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Nuclear transport of peroxisome-proliferator activated receptor α

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Peroxisome-proliferator activated receptor α (PPARα) is a ligand-activated transcription factor, playing a key role in several essential pathways including lipid metabolism. Although nuclear localization of PPARα is essential for its transactivation activity, mechanisms underlying intracellular traffics of PPARα remain undefined. We here identify and characterize a nuclear localization signal (NLS) residing in the junction between DNA-binding domain and hinge regions of PPARα. The NLS consists of two basic-amino acid clusters locating in the sequence encompassing amino acid residues at 144–187. We evidently show by mutational analysis that the basic residues in this NLS are essential for the nuclear import. Moreover, the PPARα NLS binds well-known nuclear transporters, importin α and importin β, in a manner independent of DNA-binding activity.

Keywords: DNA-binding domain/enhanced green fluorescent protein/hinge region/importin α/β/nuclear localization signal/peroxisome-proliferator activated receptor.

Abbreviations: AF1, activation function 1; AOx, acyl-CoA oxidase; DBD, DNA-binding domain; β-gal, β-galactosidase; EGFP, enhanced green fluorescent protein; GR, glucocorticoid receptor; GST, glutathione-S-transferase; NLS, nuclear localization signal; NP, nucleoplasmin; PPAR, peroxisome-proliferator activated receptor; PPRE, peroxisome-proliferator response element; RXR, retinoid X receptor.

Peroxisome-proliferator activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear receptor superfamily (*1*). Three distinct PPARs, α, γ and β/δ are identified in several species such as frog, rat, mouse and humans (*2*, *3*). PPARs play a critical role as lipid sensors and regulators of lipid metabolism (*4*). PPARα is particularly important in the lipid catabolism in liver, up-regulating the expression of a variety of genes that encode proteins involved in fatty acid oxidation, ketogenesis, lipid transport and gluconeogenesis (*5*). PPARα, γ and β/δ are encoded by different genes and exhibit distinct tissue distribution patterns (*2*, *3*). Like most of the nuclear receptors, PPARs consist of four distinct functional domains, including AF1 (activation function 1), DNA-binding domain (DBD), hinge and ligand-binding domain (LBD) harboring the C-terminal region AF2. AF1 is a ligand-independent activation domain, whereas LBD functions as a ligand-dependent transactivation domain. DBD is a highly conserved region between the receptor isoforms, consisting of two zinc fingers. PPARs bind to their target genes through the DBD forming a heterodimer with a retinoid X receptor (RXR) (*6*). Upon a direct binding their ligands, PPARs modulate expression of the target genes on a specific response element named peroxisome-proliferator response element (PPRE) within their promoters.

The nuclear transport is essential for PPARs to interact with the target genes. In contrast to glucocorticoid receptor (GR) that translocates to the nucleus only in the presence of its ligand, PPARs are reported to localize into the nucleus even in the absence of the ligands (*7*, *8*). However, the molecular mechanisms underlying the regulation of trafficking of PPARs to nucleus involving the transactivation of the target genes by PPARs remain enigmatic.

In the present study, we identified and characterized a nuclear localization signal (NLS) of PPAR for the first time, which consisted of basic amino acid residues but longer than any other NLS of nuclear receptors so far identified. We also address the NLS receptors involved in the PPAR transport to nucleus.

Materials and Methods

Plasmid constructions

Expression vector of mouse PPARα cDNA (*9*), pCMX-PPARα was as described earlier (*10*). pEGFP-PPARα encoding enhanced green fluorescent protein (EGFP)-fused to the N-terminus of PPARα was constructed by PCR amplification of full-length *PPARα* devoid of the first ATG codon with pCMX-*PPARα* as a template, using primers with BamHI and NotI sites at 5′- and 3′-sides, respectively. The amplified fragment was inserted into the EcoRI-BamHI site of pEGFP-C1 (Clontech) with a synthetic linker to generate BamHI-NotI site. pGEX-*PTAK58* (*11*), pGEX-*Ran* and pGEX-*RanQ69L* were a gift from Dr Yoneda. Osaka University, Japan. pGEX-*importin β* was gift form Dr Adam, Northwestern University, Chicago, pGST-*importin 7* was constructed by PCR amplification of the importin 7 cDNA with pQE70-*RanBP7*, a gift from Dr D. Görlich, Max Planck Institute for Biophysical Chemistry, Germany, as a template and subcloning into pGEX6p-1 (GE Healthcare).

Plasmids for C-terminal-deletion mutants of PPARα were constructed by PCR amplification using pCMX-*PPARα* as a template for fragments corresponding to the sequences for amino acid residues at 1–272, 1–166 and 1–100, with primers containing a BamHI site and an in-frame Kozak sequence at the 5′-end and a SalI site at the 3′-end. PCR products were inserted to the upstream of the sequence for tandem EGFP. N-terminal deletion mutants were similarly constructed by amplification for fragments corresponding to the sequences for amino acid residues at 101–468, 167–468 and 273–468 of PPARα, with primers harboring 5′ BamHI- and 3′ SalI-sites with a stop codon at 3′-end. PCR products were inserted into the BamHI-SalI site downstream the sequence for tandem EGFP.

Plasmid pSRα-*β-gal* encoding β-galactosidase (β-gal) under SRα promoter (*12*) was a gift from Dr H. Shida, Hokkaido University. Plasmids coding for β-gal-EGFP fusion proteins were constructed by PCR amplifications using pCMX-*PPARα* as a template for fragments corresponding to the sequences for amino acid residues at 101–272, 144–272, 167–272, 101–187, 101–166, 144–187, respectively, with respective primers. PCR products were inserted to the sequence for β-gal-EGFP.

Plasmids encoding PPARα mutants with point mutations were constructed by PCR amplification using pCMV-*PPARα* as a template with the reverse primer 187*Sal*IRv (5′-CGTCGTCGACTTCT GCTTTCAGTTTTGCTTTTT-3′) and distinct BamHI forward primers: 5′-CGGGATCCGCGATTCAGAAGAAGAACCGG-3′, 5′-GCGGATCCAAGATTCAGGCAGCGAACCGGAACAAAT GCC-3′ and 5′-GCGGATCCAAGATTCAGAAGAAGAACGCG AACGCATGCCAGTACTGCCG-3′, to generate K144A, K147A/K148A, R150A/K152A mutants, respectively (underlined, codons for alanine). Each amplified fragment was inserted into the BamHI-SalI sites of p*β-gal-EGFP*. To construct a plasmid coding for R157A/K160A double-mutant, used were BamHI-FspI and FspI-SalI fragments made by PCR amplification using a set of primers: a forward 144BamHIFw (5′-CGGGATCCAAGATTCAG AAGAAGAACCGG-3′) and a reverse FspI primer (5′-GAAATGC GCAGTACTGGCATTTGTT-3′) and another set: a forward FspI primer (5′-TACTGCGCATTTCACGCGTGCCTGTCTGTCGG-3′) and the 187SalIRv primer, respectively. Two fragments were ligated at FspI site and then inserted into the BamHI-SalI sites of p*β-gal-EGFP*. Plasmid encoding R172A/R175A double-mutant was also constructed with two fragments, BamHI-HaeI and HaeI-SalI fragments that had been PCR-amplified using a pair of primers: the 144BamHIFw primer and a reverse HaeI primer (5′-CAT AGCGCCAAATGCAATTGCATTGTGTGACATCC-3′) and another pair: a forward HaeI primer (5′-TTTGGCGCTATGCCA AGATCTGAAAAAGC-3′) and the 187SalIRv primer, respectively. The two fragments were ligated at HaeI site and then inserted into the BamHI-SalI sites of p*β-gal-EGFP*. Plasmids coding for R178A/K181A and K183A/K185A mutants were constructed by PCR amplification using the same forward primer 144BamHIFw and distinct SalI reverse primers (5′-CGCGTCGACTTCTGC TTTCAGTTTTGCTGCTTCAGATGCTGGCATTCTTCCAAAG CG-3′) and (5′-CGCGTCGACTTCTGCTGCCAGTGCTGCTTTT TCAGATCTTGGC-3′), respectively. Respective amplified fragments were separately inserted into the BamHI-SalI sites of p*β-gal-EGFP*.

Cell culture and transfection

COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% foetal calf serum, 50 μg/ml streptomycin, 50 U/ml penicillin at 37°C under 5% CO_2-95% air.

To examine sub-cellular localizations of transiently expressed proteins, 3×10^4 COS7 cells (3×10^4) were plated in 12-well cell culture cluster (Costar) and transfected 24 h later with 0.2 μg plasmids using LipofectAMINE (Invitrogen). Cells were fixed in 4% paraformaldehyde at 24 h post-transfection and permeabilized with 0.5% Nonidet P-40 (NP-40). For immunofluorescence staining, cells were then incubated with anti-PPARα antibody (N-19, Santa Cruz Biotechnol.) after blocked with 2% bovine serum albumin, and subsequently incubated with rhodamine-labelled donkey anti-goat IgG antibody (Chemicon) as secondary antibody. All images were captured using an LSM 510 confocal microscope (Carl Zeiss).

Quantification of sub-cellular distribution

Cells were classified into five categories (N, N > C, N = C, C < N and C), depending upon the localization of EGFP-tagged proteins. Briefly, for cells classified as N, the EGFP signal was localized entirely to the nucleus, with no detectable fluorescence in the cytoplasm. For cells classified as N > C, fluorescence was predominantly nuclear, with a lower level of fluorescence detectable in the cytoplasm. Cells with fluorescence evenly distributed throughout the cell were classified as N = C, while cells with fluorescence predominantly in the cytoplasm were characterized as C < N. Finally, cells in which no EGFP fluorescence was detectable in nucleus were scored as C.

Oligonucleotide pull-down assay

Oligonucleotide pull-down assay was performed as described (*13*). Biotinylated acyl-CoA oxidase (AOx) gene PPRE was synthesized as 3′-biotinated oligonucleotides: 5′-AGGGGACCAGGACAAAGGT CACGTTCGGGA-3′ and 5′-TCCCGAACGTGACCTTTGTCCT GGTCCCCT-3′, annealed and used for the pull-down assay. COS7 cells (1×10^7) were transfected with 3.5 μg of plasmid coding for β-gal-PPARα-EGFP. Cells were lysed on the next day with lysis buffer containing 1% NP-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and aprotinin (Sigma). Cell lysates were incubated for 1 h with 30 nM biotinylated oligonucleotides and 0.02 U/ml Poly(dI-dC)/Poly(dI-dC) (Amersham Pharmacia Biotech.), and subsequently with 25 μl avidin D-bound agarose (Vector laboratories) for another 1 h. DNA-PPAR complexes were analysed by SDS-PAGE using 7.5% polyacrylamide gel and western blotting with anti-GFP antibody.

In vitro *binding to glutathione-S-transferase fusion protein*

Glutathione-S-transferase (GST) pull-down assay was performed as follows. GST fusion proteins (2 μg) bound to 10 μl glutathione-Sepharose beads were incubated with lysates of COS7 cells expressing Flag-EGFP, Flag-PPARα144-187-EGFP and Flag-PPARamt144-187-EGFP for 4 h at 4°C in pull-down buffer: 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10% glycerol, 20 μg/ml BSA, 2 mM dithiothreitol, 0.01% NP40, 1 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml antipain, 2.5 μg/ml leupeptin and 2.5 μg/ml aprotinin, essentially as in (*14*). Sepharose beads were washed three times with binding buffer, and bound proteins were analysed by SDS-PAGE and western blotting. For the RanGTP and RanGDP release experiment, after pull-down assay of Flag-PPARα144-187 using GST-importin β, Sepharose beads were washed by pull-down buffer, spun down at 2,000 rpm for 1 min in a Hitachi CF15RXII centrifuge, and suspended in 100 μl of Ran buffer: 20 mM Na_2HPO_4, 50 mM NaCl, and 1 mM Mg acetate (*15*). The beads were then incubated at 25°C for 30 min in the presence of buffer alone, or 2 and 4 μg each of RanQ69L-GTP or RanGDP. For NLS competition, EGFP and nucleoplasmin-NLS-EGFP (NPNLS-EGFP) fused to GST were expressed in *Escherichia coli*, purified by cleavage with PreScission protease. Eluted NPNLS-EGFP was dialysed on Centricon (Millipore) with pull-down buffer and frozen until use. EGFP and NPNLS-EGFP (80 μg each) were added to the pull-down reaction mixtures.

Results

EGFP-PPARα is functional and localized in the nucleus

As a step to delineating the mechanism underlying nuclear transport of PPAR, we first ectopically expressed PPARα and EGFP-fused PPARα in COS7 cells. Visualization of PPARα and EGFP-PPARα by immunostaining and EGFP fluorescence indicated that both types of PPARα were exclusively localized in nucleus in COS7 cells (Fig. 1A, lanes a and b). Nuclear localization of PPARα and EGFP-PPARα was likewise observed in NIH 3T3, CHO-K1 and HeLa cells (data not shown). Proper expression of PPARα and EGFP-PPARα was confirmed by immunoblotting of cell lysates (Fig. 1B, lanes 1 and 2).

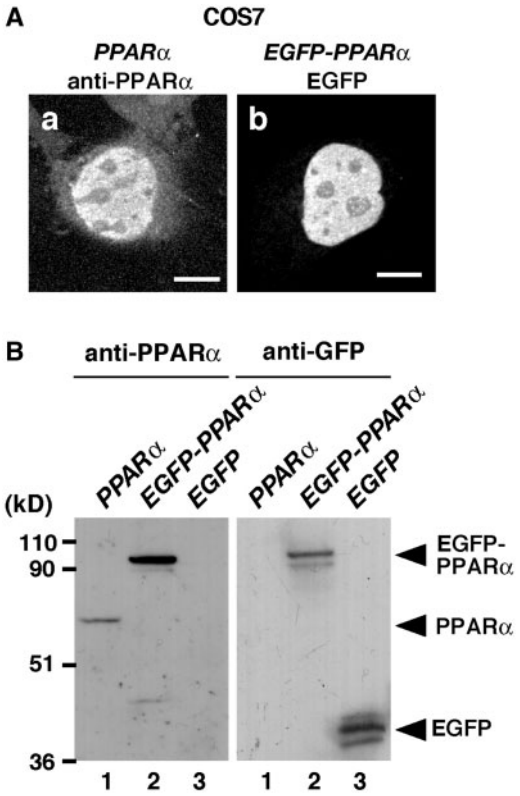

Fig. 1 Sub-cellular localization of PPARα and EGFP-PPARα in COS7 cell. (A) COS7 cells were transiently transfected with p*CMX-PPARα* (a) or p*CMX-EGFP-HPPARα* (b). At 24-h post-transfection, cells were fixed and immunostained with antibodies to PPARα (a) and GFP (b). Scale bar, 10 μm. (B) PPARα, EGFP-PPARα and EGFP were transiently expressed as in (A) and verified by SDS-PAGE and immunoblotting with antibodies to PPARα and GFP.

Moreover, EGFP-PPARα was potent in ligand-dependent transactivation of the luciferase reporter assay, although it was somewhat lower than PPARα (data not shown), consistent with the earlier report (*7*). Therefore, ~60 kDa PPARα and ~100 kDa biologically potent EGFP-PPARα are transported to nucleus, implying that PPARα likely translocates to the nucleus in a manner dependent of NLS.

An NLS is located at the junction between DBD and hinge domain

Next to identify an NLS of PPARα, we constructed, expressed in COS7 cells various deletion mutants of PPARα N- and C-terminally fused to EGFP, termed PPARα-2EGFP, and verified their subcellular localizations. Full-length PPARα-2EGFP was predominantly localized in the nucleus (Fig. 2). In contrast, several mutants deleted in hinge or DBD domains were equally distributed in the nucleus and the cytoplasm as 2EGFP, thereby suggesting that DBD and hinge domains contain an NLS.

We further dissected DBD and hinge domains of PPARα to delineate the amino acid sequence responsible for the nuclear import. We expressed in COS7 cells PPARα mutants each fused N-terminally with bacterial β-galactosidase and C-terminally to EGFP to avoid passive diffusion of smaller size proteins across the nuclear pore complexes. The sub-cellular localizations of these proteins were verified in over 100 transfected cells. β-gal-EGFP was shown to be larger than 150 kDa by western blotting (Fig. 3A, right panel, lane a) and localized exclusively in the cytoplasm in ~90% of transfected cells (Fig. 3B, lane a). In contrast, PPARα DBD and hinge domains inserted into β-gal-EGFP were exclusively localized in nucleus (Fig. 3B, lane b), suggesting that the nuclear import activity of this region is strong enough to carry cytoplasmic protein, β-gal-EGFP into the nucleus. Based on further truncation analyses, the region encompassing 44 amino acid residues at 144–187 at the junction of DBD-hinge domains possesses the nuclear import activity (Fig. 3B, lanes c–g). This region is more likely a minimal sequence for the efficient nuclear import, since further deletion mutants including β-gal-EGFP fused with residues at 151–187 or 144–181 showed cytoplasmic localization (data not shown). Furthermore, a mutant deleted in the 44 residues was completely abolished in the nuclear import ability (Fig. 3B, lane h), indicating that these 44 residues are required for nuclear import of PPARα, termed an NLS of PPARα.

DNA binding is not required for the nuclear import

The NLS of PPARα partly includes the second zinc finger loop of DBD (Fig. 4A). To assess whether PPARα NLS interacts with DNA in the nucleus, we performed the oligonucleotide pull-down assay using biotinylated oligonucleotides, AOx PPRE sequence, with lysates of cells expressing β-gal-PPARα–EGFP variants. β-gal-α(144–187)-EGFP harboring only the NLS region bound a little to the PPRE, whilst β-gal-α(101–187)-EGFP containing the intact DBD strongly bound to the DNA (Fig. 4B, lanes 2 and 3). β-gal-EGFP showed no binding to the PPRE (lane 1). These results strongly suggest that the binding to DNA is not responsible for the nuclear import of PPARα.

All of the basic residues in the NLS co-operate for the nuclear import

PPARα NLS contains about a dozen basic amino acids as shown in Fig. 5A. As reported, classical NLSs comprise a cluster of basic amino acids essential for the nuclear import. We therefore investigated whether the basic residues in the NLS of PPARα are prerequisite for the nuclear import. Subcellular localizations of mutant proteins of β-gal-α(144–187)-EGFP harboring one or two basic residues mutated to Ala in COS7 cells were assessed by EGFP fluorescence (Fig. 5B). Double mutations, K147A/K148A and R150A/K152A, strongly affected nuclear accumulation of β-gal-α(147–187)-EGFP (Fig. 5B, lanes c and d), while the other mutations showed weaker but significant reduction of nuclear import (lane b and e–h). In contrast, mutations of N-terminal 7 or C-terminal 6 basic residues or both clusters completely abrogated the nuclear import activity (Fig. 5B, lanes i–k). Taken together, all of the basic residues, but not any individual ones, are most likely to play important

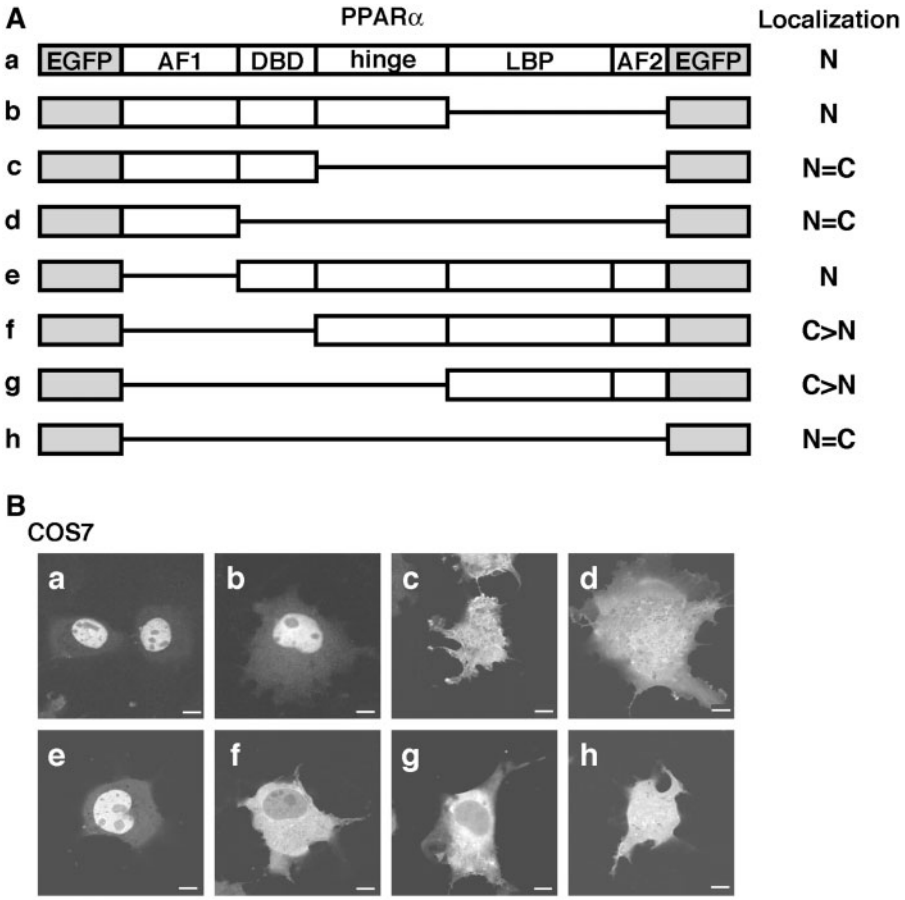

Fig. 2 Domain mapping responsible for nuclear localization of PPARα. (A) Constructs of functionally distinct domains of PPARα that are N- and C-terminally fused to EGFP. N, nucleus; C, cytoplasm. (B) EGFP-fusion proteins designed in (A) were expressed in COS7 cells and their subcellular localization was assessed by EGFP fluorescence at 24-h post-transfection. Scale bar, 10 μm. Intracellular localization of EGFP-PPARα variants was also shown in (A).

roles in the NLS activity. As one of N- and C-terminal clusters is not sufficient for the NLS function (Fig. 5B, lanes i, j, l and m), the NLS of PPARα is most likely a bipartite NLS. The distance separated by a spacer region between two basic elements is important for the nuclear import in the bipartite NLSs since these regions are considered to bury a space between two pockets in importin α in substrate-importin α interaction (*16*). However, deletion of the spacer region consisting of 11 neutral amino acids between the basic motifs of the PPARα NLS did not interfere with nuclear localization the fusion protein (Fig. 5B, lane n), suggesting that neither the amino acid sequence nor the space of this uncharged region was required for the nuclear import.

PPARα interacts with importin α and importin β

Classical NLSs consisting of a cluster of basic amino acid residues interact with importin α and their complexes with importin α/β are imported into the nucleus. We performed pull-down assay to assess whether PPARα(144–187) rich in basic residues bind to importin α and importin β. Flag-PPARα144–187-EGFP (Flag-144–187-EG) was pulled down specifically with GST-importin α and GST-importin β, but not with GST (Fig. 6A, lanes 6–8), whilst Flag-EGFP was not pulled down (lanes 2–4). In contrast, mutant of PPARα144–187 (Flag-PPARαmt 144–187-EGFP) in which N-terminal 7 basic residues were substituted with alanine was not pulled down (lanes 10–12). Next, we verified the specificity of the interaction between importin α and PPARα144–187 by competition in the pull-down assay. Nucleoplasmin NLS is a typical classic NLS that binds importin α (*17*). Addition of NPNLS-EGFP to the pull-down reaction mixture apparently interfered with the binding of PPARα144–187 to importin α, whilst EGFP did not affect it (Fig. 6B, lane 3 and 4).

The nuclear import receptors such as importin β or transportin bind their substrates including importin α in the cytoplasm where the RanGTP level is low and release the cargoes upon interaction with RanGTP in the nucleus where the RanGTP concentration is high (*18*). Therefore, if the binding of PPARα144–187 to importin β via importin α is involved in the nuclear import, this interaction would also be compromised by RanGTP. Flag-PPARα144–187-EGFP was indeed released from GST-importin β by RanGTP, not RanGDP, in a manner dependent on the RanGTP concentration (Fig. 6C, lanes 3–6), thereby suggesting that the PPARα144–187 and importin α/β complexes lead to the nuclear import of PPARα.

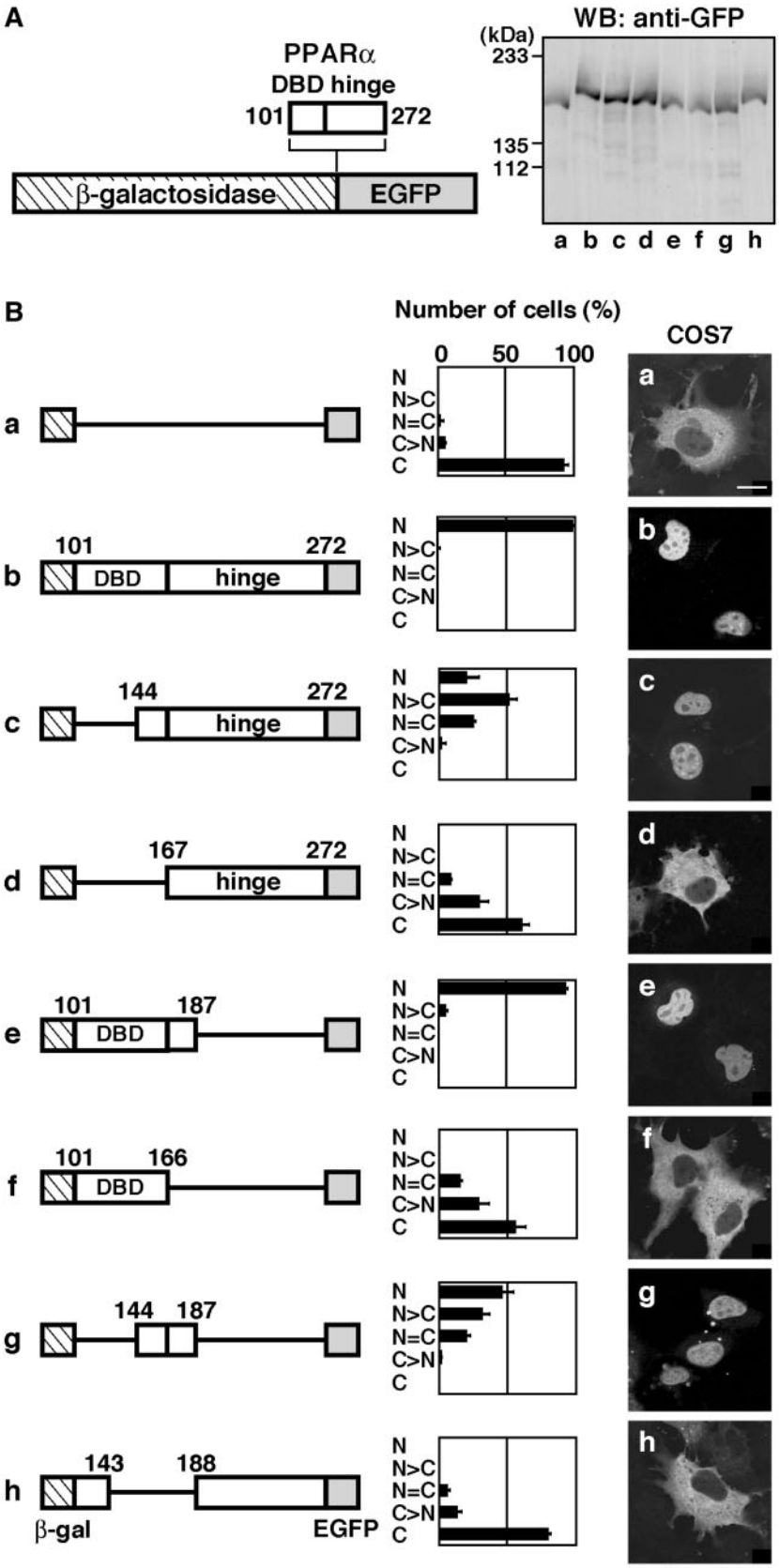

Fig. 3 An NLS of PPARα comprises 44 amino acid residues in the junction of DBD and Hinge regions. (A) Various deletion mutants of PPARα were fused to β-galactosidase and EGFP at their C- and N-terminus ends, respectively, as shown in the left panel. Whole lysates of COS7 cells separately expressing these fusion proteins were analysed by western blotting with anti-GFP antibody (right panel). (B) Sub-cellular localization of the chimera proteins was assessed and quantified by EGFP fluorescence at 24-h post-transfection of respective expression plasmids. Scale bar, 20 μm. Cells were scored into five categories from exclusively nuclear to exclusively cytoplasmic (N, N > C, = C, C < N and C) and plotted. More than 100 transfected cells were examined. Values are means ± SD of three independent experiments.

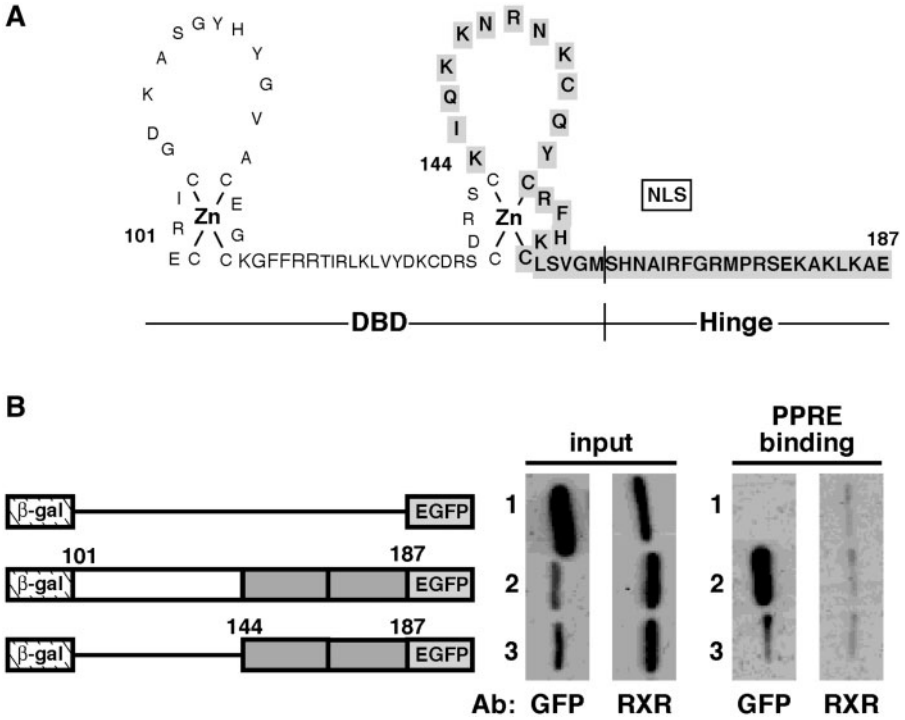

Fig. 4 DNA-binding activity of NLS. (A) NLS of PPARα encompasses the sequence including the second zinc finger loop. The NLS is shown with bald and shaded letters. (B) N-terminally β-galactosidase- and C-terminally EGFP-fused PPAR NLS: β-gal-PPARα(101–187)-EGFP [β-gal-α(101–187)-EGFP, lane 1], β-gal-PPARα(144–187)-EGFP [β-gal-α(144–187)-EGFP, lane 2] and a control β-gal-EGFP (lane 1) were transiently expressed in COS7 cells. After 24-h culture, cells were lysed, subsequently incubated with biotinylated oligonucleotides including PPRE sequence of *AOx* gene. Pulled-down fractions were assessed with anti-GFP antibody and for endogenous RXRα with anti-RXRα antibody as an internal control.

Moreover, ectopically expressed Flag-PPARα was immunoprecipitated with HA-importin α (data not shown). Taken these results together, we conclude that the nuclear import of PPARα is mediated by importin α and importin β.

Discussion

PPARs are nuclear receptors that translocate into nucleus to transactivate their target genes in response to their ligands. We attempted to address how PPARs are transported into the nucleus. In the present work, we identified and characterized an NLS of PPARα at the junction of DBD and hinge domain. The PPARα NLS consists of two positively charged clusters. Based on the results of mutational analysis, all of the positively charged residues in both N- and C-terminal clusters contribute to the nuclear import of PPARα. In other nuclear receptors such as GR, mineralcorticoid receptor and androgen receptor, GR and mineralcorticoid receptor harbour one of their NLSs in DBD and androgen receptor does in the hinge domain (*19–21*). However, all of these NLSs are classical monopartite or bipartite NLS and are dissimilar to that of PPARα. Vitamin D receptor also contains a bipartite-type NLS in its hinge domain that is not classical one but contains PPXR motif (*22*). Bipartite NLS of this type is also distinct from the PPARα NLS.

As shown by the oligonucleotide pull-down and nuclear import assays (Figs 4B and 5B, lane a), DNA-binding activity of PPARα is not required for the nuclear import of PPARα as noted that its NLS shows weak DNA-binding ability and the DBD does not localize to the nucleus (Fig. 3B, lane f). The NLS of PPARα readily translocate β-galactosidase-EGFP to nucleus without DNA-binding activity.

PPARα specifically interacts with importin α and importin β *in vitro*, where the basic amino acid residues are responsible for the interaction (Fig. 6). The binding of PPARα to importin α/importin β and its release from the complexes by RanGTP strongly suggest that PPARα is imported into the nucleus as a trimeric complex with importins α and β via a Ran-gradient system as for many nuclear proteins (Fig. 6). Importin binds to a classical NLS in the GR (*19*). Recently, importins 7 and 8 also are reported to interact with GR (*14*). Likewise, PPARα may possibly interact with other importins. A deletion mutant of the PPARα NLS reported here significantly eliminates nuclear localization but does not show its complete abrogation (T. Umemoto and Y. Fujiki, unpublished data), hence suggesting another NLSs. As in other nuclear receptors, it is plausible that PPARα is transported into nucleus by multiple NLS and their transporters.

Exclusively cytoplasmic localization of EGFP-LBD(AF2)-EGFP (Fig. 2, lane g) suggest that PPARα likely contain a nuclear export signal in its LBD (AF-2)

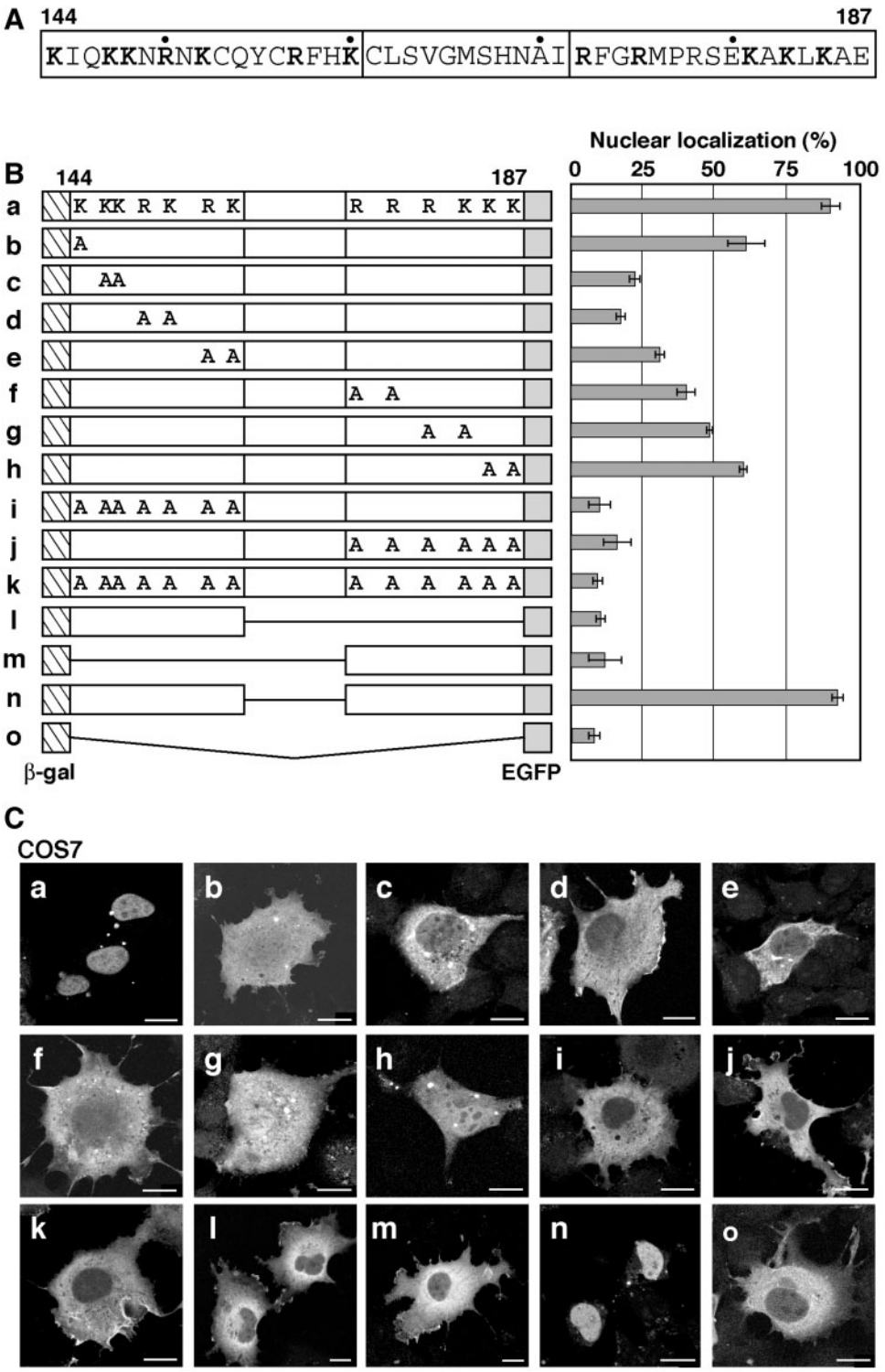

Fig. 5 Basic amino acid residues of the NLS of PPARα are essential for its nuclear import activity. (A) NLS of PPARα is shown. The NLS consists of two basic clusters at the N- and C-terminal and a spacer region in the middle. Basic amino acid residues are shown designated as bald and shaded letters. (B and C) Positively charged amino acids were mutated to alanines. Fusion proteins were separately expressed in COS7 cells and their sub-cellular localization was verified by EGFP fluorescence at 24-h post-transfection. Nuclear accumulation of NLS mutants was quantified and plotted as in Fig. 3. Scale bar, 20 μm.

domain, consistent with the report by Burgermeister *et al*. (*23*). Collectively, PPARα is more likely a shuttling protein between nucleus and the cytoplasm. The regulation of such shuttling may be possibly and tightly linked to PPARα-mediated transactivation of the transcription of target genes. The findings in regard to the NLS reported here would open a way to elucidate the mechanisms underlying PPAR functions.

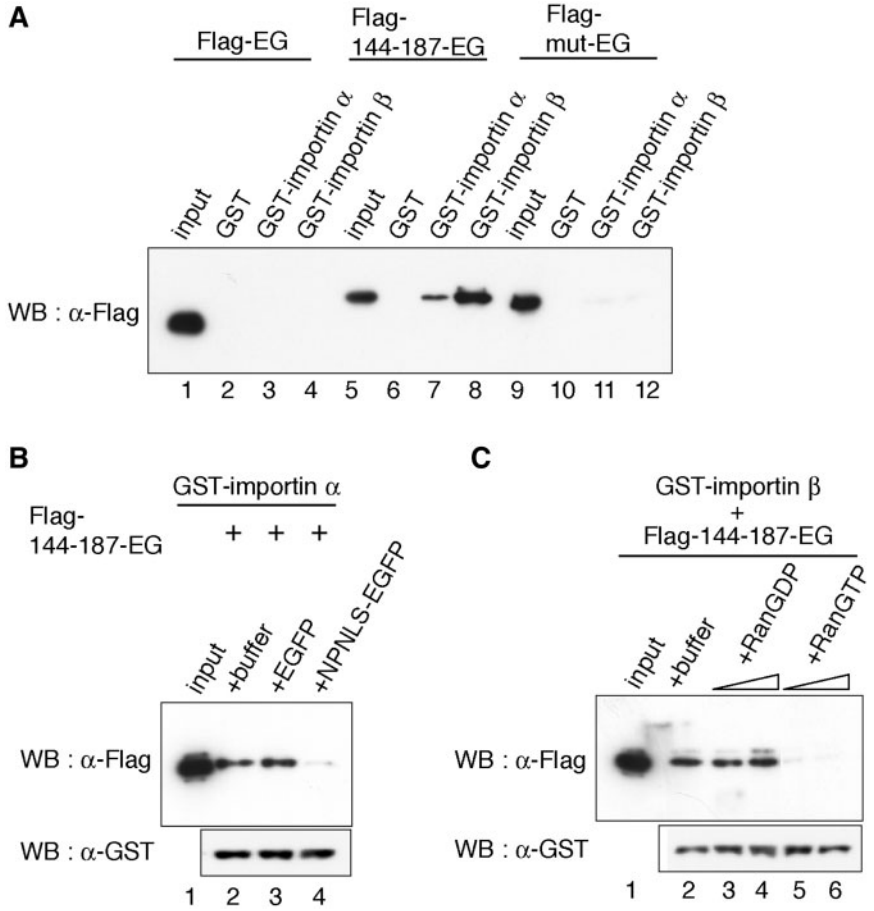

Fig. 6 PPARα binds to importin α and importin β. (A) GST, GST-importin α or GST-importin β (2 μg each) were immobilized on glutathione–sepharose resin and incubated at 4°C for 2 h with lysates of COS 7 cells expressing Flag-PPARα144–187-EGFP, Flag-PPARα-144–187-EGFP or Flag-PPARαmt144–187-EGFP. Bound proteins were resolved by SDS-PAGE and immunoblotted with Flag-specific antibody. Input, one 25th aliquot used for binding assay. (B) Pull-down assay of Flag-PPARα144–187-EGFP using GST-importin α (2 μg each) immobilized on glutathione–sepharose was performed in the presence of EGFP or nucleoplasmin-NLS-EGFP (NPNLS-EGFP). Bound Flag-PPARα144–187-EGFP was analysed as in (A). Input, one-fiftieth aliquot used for binding assay. (C) After pull-down assay of Flag-PPARα144–187-EGFP by GST-importin β (2 μg each), the Sepharose beads were incubated in buffer alone (lane 2) or with 2 or 4 μg of Ran-GDP (lanes 3 and 4) or RanQ69L-GTP (Ran-GTP, lanes 5 and 6). After thorough washing, the bound Flag-PPARα144–187-EGFP was analysed by SDS-PAGE and immunoblotting as in (A). Input, one 25th aliquot used for binding assay.

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Conflict of interest

None declared.

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