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Nuclear Translocation of SHP and Visualization of Interaction with HNF-4α in Living Cells

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Mutations in small heterodimer partner (SHP) and hepatocyte nuclear factor 4α (HNF4α) are associated with mild obesity and diabetes mellitus, respectively. Both receptors work together to determine the normal pancreatic β-cell function. We examined their subcellular localization and interaction in living cells by tagging them with yellow and cyan variants of green fluorescent protein (GFP) variants. Expressed SHP resided only in the cytoplasm in COS-7 cells which lacks HNF4α, but predominantly in the nucleus in insulinoma cells (MIN6). HNF4α was localized exclusively in the nuclei of both cells, coexpressed with HNF4α in COS-7 cells, redistributed in the nucleus, depending on the amount of HNF4α. We found fluorescence resonance energy transfer between GFP-tagged SHP and HNF4α, indicating a specific close association between them in the nucleus. The results strongly suggest that SHP exists primarily in the cytoplasm and is translocated into the nucleus on interacting with its nuclear receptor partner HNF4α.

Key Words: **small heterodimer partner; hepatocyte nuclear factor 4α; fluorescence resonance energy transfer; subcellular localization; diabetes mellitus.**

The orphan nuclear receptor small heterodimer partner (SHP) functions as a negative regulator of nuclear receptor signaling pathways (1–4). SHP lacks the DNA-binding domain (1) and interacts with various nuclear receptors including estrogen (2, 3) and retinoic acid receptors inhibiting their activity presumably by coactivator competition or direct repression (4). The orphan receptor hepatocyte nuclear factor 4α (HNF4α), a key regulator of gene expression in the liver, insulin-secreting pancreatic β-cell and other tissues (5–8), is also a target of SHP (4). In the pancreatic β-cell, HNF4α regulates the expression of a number of several proteins implicated in the regulation of insulin secretion by glucose including glucose transporter-2, aldolase B and L-pyruvate kinase (9). Loss-of-function mutations in human HNF4α cause one form of diabetes mellitus called maturity-onset diabetes of the young type 1 (MODY1) (10) which is characterized in part by a diminished insulin secretory response to glucose (11). It has recently been shown that loss-of-function mutations in SHP led to a childhood form of mild obesity characterized high birth weight and hyperinsulinemia (12). It has been suggested that the transcriptional activity of HNF4α in β-cells is enhanced in these patients because of release from the inhibition by SHP thereby leading to hypersecretion of insulin, stimulation of adipogenesis and obesity (12). Thus, SHP and HNF4α may function together in the regulation of insulin secretion.

Cytoplasm-to-nucleus translocation is a prerequisite first step in order for nuclear receptors to exert their actions. HNF4α possesses a nuclear localization signal in the region between the DNA-binding and putative ligand-binding domains and resides in the nucleus (13, 14). However, SHP lacks an obvious nuclear localization signal and it is unclear how SHP translocates to the nucleus.

In the present study, we examined the subcellular localization of SHP in relationship to HNF4α in monkey kidney COS-7 cells (15) and mouse insulinoma MIN6 cells (8, 9). HNF4α is endogenously expressed in insulinoma cell lines (8, 9) but not in COS-7 (15). We tagged SHP and/or HNF4α with enhanced yellow fluorescent protein (EYFP) (16) or enhanced cyan fluorescent protein (ECFP) (17) to examine the association of the two receptors *in situ* by detecting fluorescence resonance energy transfer (FRET) (18) between them. We

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show that the cytoplasm-to-nucleus translocation of SHP depends on the presence of HNF4α.

MATERIALS AND METHODS

Expression constructs. The enhanced yellow fluorescent protein (EYFP) (16), enhanced cyan fluorescent protein (ECFP) (17), or FLAG (DYKDDDDKG)-tagged SHP (GenBank Accession No. AF044316) and HNF4α2 (No. U72969) were constructed by standard molecular biological techniques. PCR-generated fragment of SHP or HNF4α2 was cloned in a frame into *Nhe*I and *Kpn*I sites of pEGFP-N3 (Clontech Lab. Inc., Palo Alto, CA), fused to the N terminus of EGFP. Then EGFP was replaced by PCR fragment of EYFP or ECFP generated from pEYFP-C1 or pECFP-C1 (Clontech) at *Kpn*I and *Not*I sites of SHP- or HNF4α2-EGFP (Fig. 1). The cDNAs of FLAG-SHP and FLAG- HNF4α2 (Fig. 1) were constructed in pBK-CMV (Stratagene, Cedar Creek, TX). The sequences of all constructs were confirmed by DNA sequencing. HNF4α2 is referred to as HNF4α throughout this paper.

Cell culture and transient transfections. COS-7 and MIN6 cells were cultured in Dulbecco's modified Eagle medium (Sigma, St. Louis, MO) containing 70 μM 2-mercaptoethanol and 10% or 20% fetal bovine serum, respectively. Cells were grown in glass-bottomed dishes. Plasmid DNA was introduced into cells by transfection using FuGENE 6 reagent (Roche Molecular Biochemicals, Mannheim, Germany). In some experiments, SHP-EYFP and HNF4α were co-introduced into COS-7 cells with 4 μg of total DNA at different SHP-EYFP/HNF4α ratios (300, 100, 30, 10, 3, and 1).

Determination of the subcellular localization of expressed proteins. Twenty-four hours after transfection, cells were washed twice with PBS(−), and the subcellular localization of EYFP-tagged proteins was observed by a confocal laser scanning microscope (LSM310; Carl Zeiss, Oberkochen, Germany) using a 488-nm argon laser. Details have been described previously (19). FLAG-tagged proteins were observed by an immunohistochemical method with an anti-FLAG monoclonal antibody (Sigma, St. Louis, MO) and Cy3-labeled secondary antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK) in cells permeabilized by 0.1% Triton X-100.

For analysis of fluorescence images, cells were observed under a microscope (IX-70; Olympus, Tokyo, Japan) equipped with a silicon intensifier target camera (C2400-08; Hamamatsu Photonics, Hamamatsu, Japan). Using band-pass filters, excitation light was set at 480 ± 10 nm for EYFP or 420 ± 15 nm for ECFP, and emission light was detected at 525 ± 13 nm for EYFP or 480 ± 15 nm for ECFP. To examine FRET, 420 and 480 nm excitation lights were separated by 455-nm dichroic mirror (Omega Optical, Inc.; Brattleboro, VA). Images were acquired using an image processor (XL-20; Olympus) and processed using Adobe Photoshop software (Adobe System Inc., San Jose, CA).

Calculation of the fraction of fluorescence in the nucleus. To quantify the effect of HNF4α on the nuclear localization of SHP-EYFP, fluorescence images under a conventional fluorescence microscope were digitally acquired into two-dimensional array of picture elements (pixels; 0.35 × 0.35 μm squares), and the fluorescence intensity (F) of SHP-EYFP in each pixel was transduced to arbitrary values from 0 (black) to 65536 (white) using a linear range. We transfected COS-7 cells with different ratios between SHP-EYFP and HNF4α DNAs keeping the total amount of DNA constant. The areas of the cell and the nucleus were determined by manually tracing the contour on the corresponding blight field image, and F was integrated in each area. We calculated the ratio of F integrated in the nucleus (F_{nucleus}) to total F in the whole cell (F_{total}) in an optical section ($F_R = F_{\text{nucleus}}/F_{\text{total}}$) using IPLab software (Signal Analysis Co., Vienna, VA). The amount of expressed SHP-EYFP in the whole nucleus might be underestimated when presented by F_{nucleus}, because the nucleus has a certain thickness while the cytoplasm was flattened in cultured cells.

Data are mean ± SD, and one-way analysis of variance (ANOVA) and Bonferroni's t test were used to evaluate the statistical significance.

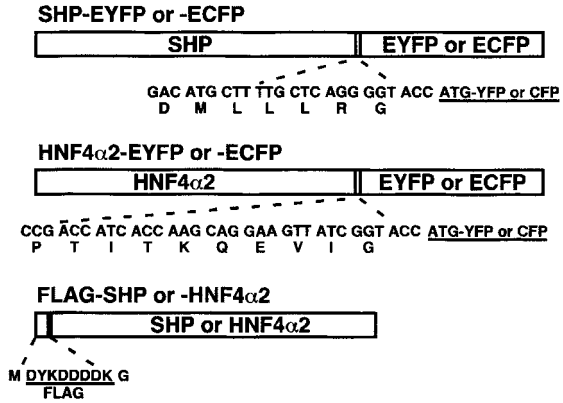

FIG. 1. Schematic presentation of EYFP-, ECFP-, or FLAG-tagged SHP and HNF4α constructs.

RESULTS

Subcellular Localization of SHP and HNF4α

We examined COS-7 and MIN6 cells by confocal microscopy 24 h after transfection with constructs encoding SHP-EYFP or HNF4α-EYFP (Fig. 1). SHP-EYFP was present in the cytoplasm in living COS-7 cells (Fig. 2A). As the cytoplasm was flattened on the bottom of a culture dish, most of EYFP fluorescence could be observed in a focal plane. SHP-EYFP in the cytoplasm showed an inhomogeneous distribution consisting of sporadic granular spots of various shapes and sizes (Fig. 2A). It should be noted that no substantial SHP-EYFP was found in the nucleus at any focal plane. On the other hand in MIN6 cells, SHP-EYFP was located predominantly in the nucleus although it was also present in the cytoplasm (Fig. 2B). It showed a punctuate distribution in the cytoplasm as seen in COS-7 and accumulated in a non-homogeneous fashion in the nucleus. The EYFP tag, a relatively large moiety at the C-terminus of SHP, did not affect the localization of SHP. A similar distribution was observed using a FLAG tag (a small peptide of 8 amino acids) at the N-terminus of SHP as a marker (Fig. 1) and visualization with an anti-FLAG monoclonal antibody and a Cy3-labeled secondary antibody in the cells permeabilized by Triton X-100 (data not shown).

In contrast to SHP-EYFP, HNF4α-EYFP was localized exclusively in the nucleus in both COS-7 and MIN6 cells (Figs. 2C and 2D). HNF4α-EYFP distributed throughout the nucleus with the exception of the nucleoli (dark spots in Fig. 2C and 2D) in such a way that the contour of the nucleus was clearly discernible. The distribution of HNF4α-EYFP in the nucleus was relatively homogeneous although granular spots were

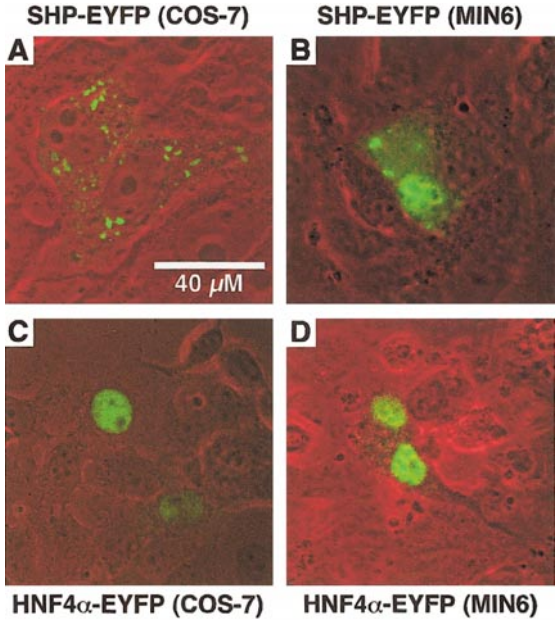

FIG. 2. Subcellular localization of SHP-EYFP (A and B) and HNF4α-EYFP (C and D) in COS-7 and MIN6 cells observed by confocal microscopy.

observed in some cells (see Fig. 3B). The nuclear localization of HNF4α-EYFP (tagged at the C-terminus) in COS-7 and MIN6 was similar to that of EGFP-HNF4α (i.e., HNF4α tagged at the N-terminus) in HIT-T15 and HeLa cells in the studies of Laine *et al.* (14). Thus, the location of the GFP tag (i.e., C- or N-terminus) does not affect the subcellular distribution of HNF4α.

Dependence of SHP Translocation on the Presence of HNF4α

The preferential localization of SHP-EYFP in the cytoplasm in COS-7 cells and the nucleus in MIN6 cells suggests that the nuclear translocation of SHP may rely on the presence of a target such as HNF4α. To examine this possibility, we coexpressed SHP-EYFP and wild-type HNF4α (i.e., HNF4α without a GFP tag) in COS-7 cells. The majority of the SHP-EYFP resided in the nucleus and was distributed throughout the nucleus when it was coexpressed with HNF4α in COS-7 cells (Fig. 3A). Some fluorescence-dense spots were recognized either in the periphery of the nucleus or in the perinuclear cytoplasmic region (Fig. 3A), the precise localization of these spots was not determined. In these co-expressing cells, SHP does not interfere with the translocation of HNF4α to the nucleus, since HNF4α-EYFP was localized in the nucleus when it was coexpressed with non-EYFP-tagged SHP (Fig. 3B).

We transfected COS-7 cells with different ratios between SHP-EYFP and HNF4α DNAs keeping the total amount of DNA constant. To quantify the effect of HNF4α on the nuclear localization of SHP-EYFP, we

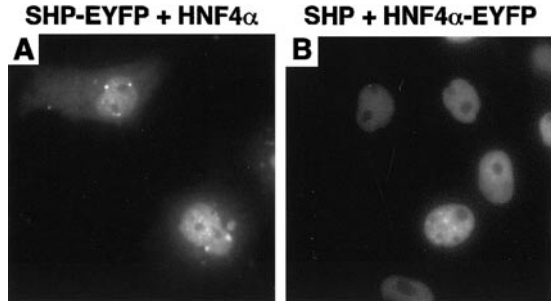

FIG. 3. Localization of SHP and HNF4α in coexpressing COS-7 cells observed by fluorescence microscopy. (A) SHP-EYFP coexpressed with non-EYFP-tagged HNF4α. (B) HNF4α-EYFP coexpressed with non-EYFP-tagged SHP.

calculated the ratio of F integrated in the nucleus (F_{nucleus}) to total F in the whole cell (F_{total}) in an optical section ($F_R = F_{\text{nucleus}}/F_{\text{total}}$). The F_R was about 20% in the absence of HNF4α (Fig. 4, left column). This was considered to be partly due to background fluorescence and stray fluorescence from the cytoplasm. The F_R increased in a dose-dependent manner depending upon the relative ratio (μg/μg) of HNF4α/SHP-EYFP DNA added (Fig. 4). F_R was significantly elevated even when the DNA ratio between SHP-EYFP and HNF4α was 300:1 ($P < 0.001$). F_R was 80% for when equal amounts of HNF4α and SHP-EYFP DNA (i.e., 1:1) were added which was comparable to the F_R for expression of

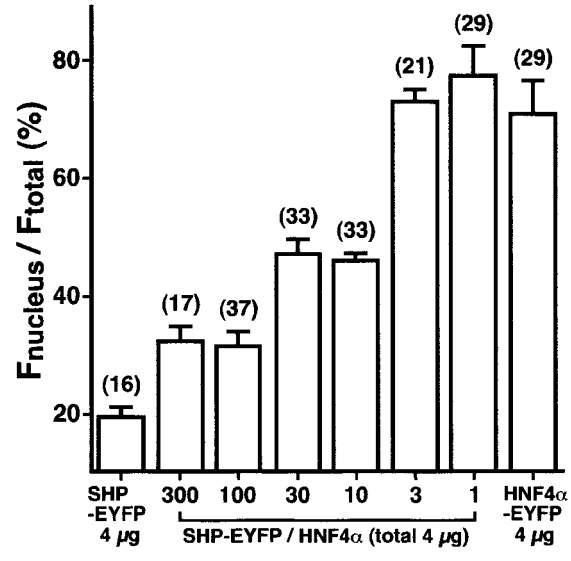

FIG. 4. The dose dependence of SHP-EYFP redistribution in the nucleus depends on the magnitude of HNF4α expression. COS-7 cell were transfected with the same amount of DNA (4 μg) but different ratios of SHP-EYFP/non-EYFP-tagged HNF4α DNAs (300, 100, 30, 10, 3, and 1), SHP-EYFP DNA alone (left column), or HNF4α-EYFP DNA alone (right column). The ordinate indicates the ratio (%) of fluorescence intensity (F) of EYFP in the nucleus to total F in the whole cell (mean $\pm$ SD). The numbers in the parentheses indicate the numbers of cells examined.

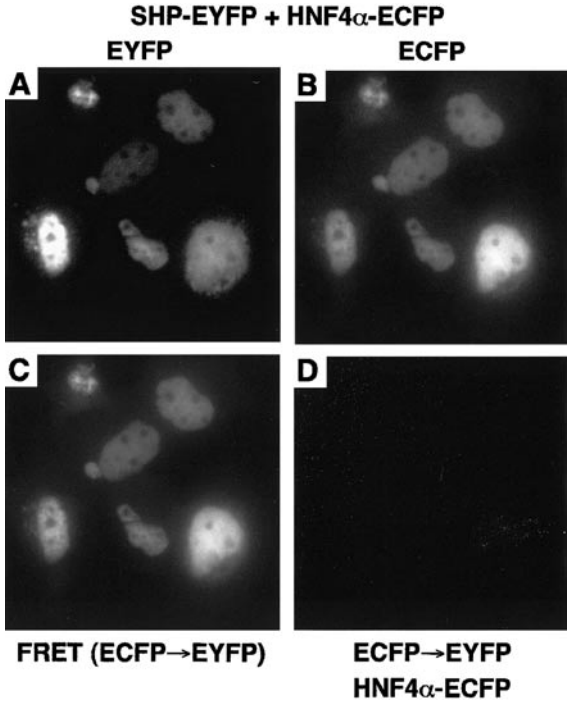

FIG. 5. Association of SHP-EYFP and HNF4α-ECFP in the nuclei of coexpressing COS-7 cells examined by FRET microscopy. (A) SHP-EYFP excited by 480 nm light and recorded by 525 nm EYFP channel. (B) HNF4α-ECFP excited by 420 nm light and recorded by 480 nm ECFP channel. (C) SHP-EYFP excited indirectly by excitation of ECFP (FRET) and recorded by EYFP channel. (D) A control image showing that no fluorescence of ECFP was recorded by EYFP channel upon excitation of ECFP in cells transfected with HNF4α-ECFP DNA alone.

HNF4α-EYFP alone (Fig. 4, right column). The results indicate that the redistribution of SHP from the cytoplasm to the nucleus depends on the presence of HNF4α as well as the relative amounts of SHP and HNF4α.

Detection of Association between SHP and HNF4α by FRET

We examined the direct interaction between SHP and HNF4α in living cells by accessing FRET between two molecules. This technique is based on the principle that energy transfer occurs between two fluorophores when they are in close proximity, 10–100 Å for the case of GFP-based fluorescent probes (18), and the emission spectrum of the first fluorophore (the donor) overlaps the excitation spectrum of the second (the acceptor). To examine FRET, SHP-EYFP and HNF4α-ECFP were co-expressed in COS-7 cells. Both SHP indicated by EYFP (excitation light, 480 nm; emission, 525 nm) and HNF4α indicted by ECFP (excitation, 420 nm; emission 480 nm) were located in the nucleus (Figs. 5A and 5B, respectively), consistent with coexpression of an EYFP-tagged receptor together with its non-EYFP-tagged partner (Figs. 3A and 3B). Interestingly, when HNF4α-ECFP was excited by 420 nm light, its emission of 480 nm was found to excite SHP-EYFP as recorded at 525 nm (Fig. 5C). In this recording system, interference between EYFP and ECFP fluorescence was eliminated by filters, as shown in Fig. 5D in which no fluorescence was visible at 525 nm upon excitation of ECYP in the cells transfected with HNF4α-ECFP alone. No fluorescence was observed in SHP-ECFP expressing cells upon excitation at 420 nm and recording at 525 nm (not shown). The distribution of SHP-EYFP observed by FRET (Fig. 5C) was almost identical with that of HNF4α-ECFP by excitation of ECFP (Fig. 5B). It was also similar to the distribution of SHP-EYFP observed by excitation of EYFP (Fig. 5A), although the difference in the fluorescence intensity between the two images (Figs. 5A and 5C) was variable in each cell probably because of different amounts of SHP-EYFP and/or HNF4α-ECFP. These results described above were also obtained by co-expression of SHP-ECFP and HNF4α-EYFP (data not shown). Thus, SHP and HNF4α are closely associated in the nucleus of living cells.

DISCUSSION

The present study demonstrates that SHP-EYFP is located only in the cytoplasm in the absence of its nuclear receptor partner HNF4α and that it translocates to the nucleus in a manner dependent on the amount of HNF4α. The results which were obtained by artificial over-expression of the nuclear receptors suggest that SHP lacks a nuclear localization signal and resides primarily in the cytoplasm at the resting state although the molecular basis for the unusual punctuate distribution pattern in the cytoplasm is unknown. They also suggest that SHP translocates to the nucleus at the activated state when cells also express a transcription factor that has the DNA-binding domain and is negatively regulated by SHP. The present experiments show that the majority of SHP molecules are able to redistribute in the nucleus when coexpressed with HNF4α at 1:1 DNA ratio and only a partial fraction of SHP molecules translocate to the nucleus with lower levels of HNF4α (Fig. 4). The value of F_R for 3:1 and 1:1 DNA ratios and for HNF4α-EYFP alone were not significantly different ($P > 0.05$). This suggests that more than one SHP molecules may bind one HNF4α molecule. The distribution of some fraction of SHP-EYFP in the nucleus of MIN6 cells is likely to be at least partly due to endogenous HNF4α. It is now essential to examine whether the HNF4α-dependent translocation of SHP operates in normal cells under normal physiological conditions.

HNF4α possesses a nuclear localization signal (14) and was always located in the nucleus, irrespective of the level of expression. No fluorescence of HNF4α-

EYFP was found in the cytoplasm suggesting that HNF4α molecules translocate to the nucleus as soon as they are synthesized. SHP is known to bind HNF4α and inhibits its transcriptional activity (4). In coexpression experiments, SHP did not interfere the translocation of HNF4α (Fig. 3B) indicating that the inhibitory effect of SHP is not due to its preventing HNF4α from trafficking to the nucleus. Experiments using FRET microscopy indicated a specific close association between SHP and HNF4α inside the nucleus of living cells. Presumably, the two molecules bind one another, forming a complex. Further studies are necessary to determine the stoichiometry of this interaction.

It is interesting that the nuclear redistribution of a regulatory nuclear receptor (SHP) depends on the interaction with its target transcription factor (HNF4α), although the mechanism is unknown. SHP may spontaneously shuttle between the cytoplasm and nucleoplasm through nuclear pores at a low rate and be trapped by HNF4α in the nucleus. Alternatively, SHP may bind HNF4α in the cytoplasm and access the nucleus by means of translocation signal present in HNF4α. GFP-tagged constructs may be useful in further analyzing the interaction of nuclear receptor partners in term of the regulation of translocation and dissecting the roles of SHP and HNF4α in determining normal pancreatic β-cell function *in vivo*.

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