Identification of a nuclear localization signal, RRMKWKK, in the homeodomain transcription factor PDX-1

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Received 29 September 1999

Abstract Pancreatic duodenal homeobox-containing transcription factor 1 (PDX-1) plays a crucial role in pancreas development and β-cell gene regulation. Absence of PDX-1 leads to pancreas agenesis and its malfunction causes MODY4 diabetes mellitus. PDX-1 has been suggested to be involved in the glucose-dependent regulation of insulin gene transcription. Whereas DNA-binding and transactivation domains of PDX-1 are in the process of being characterized, protein sequences responsible for its nuclear translocation remain unknown. By combining site-directed mutagenesis of putative phosphorylation sites and nuclear localization signal (NLS) motifs with on-line monitoring of GFP-tagged PDX-1 translocation, we demonstrate that the NLS motif RRMKWKK is necessary and in conjunction with the integrity of the 'helix 3' domain of the PDX-1 homeodomain is sufficient for the nuclear import of PDX-1. Furthermore, we show that there is no glucose-dependent cytoplasmic-nuclear cycling of PDX-1.

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Key words: PDX-1; Transcription factor; Gene expression regulation; Nuclear localization signal

1. Introduction

Nuclear translocation of transcription factors is a crucial requirement for their action and stimulus-dependent nuclear translocation can serve as a mechanism to regulate gene expression at the level of transcription initiation. The pancreatic duodenal homeobox (PDX)-containing transcription factor PDX-1 (identical or homologue to IPF-1 [1], IDX-1 [2], STF-1 [3], GSF [4], GSTF [5] and IUF-1 [6]) has been shown to have a dual function in gene regulation. During ontogenesis, PDX-1 is crucial for pancreas development and the maturation of premature β-cells into mature insulin-producing β-cells in the islet of Langerhans [7,8]. Absence of PDX-1 leads to agenesis of the pancreas [7,8] and its malfunction has been shown to cause diabetes in animals [9,10] and leads to the development of the MODY-type of diabetes in man, i.e. MODY4 [11]. In the adult, PDX-1 has been discussed to be involved in the transcriptional control of pancreatic islet β-cell-expressed genes, such as insulin, glucokinase, amylin, the islet δ-cell-expressed somatostatin gene (for review, see [12]) and has recently been reported to contribute to pancreatic exocrine elastase gene expression [13]. In insulin gene transcriptional control, PDX-1 has been shown to bind to A-box motives (for nomenclature, see [14]) and has been suggested to be involved in the glucose-stimulated up-regulation of insulin gene transcription. We have recently shown that glucose acutely activates insulin gene transcription [15] and that mutation of the PDX-1-binding A1-box in the rat insulin I promoter abolishes short-term glucose-stimulated insulin gene transcription [16]. As a possible mechanism of short-term activation of insulin gene transcription by PDX-1, the glucose-dependent nuclear translocation of the transcription factor has been discussed [17,18]. Although the molecular mechanisms of PDX-1 action are in the process of being addressed, i.e. its general domains such as the N-terminally located transactivation domain, the DNA-binding homeobox and the C-terminal domain have been described (for review, see [12]), the molecular basis of its nuclear translocation remains poorly understood.

In the present paper, we aimed to identify amino acid sequences that are responsible for the nuclear translocation of mouse PDX-1. Site-directed mutagenesis of putative phosphorylation sites and of positively charged amino acid residues in putative nuclear localization signal (NLS) motifs of GFP-tagged PDX-1 revealed that not a specific phosphorylation site but the presence of the NLS motif RRMKWKK is necessary and in conjunction with the integrity of the 'helix 3' domain of the PDX-1 homeodomain, sufficient for nuclear translocation of PDX-1 in insulin-producing MIN6 cells.

2. Materials and methods

2.1. Plasmids

The cDNA of mouse PDX-1 (i.e. mouse IPF-1) was kindly provided by Dr H. Edlund (Dept. Microbiology, University of Umeå, Sweden). To generate pB.RSV.PDX-1∼GFP, the *Pst*I site in the PDX-1 cDNA was used for in-frame fusion of GFP. To obtain GFP∼PDX-1, we introduced a *Hpa*I site into the PDX-1 cDNA by substituting nucleotides encoding Met[1], i.e. ATG for TTG. Following digestion with *Hpa*I/*Bam*HI, the PDX-1-SV40-polyA cassette was introduced into *Sma*I/*Bam*HI-opened pB.CMV.GFP0, thus generating pB.CMV.GFP∼PDX-1. To obtain pB.CMV.GFP∼PDX-$1_{185-209}$ and pB.CMV.GFP∼PDX-$1_{193-209}$, an *Eco*RV site was introduced into the PDX-1 cDNA exchanging codons for Ala[19] and Phe[20] for GATATC. Following further introduction of a *Hpa*I site by changing codon TTG (Leu[184]) for GTT or of an *Eco*RV site by changing codon AAA (Lys[192]) for GAT, sequences encoding amino acids 20–183 and 20–192, respectively, were deleted by restriction enzyme digestion and re-ligation. The stop codon after amino acid Lys[209] was introduced by changing codon CGT (Arg[210]) for TGA. Plasmid pB.PDX-$1_{194-208}$∼GFP was generated by changing Ile[193] into Met, thus providing a translation start site, and fusing PDX-1 cDNA encoding amino acids M∼WFQN**RRMKWKK**EEDK[208] in-frame with the GFP cDNA. The PDX-$1_{194-208}$∼GFP expression cassette was subcloned into a pRcCMVi backbone plasmid, i.e. pRc/CMV (Invitrogen) shortened by *Bam*HI digestion/re-ligation. As the control plasmid served pRcCMVi.GFP, i.e. pRcCMVi containing the

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PII: S0014-5793(99)01446-5

'humanized' enhanced version of GFP (L64F, S65T). All mutations were performed by employing the QuikChange Mutagenesis kit (Stratagene) and respective oligonucleotides purchased from Genset (France). All constructions were verified by DNA sequence analysis. Plasmid pK7-GR-GFP [19] for expression of GFP-tagged glucocorticoid receptor was kindly provided by Dr I. Macara (Dept. Pathology, University of Vermont, Burlington, VT, USA).

2.2. Cell culturing and gene expression

MIN6 cells [20] were obtained from Dr J. Miyazaki (Dept. Nutrition and Physiol. Chemistry, Osaka University School of Medicine, Osaka, Japan) and were adopted to culture at 11.1 and 5.5 mM glucose in Dulbecco's modified Eagle medium (DMEM). Cells were grown on 24 mm glass coverslips and transfected by the lipofectamine technique as previously described [21] with the following modification. Transfection was carried out in a 35 mm culture dish using 2.5 μg DNA and 7.5 μl lipofectamine. Following transfection, cells were cultured for a further 24 h in DMEM containing the glucose concentrations indicated in the text, 10% fetal calf serum, 100 μg/ml streptomycin and 100 U/ml penicillin, at 5% CO_2 and 37°C.

2.3. Laser scanning confocal microscopy

Laser scanning confocal microscopy was performed as described previously [15], using a Leica CLSM (Leica Lasertechnik, Heidelberg, Germany). Cells were prepared and treated as described above. Coverslips were placed in a perifusion chamber, which was mounted on a Leica Fluovert FU inverted microscope (Leica Lasertechnik, Heidelberg, Germany). The temperature was kept at 37°C. For the confocal microscope, the following settings were used: 100×/1.30 oil Leitz Fluotar objective lens, excitation wavelength 488 nm (krypton/argon laser), excitation filter HQ470/40, dichroic mirror Q495LP and emission filter HQ525/50. Presentation images were generated using Adobe Photoshop version 4.0.

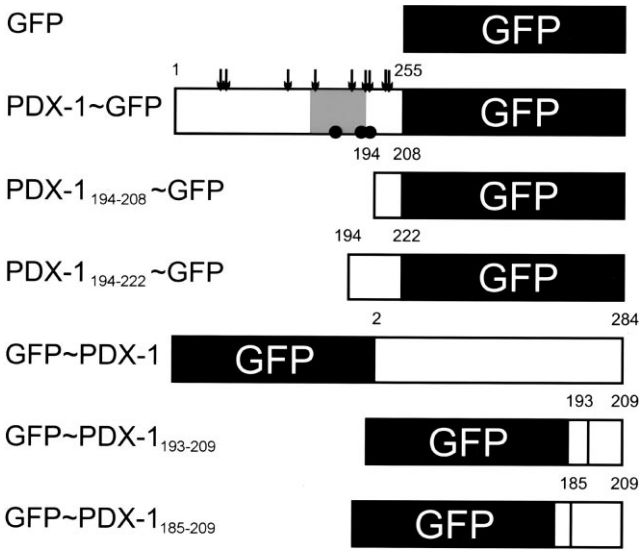

Fig. 1. Schematic representation of GFP-tagged PDX-1 fusion proteins. The GFP moiety is shown as a black bar, the PDX-1 part is shown as a white bar. Numbers above the PDX-1 part indicate the respective PDX-1 amino acids according to mouse PDX-1. The gray area in PDX-1∼GFP delineates the homeodomain of PDX-1. Arrows show the localization of mutated putative phosphorylation sites, filled circles indicate the position of mutated putative NLS motifs.

3. Results and discussion

Nuclear translocation of proteins can be accomplished by a diversity of mechanisms (for review, see [22]). In general, the cargo is recognized in the cytoplasm by a receptor via a nuclear location signal, NLS, presented by the cargo. Following binding to the receptor, often called importin or karyopherin, the cargo-receptor complex is recognized by Ran, translocated through the nuclear pore complex and the cargo is released inside the nucleus. The translocation of the cargo-receptor complex requires Ran's GTPase cycle. In case that the cargo itself does not possess a NLS, it can be imported by a piggyback mechanism after binding to a NLS-containing piggyback partner. Data from a recent report by Macfarlane et al. [17] suggest that when PDX-1 is translocated to the nucleus, it undergoes a modification that results in a 15 kDa shift in Western blot analysis and that translocated PDX-1 is phosphorylated. Both phosphorylation/dephosphorylation and the presence of a NLS, very often formed by a stretch of the positively charged amino acids arginine and lysine, have been shown to be involved in nuclear translocation of transcription factors (see for review [23]).

In order to identify amino acid residues in PDX-1 that are responsible for its nuclear translocation, we performed site-directed mutagenesis of putative phosphorylation sites as well as of stretches of positively charged amino acids in the GFP-tagged PDX-1 (see Fig. 1) and studied the nuclear translocation of the respective mutants by laser scanning confocal microscopy. In addition, this approach allows us to monitor online stimulus-induced nuclear translocation of transcription factors.

The predominant intranuclear localization of GFP-tagged PDX-1 (compare with cytoplasmic and nuclear localization of GFP-tagged glucocorticoid receptors, Fig. 2A), carrying the tag at either the N-(GFP∼PDX-1) or the C-terminus (PDX-1∼GFP), shows that the tag did not disturb the nuclear translocation of the chimera and that the chosen conditions did not lead to a saturation of the import mechanism (Fig. 2B,C).

3.1. The involvement of putative phosphorylation sites in nuclear translocation of PDX-1

Phosphorylation of transcription factors is one of the mechanisms to unmask a hidden NLS and thereby allow for binding of the cargo to its receptor. Computer analysis of the mouse PDX-1 amino acid sequence by employing the PROSITE software package revealed the presence of nine putative phosphorylation sites. Each of the respective putative phosphorylation sites was changed by site-directed mutagenesis into an alanine (Ser^{61}, Ser^{66}, Ser^{125}, Thr^{152}, Thr^{187}, Ser^{211}, Thr^{231}, Ser^{232}) or arginine residue (Ser^{211}) in C-terminally GFP-tagged mouse PDX-1. Transient expression and subcellular localization of the respective PDX-1∼GFP chimeras in MIN6 cells was analyzed by laser scanning confocal microscopy. As shown in Fig. 2D–L, none of the mutated putative phosphorylation sites alone abolished the translocation of PDX-1∼GFP to the nucleus.

3.2. The involvement of Arg/Lys stretches in nuclear translocation of PDX-1

Classical NLSs have been suggested to be formed by a hexapeptide (1) that consists of four or more positively charged amino acids (Arg and/or Lys), (2) that contains no bulky amino acids, such as Phe, Trp, Tyr, (3) that contains no Asp and Glu residues, (4) that is flanked by acidic residues proline and glycine that break α-helices and (5) that contains no hydrophobic residues in the core NLS flanking region [23]. Analysis of the mouse PDX-1 amino acid sequence did not

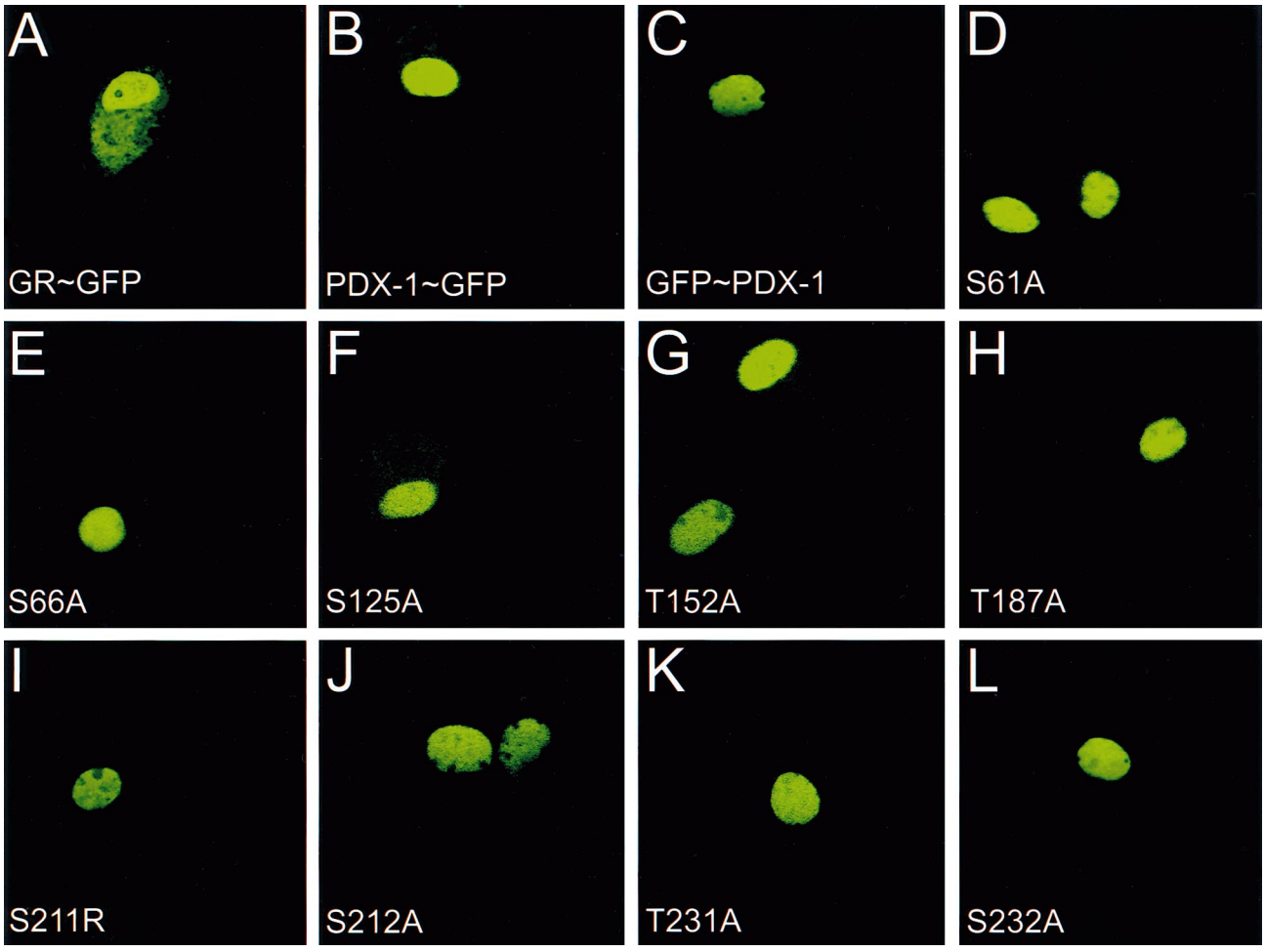

Fig. 2. The role of phosphorylation sites in nuclear translocation of PDX-1. A: Intracellular distribution of GFP-tagged glucocorticoid receptor 12 min following stimulation with 1 μM dexamethasone. B,C: Localization of C-terminally (PDX-1∼GFP) and N-terminally (GFP∼PDX-1) tagged PDX-1. D–L: Influence of indicated mutations on intracellular localization of GFP-tagged PDX-1. Representative images (out of a total of 20) are shown.

reveal a classical NLS that fulfills all the named requirements. However, three amino acid stretches came close to a NLS and were studied as putative NLS of PDX-1: K^{170}YISRPRRVE^{179}, R^{198}RMKWKKEED^{207} and E^{205}EDKKRSSG^{213}. Whereas mutations R174A+R177A (Fig. 3B) or K208N+K209Q+R210L (Fig. 3C) did not change the translocation pattern of GFP-tagged PDX-1, mutation of amino acids R199A+K203A in the R^{198}RMKWKKEED^{207} sequence (Fig. 3D) clearly shifted the PDX-1∼GFP distribution pattern from almost exclusively nuclear to that very similar of GFP alone, i.e. throughout the cell (Fig. 3E). Because the PDX-1∼GFP fusion protein with a molecular weight of 58 kDa still remains below the cut-off for nuclear import by diffusion [22], the destruction of the NLS in a PDX-1∼GFP fusion protein was expected to lead to the obtained distribution pattern (Fig. 3D), i.e. throughout the cell, rather than to result in a 'spared-out-nucleus' pattern.

The identified putative NLS consists of RRMKWKK and forms, according to Boulikas [23], a θθxθxθ or θxθxθθ motif, where θ represents Arg or Lys and x stands for any amino acid residue except Phe, Trp, Tyr, Glu and Asp. The fact that the stretch of positively charged amino acids is interrupted by two hydrophobic amino acid residues (Met and Trp), where Trp in addition is rather bulky, and is flanked by hydrophobic residues Trp-Phe makes the by us identified putative NLS atypical.

In order to test whether this putative NLS is able to confer NLS function to a heterologous protein, we tested the PDX-1 sequence WFQN**RRMKWKK**EEDK as an NLS-tag for the green fluorescent protein. Indeed, N-terminal fusion of GFP with the PDX-1 NLS-containing sequence shifted the GFP distribution pattern from throughout the cell to strongly nuclear-localized (Fig. 3E,F). The fact, that the PDX-$1_{194–208}$ sequence did not lead to an exclusive translocation of GFP to the nucleus could be explained by (1) an improper localization of the NLS within the NLS∼GFP molecule or (2) the fact that the by us identified NLS sequence represents only a part of a complete PDX-1 NLS. The involvement of the adjacent located KKR^{210} motif in the formation of a putative bipartite basic NLS can be excluded by the fact that mutation of this motif per se did not lead to an impaired nuclear translocation of PDX-1∼GFP (Fig. 3C). To test whether improper localization of the PDX-$1_{194–208}$ sequence in the PDX-1∼GFP molecule caused its incomplete nuclear translocation, we extended the PDX-1 sequence to 194–222, thereby spacing it further from the GFP moiety. As an alternative approach, we fused the NLS motif-containing PDX-1 sequence 193–208 to the C-terminus of GFP, also spaced by 20 amino acids.

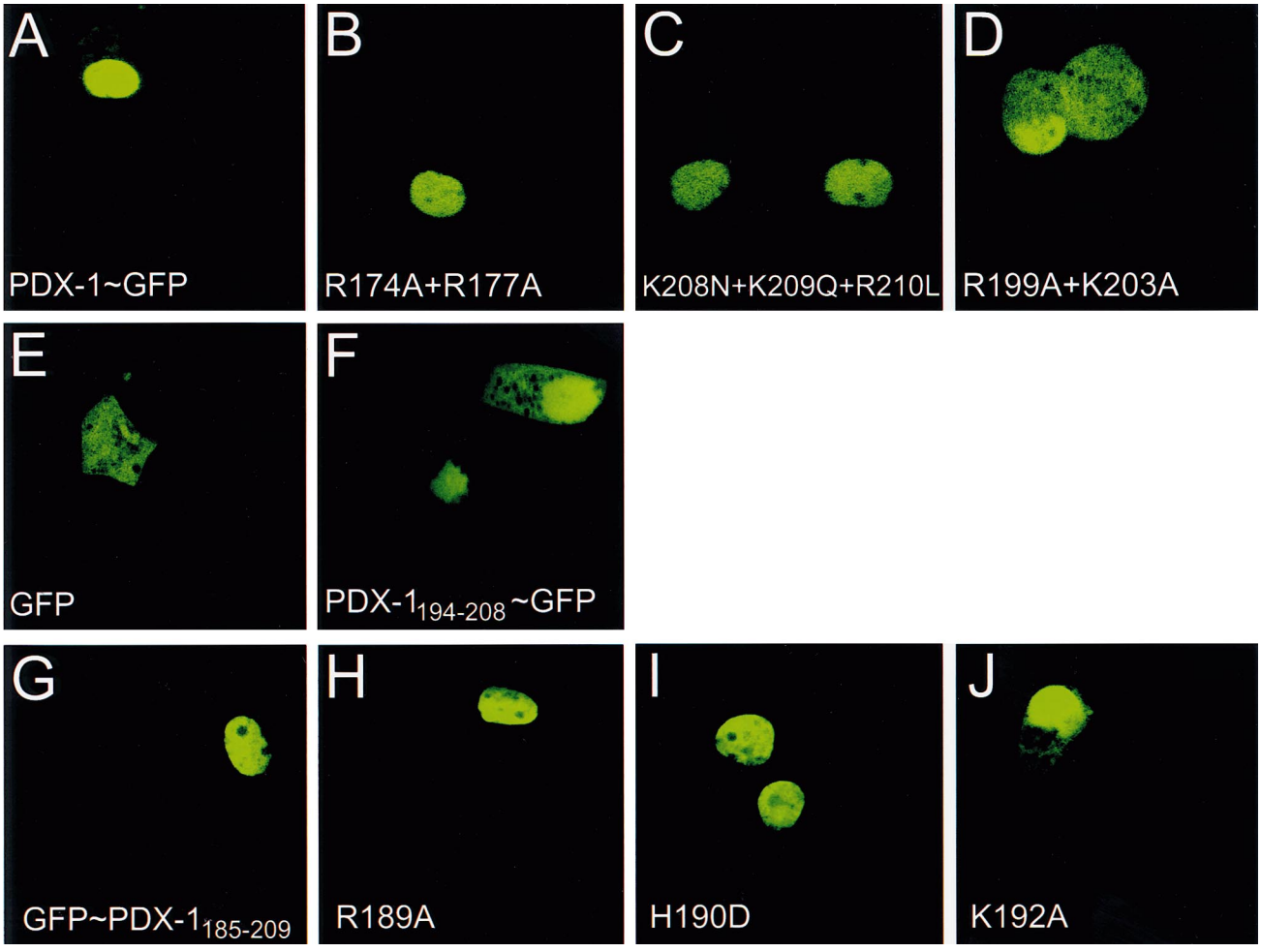

Fig. 3. The role of stretches of positively charged amino acids in nuclear translocation of PDX-1. A: Nuclear localization of PDX-1∼GFP. B–D: Influence of indicated mutations on the intracellular localization of GFP-tagged PDX-1. E: Intracellular distribution of GFP. F: Influence of N-terminal fusion of GFP with the putative PDX-1 NLS motif WFQN**RRMKWKK**EEDK[208]. G–J: Influence of C-terminal fusion of GFP with wild-type and mutated PDX-1 amino acids 185–209. Representative images (out of a total of 20) are shown.

However, both attempts did not lead to an exclusive nuclear translocation of the created fusion proteins. Exclusive nuclear translocation was achieved when extending the PDX-1 sequence to 185–209 (Fig. 3G). Inclusion of Lys[209] into the PDX-$1_{184–209}$ sequence was unlikely to be the reason for this effect since mutation of this amino acid did not hinder nuclear translocation of PDX-1∼GFP (Fig. 3C). The N-terminal extension from amino acid 193 to 185 caused two effects: (1) it completed the 'turn-helix 3' domain of the PDX-1 homeodomain, i.e. amino acids 185–205, and (2) it introduced three positively charged amino acids, Arg[189], His[190] and Lys[192]. To test whether the introduction of these three positively charged amino acids caused the exclusive nuclear translocation of GFP∼PDX-$1_{185–209}$, we generated separate mutations, R189A, H190D and K192A, which allowed for the maintenance of the helical structure of the 'helix 3' domain. Whereas mutations R189A and H190D did not alter the nuclear translocation of GFP∼PDX-$1_{185–209}$, mutation K192A led to a less efficient nuclear translocation (Fig. 3H–J). Interestingly, Lys[192] is part of the most highly conserved region of the homeodomain, KIWFQN [24]. That the integrity of this motif per se is not a requirement for exclusive nuclear translocation was tested by exchanging Ile[193] versus Gln, a mutation that abolished DNA-binding [25]. This did not hinder nuclear translocation of PDX-1∼GFP (data not shown).

3.3. Glucose-dependent translocation of GFP-tagged PDX-1

Two recent reports addressed the question of glucose-dependent translocation of PDX-1 in insulin-producing MIN6 cells with different results [17,18]. Whereas Macfarlane et al. [17] demonstrated a glucose-dependent cytoplasmic-nuclear translocation of PDX-1, Rafiq et al. [18] reported an intranuclear change of PDX-1 distribution but did not observe a glucose-dependent cytoplasmic-nuclear translocation.

We aimed to address this question by monitoring on-line the potential glucose-induced cytoplasmic-nuclear translocation of GFP-tagged PDX-1. That stimulus-induced cytoplasmic-nuclear translocation can be studied in MIN6 cells by this approach has been proven by the dexamethasone-induced translocation of GFP-tagged glucocorticoid receptor (Fig. 4A–D). To analyze the potential glucose-dependent translocation of PDX-1, we looked for a condition where PDX-1 is enriched in the cytoplasm, i.e. where it is not yet translocated to the nucleus or has left the nucleus. However, neither short-term (up to 1 h) nor long-term (6–24 h) glucose starvation (0.5–2 mM glucose) following transfection led to an enhanced

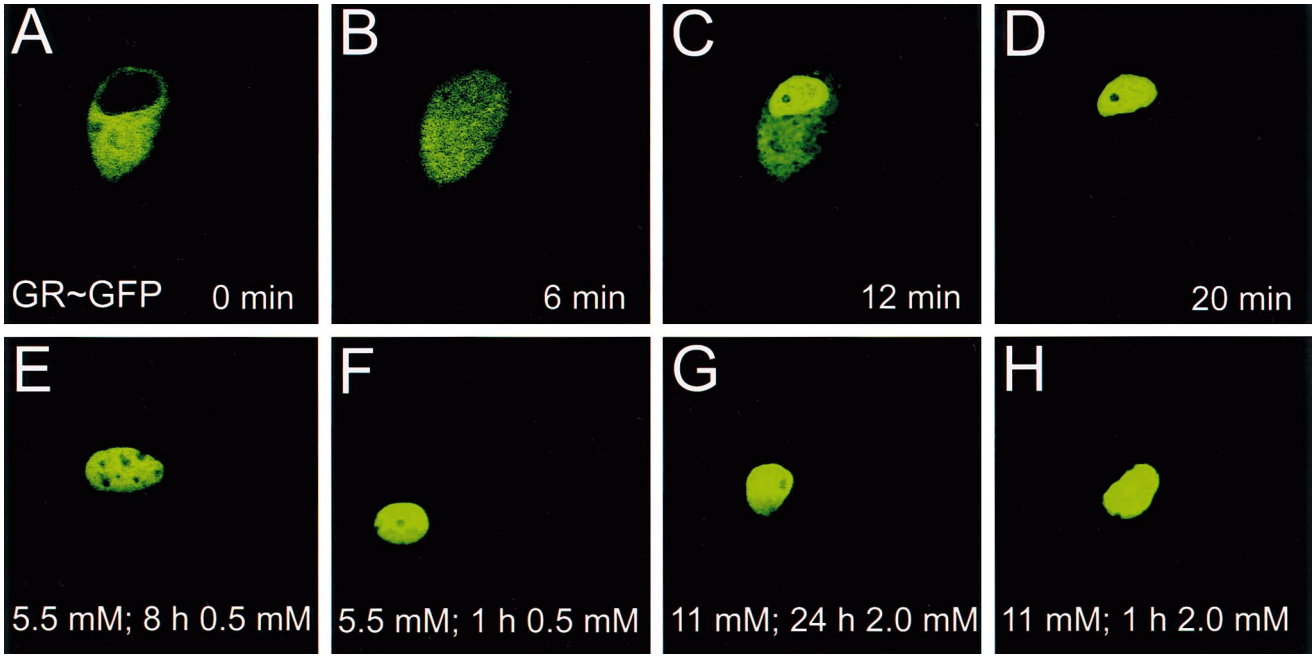

Fig. 4. Stimulus-induced nuclear translocation of glucocorticoid receptors and PDX-1. A–D: On-line monitoring of nuclear translocation of GFP-tagged glucocorticoid receptors following stimulation with 1 μM dexamethasone. Prior to and during stimulation with dexamethasone, the MIN6 cells were incubated in DMEM without phenol red and containing 10% charcoal-treated serum. E,F: Localization of GFP-tagged PDX-1 in transfected MIN6 cells that were cultured at 5.5 mM glucose and starved at 0.5 mM glucose for 8 and 1 h, respectively. G,H: Localization of GFP-tagged PDX-1 in transfected MIN6 cells that were cultured at 11.1 mM glucose and starved at 2.0 mM glucose for 24 and 1 h, respectively. Representative images (out of a total of 20) are shown.

localization of GFP-tagged PDX-1 in the cytoplasm (Fig. 4E–H). This was the case for MIN6 cells that were long-term-cultured at either 11 mM (starved at 2 mM glucose) or 5 mM glucose (starved at 0.5 mM glucose). This means, that we could not observe a nuclear to cytoplasm export of PDX-1 at reduced glucose concentrations. That the GFP-tag does not hinder the export of proteins from the nucleus has previously been demonstrated for GFP-tagged glucocorticoid receptors which return to the cytoplasm following removal of dexamethasone [19]. This finding was confirmed in this study on MIN6 cells (data not shown). On the other hand, if PDX-1 is actively translocated into the nucleus via the importin-Ran mechanism, this should be an energy-dependent step, i.e. GTP-dependent. In addition, endogenous PDX-1 biosynthesis may be glucose-dependent. The localization of freshly translated cytoplasmic PDX-1 molecules would have been missed in our detection system, because we monitored PDX-1 ~ GFP chimeras by fluorescence, and the formation of the GFP_{S65T} fluorophore after translation takes 30–60 min [26]. Therefore, the observed glucose-dependency in [17] may reflect the combination of the glucose-dependency of PDX-1 biosynthesis and its energy-dependent nuclear translocation rather than a glucose-dependent cytoplasmic-nuclear cycling of PDX-1.

3.4. Conclusion

Whereas nuclear import of PDX-1 does not seem to be dependent on the phosphorylation of a single specific phosphorylation site of PDX-1, it is dependent on the presence of the NLS motif RRMKWKK. This motif is conserved in mouse, rat and human PDX-1 sequences. The position of the NLS sequence, i.e. being part of the homeodomain, is in agreement with a previous observation by Lu et al. [25] that was obtained by deletional analysis. In fact, for exclusive nuclear translocation, the structural integrity of the 'helix 3' domain of the PDX-1 homeodomain seems to be a requirement and the presence of Lys^{192} facilitates this process. Our data do not explain the shift in molecular weight of PDX-1 from 31 kDa prior to translocation to 46 kDa after translocation, as reported by Macfarlane et al. [17]. However, the identification of a NLS in PDX-1 makes a piggyback mechanism, where the NLS is provided by the piggyback partner, unlikely. Although biosynthesis and nuclear import of PDX-1 are generally energy-dependent, our data show that PDX-1 does not cycle between the nucleus and cytoplasm in a glucose-dependent manner. Nuclear translocation of PDX-1 is a requirement for its action. Hence, the region encoding the 'helix 3' domain of the PDX-1 homeodomain is a further candidate region for MODY4.

Acknowledgements: We thank Thomas Schwarz-Romond, Zhang Fan and Sabine Kemper for excellent technical assistance. This work was supported by funds from the Karolinska Institutet, the Berth von Kantzows Foundation, the Swedish Medical Research Council (72X-12549, 72X-00034, 72X-09890 and 72XS-12708), The Novo Nordisk Foundation and the Juvenile Diabetes Foundation International (JDFI).

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