Oxidative Stress Induces Nucleo-Cytoplasmic Translocation of Pancreatic Transcription Factor PDX-1 Through Activation of c-Jun NH₂-terminal Kinase

Dan Kawamori,¹ Yoshitaka Kajimoto,¹ Hideaki Kaneto,¹ Yutaka Umayahara,¹ Yoshio Fujitani,¹ Takeshi Miyatsuka,¹ Hirotaka Watada,¹ Ingo B. Leibiger,² Yoshimitsu Yamasaki,¹ Hideaki Kaneto,¹ Yutaka Umayahara,¹ Yoshio Fujitani,¹ Takeshi Miyatsuka,¹ Hirotaka Watada,¹ Ingo B. Leibiger,² Yoshimitsu Yamasaki,¹ and Masatsugu Hori¹

Oxidative stress is induced in pancreatic β-cells under diabetic conditions and causes β-cell dysfunction. Antioxidant treatment of diabetic animals leads to recovery of insulin biosynthesis and increases the expression of its controlling transcription factor, pancreatic duodenal homeobox-1 (PDX-1), in pancreatic β-cells. Here, we show that PDX-1 is translocated from the nuclei to the cytoplasm of pancreatic β-cells in response to oxidative stress. When oxidative stress was charged upon β-cell-derived HIT-T15 cells, both endogenous PDX-1 and exogenously introduced green fluorescent protein–tagged PDX-1 moved from the nuclei to the cytoplasm. The addition of a dominant negative form of c-Jun NH₂-terminal kinase (JNK) inhibited oxidative stress–induced PDX-1 translocation, suggesting an essential role of JNK in mediating this phenomenon. Whereas the nuclear localization signal (NLS) in PDX-1 was not affected by oxidative stress, leptomycin B, a specific inhibitor of the classical leucine-rich nuclear export signal (NES), inhibited nucleo-cytoplasmic translocation of PDX-1 induced by oxidative stress. Moreover, we identified an NES at position 82-94 of the mouse PDX-1 protein. Thus, our present results revealed a novel mechanism that negatively regulates PDX-1 function. The identification of the NES, which overrides the function of the NLS in an oxidative stress-responsive, JNK-dependent manner, supports the complicated regulation of PDX-1 function in vivo and may further the understanding of β-cell pathophysiology in diabetes.

Diabetes 52:2896–2904, 2003

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From the ¹Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, Suita City, Osaka, Japan; and ²The Rolf Luft Institute, Stockholm, Sweden.

Address correspondence and reprint requests to Dr. H. Kaneto, Department of Internal Medicine and Therapeutics (A8), Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita City, Osaka Pref. 565-0871, Japan. E-mail: kaneto@medone.med.osaka-u.ac.jp.

Received for publication 8 July 2003 and accepted in revised form 18 September 2003.

H.W. is currently located at the Department of Medicine, Metabolism and Endocrinology, Juntendo University School of Medicine, Tokyo 113-8421, Japan.

ABC, avidin-biotin complex; DAPI, 4’,6-diamidino-2-phenylindole; DTT, dithiothreitol; GFP, green fluorescent protein; IB1, islet-brain-1; JNK, c-Jun NH₂-terminal kinase; MAPK, mitogen-activated protein kinase; NAC, N-acetyl L-cysteine; NES, nuclear export signal; NLS, nuclear localization signal; PDX-1, pancreatic duodenal homeobox-1; ROS, reactive oxygen species; USF-1, upstream stimulating factor-1.

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decrease of PDX-1 expression (3,4,18–20). The induction of ROS also seems to play a primary role in suppressing PDX-1 activity because the use of antioxidants can reverse the phenomenon both in vivo and in vitro (3,4).

Regulation of the intracellular localization of transcription factors is a crucial requirement for their action, and stimulus-dependent nuclear import can serve as a mechanism to regulate gene expression (21,22). This also seems true in the case of PDX-1; acute stimulation with high glucose induces import of PDX-1 from the nuclear periphery to the nucleus (23–25), and GLP-1 induces nuclear import of PDX-1 (26). In addition, a recent report showed that Foxo1, which acts as a transcriptional repressor in insulin signaling, inhibits PDX-1 function and expression by altering its intracellular localization from the nuclei to the cytoplasm (27). Thus, these observations suggest that the transcriptional activity of PDX-1 is in part physiologically regulated through posttranslational modification, which affects the intracellular localization of the protein.

On the other hand, PDX-1 translocation may also have some pathological significance. In pancreatic islets of mice fed a high-fat diet, PDX-1 was shown to be dominantly located in the cytoplasm, suggesting that the inability of the islets to adapt insulin resistance after a high-fat diet is associated with compromised translocation of PDX-1 to the nuclei (28). In addition, the reduction of nucleus-localized PDX-1 in diabetic animals is known to be markedly restored by antioxidant treatment (3,4). Because PDX-1 has a self activation mechanism, i.e., PDX-1–induced PDX-1 gene activation (29,30), a decrease of nuclear PDX-1 would instantly lead to a decrease of PDX-1 gene transcription, which would then cause further reduction of PDX-1 activity by decreasing the total amount of PDX-1 expressed in the cells.

In the present study, we investigated the possible effects of oxidative stress on the intracellular localization of the PDX-1 protein. We show here that oxidative stress induces nucleo-cytoplasmic translocation of PDX-1 through activation of the c-Jun NH2-terminal kinase (JNK) pathway. We also identified a classical leucine-rich nuclear export signal (NES) in PDX-1 that can override the function of its nuclear localization signal (NLS) in the presence of oxidative stress. The existence of a nucleo-cytoplasmic transport system supports the complicated regulation system of PDX-1 function in pancreatic β-cells and also reveals a possible target for oxidative stress in suppressing insulin gene expression and causing deterioration of the β-cell function found in diabetes.

**RESEARCH DESIGN AND METHODS**

**Materials.** Restriction enzymes, DNA polymerases, antibodies, and other modification enzymes were purchased from commercial suppliers (Toyobo, Tokyo, Japan; Takara, Kyoto, Japan; Sigma-Aldrich, St. Louis, MO; Santa Cruz Biotechnology, Santa Cruz, CA; Zymed, San Francisco, CA; and Stratagene, San Diego, CA). Tissue culture media were purchased from Nacalai Tesque, Tokyo, Japan; Takara, Kyoto, Japan; Sigma-Aldrich, St. Louis, MO; and Santa Cruz Biotechnology.

**Cell culture.** HIT-T15 cells (American Type Culture Collection no. 1777) were grown in RPMI-1640 medium containing 11.1 mmol/l glucose, 10% FCS, 100 μg/ml streptomycin, and 100 units/ml penicillin at 5% CO2 and 37°C. The HIT-T15 cells used in this study were between passages 80 and 100.

**Isolation of nuclear and whole-cell extracts and Western blot analyses.** The HIT-T15 cells were cultured in 100-mm diameter culture dishes until ~80% confluence. For isolation of nuclear extract, the cells were then collected into microtubes, centrifuged for 20 s in a microcentrifuge, and resuspended in 200 μl of 10.0 mmol/l Hepes, pH 7.9, containing 10.0 mmol/l KCl, 1.5 mmol/l MgCl2, and 0.5 mmol/l dithiothreitol (DTT). After incubation at 4°C for 15 min, cells were lysed by passing 10 times through a 22-gauge needle. Next, the cells were centrifuged for 20 s in a microcentrifuge, and the supernatant (cytoplasmic fraction) was removed and frozen in small aliquots. The pellet, which contained the nuclei, was resuspended in 150 μl of 20 mmol/l Hepes, pH 7.9, containing 20% vol/vol glycerol, 0.1 mol/l KCl, 0.2 mmol/l EDTA, 0.5 mmol/l DTT, and 0.5 mmol/l phenylmethylsulfonfyl fluoride and then stirred at 4°C for 30 min. The nuclear extracts were then centrifuged for 20 min at 4°C in a microcentrifuge. The supernatant was collected, aliquoted into small volumes, and stored at −80°C.

For isolation of whole-cell extracts, the cells were collected into microtubes, centrifuged for 1 min in a microcentrifuge, and resuspended in 150 μl of 20 mmol/l Hepes, pH 7.9, containing 20% vol/vol glycerol, 0.1 mol/l KCl, 0.2 mmol/l EDTA, 0.5 mmol/l DTT, and 0.5 mmol/l phenylmethylsulfonfyl fluoride and then stirred at 4°C for 30 min. After centrifugation for 20 min at 4°C in a microcentrifuge, the supernatant was collected as whole-cell extracts, aliquoted into small volumes, and stored at −80°C.

Western blot analyses were performed following standard procedures as described below. Nuclear, cytoplasmic, or whole-cell proteins derived from each cell sample were fractionated by SDS-PAGE, blotted onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA), and incubated at room temperature for 60 min in Tris-buffered saline buffer with a 1:5,000 dilution of anti-PDX-1 rabbit IgG antibody (16). The membrane was then incubated at room temperature for 60 min in Tris-buffered saline buffer with a 1:1,000 dilution of horseradish peroxidase–conjugated anti-rabbit IgG secondary antibody (Zymed) and then for 1 min with chemiluminescence reagent (NEN Life Science Products, Boston, MA). The antigen-antibody complex was detected by exposure to a light-sensitive film for the appropriate exposure time. Relative protein amounts were estimated by densitometry after scanning using a Fluorchem IS-8000 image analyzer system (Alpha Innotech, San Leandro, CA).

**Immunocytochemistry.** For immunostaining of cultured cells, the cells were seeded on a Lab-Tek chamber slide (Nalge Nunc International, Rochester, NY) and cultured for 2 days in appropriate medium. After rinsing with PBS three times, the cells were fixed with 4% paraformaldehyde for 20 min. For detection of PDX-1, the cells were microwaved in citrate buffer for antigen retrieval before being incubated with blocking serum. Each slide was treated with 3%,3′-diaminobenzidine tetrahydrochloride substrate (Zymed).

**Construction of expression plasmids and transient transfection.** The generation of mouse PDX-1-GFP (green fluorescent protein) fusion protein producing expression plasmids, pBSV.PDX-1-GFP, pCMV.PDX-1-GFP, and pB.CMV.PDX-1-GFP, was described previously (31). Direct mutagenesis was performed by the QuikChange XL Mutagenesis kit (Stratagene) and the required oligonucleotides purchased from Invitrogen Japan. Fidelity of sequence was confirmed by DNA sequence analyses. The pCS-JNK1 wild-type and dominant negative form (JNK1 APF) expression plasmids were kindly gifts from Dr. M. Hibi (Center for Developmental Biology, RIKEN, Japan) (32).

Twenty-four hours before transfection, HIT-T15 cells were replated in 35-mm diameter glass-bottomed tissue culture plates. One microgram of each reporter plasmid was used for the transfection of cells in each culture plate by the lipofection method using LipofectAMINE reagent (Invitrogen Japan) under the conditions recommended by the manufacturer. After transfection, cells were cultured for a further 48 h in RPMI medium until examination of the intracellular localization.

**Examination of the intracellular localization of GFP-tagged proteins.** To examine the intracellular localization of GFP-tagged proteins, the cells were exposed to the excitation wavelength for GFP (485 nm) and visualized by laser-scanning confocal microscopy using the appropriate filters. The intracellular localization of GFP was observed without fixation. To visualize the nuclei using fluorescence microscopy, 48 h after transfection, the cells were fixed for 3 h in 4% paraformaldehyde in PBS, washed twice with PBS, and incubated with 4′,6-diamidino-2-phenylindole (DAPI; diluted to 1 μg/ml in PBS) at room temperature for 60 min.

Laser-scanning confocal microscopy was performed using a Nikon Eclipse ELWD fluorescence microscope (Tokyo, Japan) and a laser scanning system with appropriate filters (Carl Zeiss LSM410; Carl Zeiss, Oberkochen, Germany). Photographs of the cells were taken and processed using a...
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Macintosh G3 with a C5910 refrigerated digital charge-coupled device camera unit (Hamamatsu Photonics, Hamamatsu, Japan) and the digital imaging system Mac SCOPE MSC-1U (Mitani, Fukui, Japan). Presentation images were generated using Adobe Photoshop version 5.0 and Aldus Persuasion version 3.0. For cell counting, photographs of the cells were randomly taken and 10 fields containing >100 positive cells/field were randomly selected for analyses. Cell counts were done by a third party that was blind to the conditions.

RESULTS

Oxidative stress induces nucleo-cytoplasmic translocation of PDX-1. To investigate the effect of oxidative stress on the expression and intracellular localization of PDX-1, we first performed Western blot analyses using nuclear and whole-cell extracts isolated from the pancreatic β-cell–derived cell line HIT-T15. Separation of the nuclear components from the cytoplasmic components was verified by the presence or absence of upstream stimulating factor-1 (USF-1) (33) and β-actin by Western blot analysis (data not shown). As shown in Fig. 1A, the amount of nuclear PDX-1 was decreased by oxidative stress (50 μmol/l H2O2). The decrease became evident within 6 h after the initiation of oxidative stress and continued for at least 24 h (Fig. 1A, upper panel). Unlike PDX-1, the expression of USF-1 in nuclei was not altered by oxidative stress (Fig. 1A, lower panel). This was in contrast to the amount of PDX-1 derived from whole-cell extracts, which did not alter significantly (Fig. 1B).

Next, we directly examined the intracellular distribution of PDX-1 by immunostaining. Agreeing with the results of Western blot analyses, PDX-1, which was localized almost exclusively in the nuclei under normal culture conditions, moved to the cytoplasm in the presence of 50 μmol/l H2O2 and was inhibited by the addition of an antioxidant, N-acetyl l-cysteine (NAC; 10 mmol/l) (Fig. 1C). Thus, these results indicated that oxidative stress changes the intracellular localization of PDX-1 from nuclei to cytoplasm.

To evaluate the mechanism underlying this phenomenon, we used a GFP-tagged PDX-1 fusion protein. Like endogenous PDX-1 (Fig. 1), GFP-tagged PDX-1 was also localized dominantly in the nuclei when transfected HIT-T15 cells were cultured under basal conditions (Fig. 2A). More than 86% of the cells revealed only nuclear localization, with only a small fraction showing cytoplasmic localization. In addition, in agreement with the results of endogenous PDX-1 (Fig. 1), the addition of H2O2 totally reversed the results. GFP-tagged PDX-1 was dispersed from the nuclei and mainly found in the cytoplasm (Fig. 2B); ∼85% of the cells showed cytoplasmic PDX-1 localization, with <3% preserving nuclear PDX-1 expression. The addition of an antioxidant (10 mmol/l NAC) partially neutralized the effects of H2O2 (Fig. 2C). Thus, the GFP-tagged PDX-1 seems to mimic endogenously expressed PDX-1 in terms of its intracellular localization in HIT-T15 cells. In addition, the GFP-tag that had been added to the COOH-terminus (Fig. 2) or NH2-terminus of PDX-1 (data not shown) did not spoil the oxidative stress–dependent PDX-1 translocation, suggesting the usefulness of this GFP-tagged PDX-1 system for the evaluation of mechanisms underlying the oxidative stress–mediated PDX-1 translocation in pancreatic β-cells. Time-dependent changes of the intracellular localization of PDX-1 by oxidative stress were also investigated using PDX-1-GFP. One hour after the oxidative stress load, the nuclear export of PDX-1 was not evident. The cytoplasmic localization of PDX-1 could be observed from 6 h after the load, and 24 h after the load, the majority of cells showed nuclear localization of PDX-1, which was the same as seen 48 h after the load (data not shown). These data are consistent with the data represented in Fig. 1.

Oxidative stress-induced PDX-1 translocation depends on JNK activation. Two major mitogen-activated protein kinase (MAPK) pathways, involving JNKs or p38 MAPKs, can be activated by a variety of extracellular stress signals, such as ultraviolet, heat, and oxidative stress, and induce various cellular responses (33,34). To clarify the mechanism underlying the oxidative stress–dependent intracellular redistribution of PDX-1, we investigated the possible
implication of the stress-related MAPK pathways in this phenomenon.

As shown in Fig. 3, when a dominant negative form of JNK (32) was expressed in HIT-T15 cells by cotransfection of a dominant negative form of JNK expression plasmid together with the PDX-1-GFP fusion protein—expressing plasmid, the H_2O_2-responsive PDX-1 redistribution to the cytoplasm was mostly inhibited (Fig. 3C). In contrast, no

FIG. 2. Oxidative stress–induced nucleo-cytoplasmic translocation of GFP-tagged PDX-1. Wild-type PDX-1-GFP fusion protein (shown at the bottom) was expressed in HIT-T15 cells by transient plasmid transfection, and the cells were exposed for 48 h to vehicle alone (A), 50 μmol/l H_2O_2 (B), or 50 μmol/l H_2O_2 and 10 mmol/l NAC (C). The fusion protein was visualized by laser-scanning confocal microscopy, and DAPI was used for nuclear staining. For cell counts shown in the graph (shown in bottom), 10 randomly selected picture fields containing >100 GFP-positive cells derived from three independent experiments were analyzed. The percentage of the cells with GFP-tagged PDX-1 in the nucleus (N), the cytoplasm (C), or both (B) were calculated and expressed as means ± SD. *P < 0.001.

FIG. 3. Oxidative stress–induced nucleo-cytoplasmic translocation of PDX-1 through activation of JNK but not p38 MAPK. Wild-type PDX-1-GFP fusion protein (shown in Fig. 2) was expressed in HIT-T15 cells by transient plasmid transfection, and the cells were exposed for 48 h to vehicle alone (A), 50 μmol/l H_2O_2 (B and C), or 50 μmol/l H_2O_2 plus 20 μmol/l SB203580 (D). C: A dominant negative form of JNK (JNK DN) was coexpressed in the cells. The fusion protein was visualized by laser-scanning confocal microscopy. For cell counts shown in the graph (bottom panel), 10 randomly selected picture fields containing >100 GFP-positive cells derived from three independent experiments were analyzed. The percentage of the cells with GFP-tagged PDX-1 in the nucleus (N), the cytoplasm (C), or both (B) were calculated and expressed as means ± SD. *P < 0.001. NS, not significant. The data shown in Fig. 2 are also shown here to facilitate comparison.
such effects were observed when the p38 MAPK pathway was blocked by addition of a specific inhibitor, SB203580 (Fig. 3D) (35). Moreover, when wild-type JNK was overexpressed by plasmid cotransfection, the GFP-tagged PDX-1 was found mostly in the cytoplasm even without oxidative stress (data not shown). These observations thus suggest that the JNK pathway, but not the p38 MAPK pathway, partially mediates the oxidative stress-induced cytoplasmic redistribution of PDX-1 in pancreatic β-cells.

**PDX-1 NLS is not oxidative stress sensitive.** Intracellular distribution of proteins is determined by a diversity of mechanisms, but in general, nuclear targeting of proteins starts with the recognition of an NLS, which resides in nuclear proteins, by a carrier protein, importin-α, in the cytoplasm (21,22). The association and dissociation between NLS and importin-α are controlled distinctively in the cytoplasm and nuclei with the aid of guanosine 5′-triphosphate (GTP) or guanosine 5′-diphosphate (GDP)-bound Ran proteins, and this enables nuclear targeting of transcription factors. Like many other transcription factors, PDX-1 also has an NLS (31,36). To identify the region of PDX-1 that is involved in the oxidative stress–induced redistribution of PDX-1, we investigated the possibility of oxidative stress altering the NLS function of PDX-1.

As shown in Fig. 4, when a GFP-tagged fusion protein containing only the NLS portion of PDX-1 (GFP-PDX-1_185–209) was expressed in HIT-T15 cells, the fusion protein was mainly located in the nuclei, regardless of the presence of oxidative stress or overexpressed JNK. Although a weak signal could also be detected in the cytoplasm, it did not seem to be affected by oxidative stress or JNK (Fig. 4). On the other hand, in expression of a PDX-1-GFP fusion protein with a disrupted NLS (31), an almost equal distribution of PDX-1 in both the nuclei and cytoplasm under the basal conditions was observed (Fig. 5A). However, when oxidative stress was charged upon the cells by adding 50 μmol/l H2O2 or when JNK expression was induced in the cells by cotransfection of an expression plasmid, GFP-tagged NLS-mutated PDX-1 responded and the amount of PDX-1 was decreased in the nuclei (Fig. 5B and C). These findings thus suggest that the NLS of PDX-1 is oxidative stress resistant and is not involved in the oxidative stress–induced nucleo-cytoplasmic redistribution of PDX-1.

**PDX-1 has a classical leucine-rich NES that responds to oxidative stress.** Because the PDX-1-GFP fusion protein that lacks the NLS could still react to oxidative stress and reduce its amount in the nuclei, we further examined the possibility of some other mechanism inducing nucleo-cytoplasmic transport of PDX-1.

Leptomycin B is an inhibitor of nucleo-cytoplasmic transport of various transcription factors that have leucine-rich type NES (37–39). It functions by inhibiting the association between NES sequences in cargo proteins (transcription factors, etc.) and an exporting molecule, CRM1 (40,41). To obtain a clue toward clarifying the mechanism of the oxidative stress–induced NLS-independent nucleo-cytoplasmic redistribution of PDX-1, we examined whether or not the phenomenon is leptomycin B sensitive. As shown in Fig. 6, addition of 2 ng/ml leptomycin B could in part restore the expression of PDX-1 in nuclei (Fig. 6B and C). Accordingly, we surveyed the whole–amino acid sequence of mouse PDX-1 and found a region that resembles the leucine-rich sequences of NES reported previously in other proteins (Table 1) (38,39,42).

To investigate whether the leucine-rich region found in PDX-1 could indeed function as an NES, we performed site-directed mutagenesis. As shown in Fig. 7, mutations introduced into the NES-like region affected the oxidative stress– or JNK-induced nucleo-cytoplasmic redistribution of PDX-1, suggesting that the region functions as an NES and, when activated by oxidative stress, the NES can override the function of NLS that also resides in the same molecule.

**FIG. 4.** No alternation in NLS function of PDX-1 by oxidative stress. GFP-PDX-1-NLS fusion protein (shown at the bottom) was expressed in HIT-T15 cells by transient plasmid transfection. **B:** Oxidative stress was induced by incubation with 50 μmol/l H2O2 for 48 h. **C:** JNK was overexpressed by cotransfection of a wild-type JNK expressing plasmid. **A:** Vehicle alone was added. The fusion protein was visualized by laser-scanning confocal microscopy.
DISCUSSION

In the present study, we showed that oxidative stress induces nucleo-cytoplasmic translocation of PDX-1 through activation of the JNK pathway. In addition, we identified a classical leucine-rich NES within PDX-1 and showed that the activation of this NES is implicated in the oxidative stress-induced nuclear export of PDX-1. When cells are subjected to oxidative stress, this NES in PDX-1 is activated in a JNK-dependent manner and overrides the function of NLS, leading to translocation of PDX-1 from the nuclei to the cytoplasm. Moreover, because the PDX-1 gene is transactivated by PDX-1 itself (29,30), the translocation may lead to a vicious cycle in which the decrease of PDX-1 expression in nuclei results in a further decrease of PDX-1 expressed in the cells. Thus, the oxidative stress-induced nucleo-cytoplasmic translocation of PDX-1 may play a crucial role in suppression of insulin gene expression and biosynthesis under diabetic conditions.

NES has characteristic sequences of hydrophobic amino acids residues, which are leucine-rich sequences (38,39). The carrier protein CRM1 binds to the NES sequence and thereby transports the target protein from the nucleus to the cytoplasm in a Ran-GTP-dependent manner (21,40,41). Leptomycin B functions as a specific inhibitor of this nuclear export system by directly binding to the CRM1 NES binding site (37). Despite these well-conserved common features of leucine-rich NES, the patterns of regulation may vary. For example, NES of cyclin B1 is phosphorylated, and this phosphorylation is essential for its affinity to CRM1, implying that NESs per se can be a target of regulation (43). On the other hand, for the case of protein kinase inhibitor (39) or influenza NS1 protein (44),...
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TABLE 1
NES sequence alignments and related amino acid sequences in PDX-1*

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1 Rev protein</td>
<td>LQLPPLERLTLTD</td>
</tr>
<tr>
<td>Protein kinase inhibitorα</td>
<td>ELALKLAGLDIN</td>
</tr>
<tr>
<td>MAPK kinase</td>
<td>ÅIQKKEEPLELD</td>
</tr>
<tr>
<td>NES consensus</td>
<td>XLX 2-3LX 2-3LXLX</td>
</tr>
<tr>
<td>PDX-1 Mouse</td>
<td>s2HLHHHHPAQLG L94</td>
</tr>
<tr>
<td>Rat</td>
<td>s1HLHHHHPAQLG L93</td>
</tr>
<tr>
<td>Human</td>
<td>s1HLHHHHPAQLLAP L93</td>
</tr>
</tbody>
</table>

*The functionally important hydrophobic residues are underlined.

It seems to be not the NESs themselves but the neighboring sequences that directly respond to stimuli. The response changes the conformation of the protein and activates the NES. Because the NES of PDX-1 lacks serine, threonine, and even tyrosine residues (Table 1), the surrounding regions may participate in the regulation of its NES.

Although this study clearly showed that JNK is involved in oxidative stress–induced PDX-1 translocation (Fig. 3), the mechanism underlying JNK-dependent NES activation has not been clarified. However, the mechanism may be similar to that for an Ets family transcription factor, Net. Net is also translocated from the nuclei to the cytoplasm in a JNK-dependent manner and has a leucine-rich NES (45), but the NES site of Net does not have potential JNK phosphorylation sites. Instead, Net has a JNK docking site and a direct phosphorylation site outside of the NES region, suggesting that the phosphorylation induces a conformational change in Net and thereby activates its NES (46). Whereas the NES region found in PDX-1 also lacks a serine-proline or threonine-proline residue that could be a phosphorylation target for JNK (Table 1), a similar mechanism may operate for JNK-dependent nucleo-cytoplasmic transport of PDX-1. Further analysis is required to clarify the detailed mechanism underlying the JNK-dependent activation of the NES in PDX-1.

Intracellular shuttling of PDX-1 has been reported to occur between the nuclear periphery and the nucleus in response to acute stimulation with high glucose (23–25). However, in contrast to this observation, our present study revealed that both endogenously and exogenously introduced PDX-1 were localized in the nucleus (both the periphery and the nucleoplasm) under basal conditions and moved from the nucleus to the cytoplasm in response to oxidative stress. We also investigated the change of intracellular localization of PDX-1 among basal (11.1 mmol/l), low (5.5 mmol/l), and high (25 mmol/l) glucose concentrations, but changes in glucose concentration did not affect the localization of PDX-1 or the response of PDX-1 to oxidative stress in HIT cells (data not shown). These results may suggest that the mechanism of oxidative stress–induced nuclear export of PDX-1 is fundamentally different from that of the glucose-mediated translocation of PDX-1 from the nuclear periphery to the nucleoplasm.

In the present study, we found that the JNK pathway but not the p38 MAPK pathway mediates oxidative stress–induced nucleo-cytoplasmic translocation of PDX-1 (Fig. 3). Because both JNK and p38 are activated by oxidative stress in pancreatic β-cells (47), this probably implies that the activation of JNK can lead to activation of the NES of PDX-1. In agreement with our present observation, a recent study has shown that JNK is involved in oxidative stress–induced suppression of insulin gene expression in β-cells (47). Indeed, the study also identified a JNK-dependent reduction of PDX-1 activity in the nuclei, suggesting that our present observation may explain the mechanism underlying the phenomenon observed in that study. Another support for the significance of JNK in the development of diabetes comes from the result of genetic analysis in humans. Islet-brain-1 (IB1), the human and rat homologue of mouse JNK-interacting protein-1 (48,49), forms complexes with components of the JNK signaling pathway, MLK3, MKK7, and JNK and selectively inhibits JNK signaling (50). Although IB1 was initially identified as an inducer of GLUT2 gene expression in pancreatic β-cells (48), it also inhibits cytokine-induced apoptosis in these cells (50,51). In addition, a missense mutation within the IB1-encoding MAPKIP1 gene (S59N) is associated with...
late-onset type 2 diabetes (51). Moreover, it has been reported that JNK is activated in several tissues of diabetic rats and humans (52). These observations thus suggest that the JNK pathway is implicated not only in β-cell apoptosis in type 1 diabetes (53) but also in deterioration of β-cell function in type 2 diabetes (51) and provide support for the pathophysiological significance of our present observations.

ROS are induced in the pancreatic islets of diabetic animals mainly due to persistent hyperglycemia (6), and suppression of oxidative stress by antioxidant in diabetic animals prevents progression of β-cell dysfunction in type 2 diabetes (3–5,7). Changes in PDX-1 activity may partially explain the phenomenon because PDX-1 is often reduced simultaneously with the progression of diabetes in various in vitro (3,19,20) and in vivo models (3,4,18), and antioxidant treatment also leads to recovery of PDX-1 expression (3,4). In our investigation, we used H2O2 for inducing oxidative stress. We previously reported that oxidative stress induces not only β-cell dysfunction but apoptosis (54). However, the number of apoptotic cells was relatively low when this strength of oxidative stress was charged. Moreover, with our examination method, apoptotic cells were excluded from the evaluation and calculation. Also, we would like to note that this concentration of H2O2 had no effect on the localization of several GFP-tagged proteins unless they had a functional NES sequence: the intracellular localization of the NES-disrupted PDX-1-GFP, NLS portion-GFP, or GFP alone was not changed in response to oxidative stress in HIT cells. These findings suggest that oxidative stress–induced nucleo-cytoplasmic translocation of PDX-1 was not relevant to reduction of cell viability. Thus, it is likely that the mechanism that we have identified in the present study occurs in vivo under diabetic conditions and can explain the decrease in PDX-1 function in diabetes. Establishment of a mouse strain carrying NES-deficient mutant PDX-1 may be a useful model for evaluating the pathophysiological significance of this phenomenon. In addition, the RIPE3b1 activator (recently defined as MaFA), which is an important transcription factor for glucose-responsive insulin gene transcription, has been reported to show reduced function after chronic exposure to high concentrations of glucose (55,56). Although the precise mechanism for this phenomenon has not been analyzed, it is possible that the phenomenon observed in our study for PDX-1 also occurs with the RIPE3b1 activator. Nucleo-cytoplasmic transport of the RIPE3b1 activator might occur in response to various stimuli, such as acute and chronic high concentrations of glucose.

Apart from its pathological implications, PDX-1 should not be equipped with an NES unless it has a certain physiological role. Although highly speculative, PDX-1 activity may need to be suppressed under certain circumstances. For example, although PDX-1 is continually expressed in the pancreas during its development, the expression rate seems to vary depending on the developmental stage (12). The NES of PDX-1 may be involved in the regulation of PDX-1 expression during development because PDX-1 is an activator of its own gene (29,30). In addition, support for this possibility comes from the observation that in some ductal cells known as potential progenitors of pancreatic β-cells, PDX-1 is mainly localized in the cytoplasm (57), suggesting that the function of PDX-1 may be regulated at the level of intracellular distribution during pancreas development.

In conclusion, we have shown that oxidative stress induces nucleo-cytoplasmic translocation of PDX-1 through activation of the JNK pathway and have identified a classical leucine-rich NES for which activation is implicated in oxidative stress–induced translocation of PDX-1. Because oxidative stress is induced in pancreatic islet cells in diabetes (6,7), the NES may mediate the suppression of PDX-1 function and thereby cause β-cell dysfunction.

ACKNOWLEDGMENTS

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by grants from the Smoking Research Foundation, Kyowa Hakko Kogyo (to Y.K. and Y.Y.), and the Swedish Research Council (to I.B.L.). H.W. and Y.F. were recipients of fellowships from the Juvenile Diabetes Research Foundation and the Japan Society for the Promotion of Science, respectively.

We thank Dr. M. Hibi (Center for Developmental Biology, RIKEN, Japan) for providing precious plasmids. We also thank Chikayo Yokogawa for her superb secretarial services and Yoko Sasaki for excellent technical assistance.

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