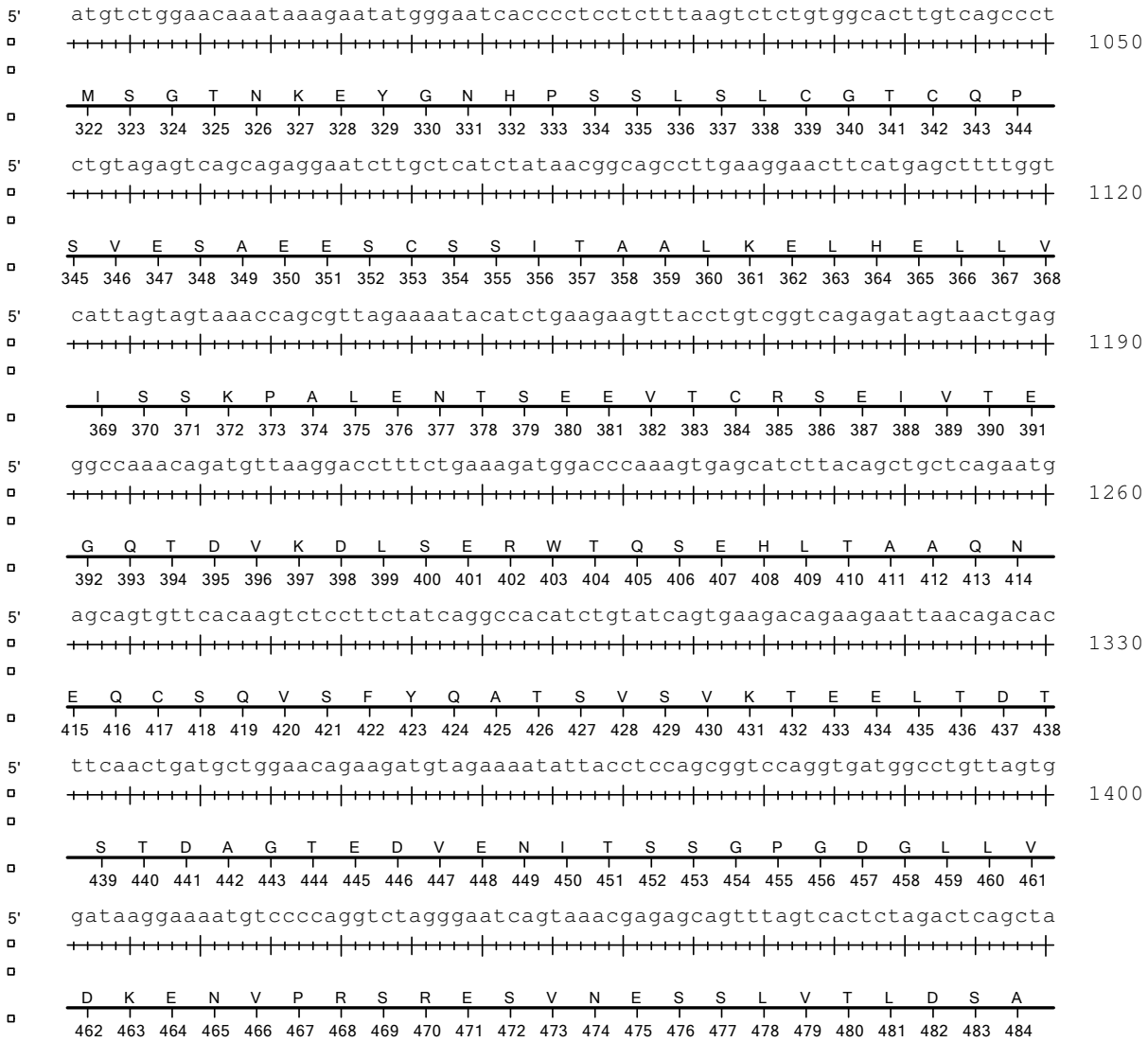
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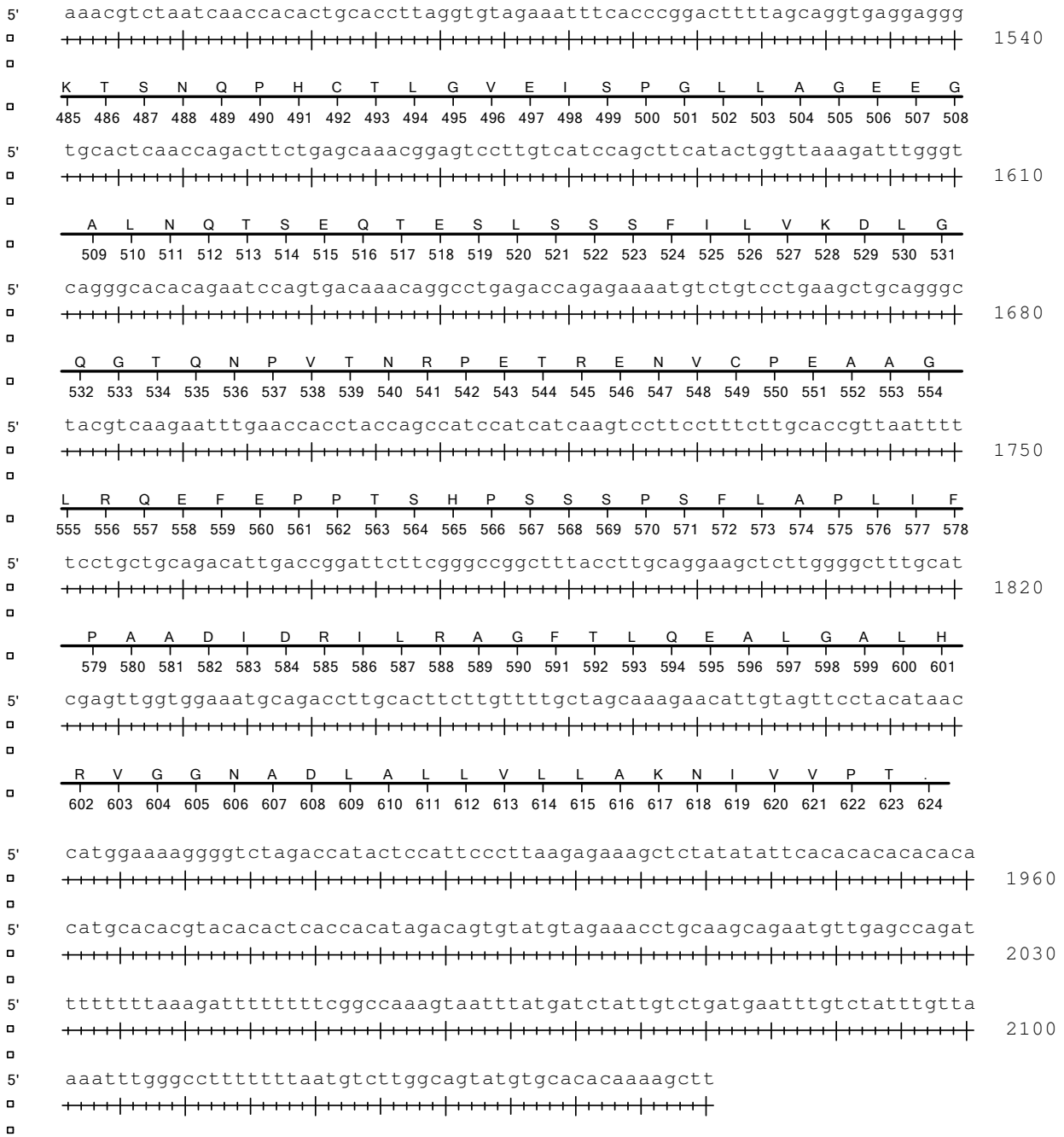
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Erklärung gemäß §4 Abs. 3 der Promotionsordnung:

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig angefertigt und keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe.

Diese Dissertation hat weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegen.

Ich habe bisher außer den mit dem Zulassungsbesuch urkundlich vorgelegten Graden keine weiteren akademischen Grade erworben oder zu erwerben versucht.

Würzburg, May 2007 Marina Leyerer