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Peroxisome Targeting of Porcine 17β-Hydroxysteroid Dehydrogenase Type IV/D-Specific Multifunctional Protein 2 Is Mediated by its C-Terminal Tripeptide AKI

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Abstract The product of the porcine *HSD17B4* gene is a peroxisomal 80 kDa polypeptide containing three functionally distinct domains. The N-terminal part reveals activities of 17β-estradiol dehydrogenase type IV and D-specific 3-hydroxyacyl CoA dehydrogenase, the central part shows D-specific hydratase activity with straight and 2-methyl-branched 2-enoyl-CoAs. The C-terminal part is similar to sterol carrier protein 2. The 80 kDa polypeptide chain ends with the tripeptide AKI, which resembles the motif SKL, the first identified peroxisome targeting signal PTS1. So far AKI, although being similar to the consensus sequence PTS1, has neither been reported to be present in mammalian peroxisomal proteins, nor has it been shown to be functional. We investigated whether the *HSD17B4* gene product is targeted to peroxisomes by this C-terminal motif. Recombinant human PTS1 binding protein Pex5p interacted with the bacterially expressed C-terminal domain of the *HSD17B4* gene product. Binding was competitively blocked by a SKL-containing peptide. Recombinant deletion mutants of the C-terminal domain lacking 3, 6, and 14 amino acids and presenting KDY, MIL, and IML, respectively, at their C-termini did not interact with Pex5p. The wild-type protein and mutants were also transiently expressed in the HEK 293 cells. Immunofluorescence analysis with polyclonal antibodies against the C-terminal domain showed a typical punctate peroxisomal staining pattern upon wild-type transfection, whereas all mutant proteins localized in the cytoplasm. Therefore, AKI is a functional PTS1 signal in mammals and the peroxisome targeting of the *HSD17B4* gene product is mediated by Pex5p. J. Cell. Biochem. 73:70–78, 1999.

Key words: peroxisomes; bile acids; steroids; protein import

Multifunctional 80 kDa Protein

Type IV 17β-hydroxysteroid dehydrogenase (gene *HSD17B4*) was originally discovered in porcine endometrium [Adamski et al., 1992b] and shown to be localized in peroxisomes [Markus et al., 1995]. Subsequent cloning of its cDNA revealed that the 32 kDa type IV dehydrogenase corresponds to an N-terminal fragment of a larger multidomain 80 kDa protein [Leenders et al., 1994a]. The N-terminal and central part of this 80 kDa protein reveal unexpected homology to the 3-hydroxyacyl-CoA dehydrogenase and 2-enoyl-CoA hydratase domains of FOX2, a multifunctional peroxisomal β-oxidation protein from yeast. The C-terminus of the 80 kDa protein is homologous to sterol carrier protein 2 (SCP2) [Leenders et al., 1994b]. Indeed, the bacterially expressed 80 kDa protein was subsequently shown to possess, beside 17β-hydroxysteroid dehydrogenase activity, 3-hydroxybutyryl-CoA dehydrogenase and crotonase activity and to be able to transport lipids and sterols [Leenders et al., 1996]. In addition to the porcine enzyme, the human [Adamski et

Abbreviations used: 17β-HSDIV/MFP-2, 17β-hydroxysteroid dehydrogenase type IV/ D-specific multifunctional protein 2; PEX5p, peroxin protein 5; PTS1, peroxisome targeting signal 1; pSCP2, C-terminal domain of the porcine 17β-HSDIV/MFP-2.

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al., 1995] and mouse [Normand et al., 1995] orthologs have now been cloned.

During studies on peroxisomal β-oxidation of pristanic acid and bile acid intermediates in rat and man, D-specific multifunctional 80 kDa proteins (named as well multifunctional protein 2, MFP2) were discovered [Novikov et al., 1994; Qin et al., 1997], characterized and cloned [Dieuaide-Noubhani et al., 1996, 1997a,b; Jiang et al., 1996, 1997; Novikov et al., 1997]. This proteins turned out to be the further orthologs of the porcine 17β-hydroxysteroid dehydrogenase IV. Since the V_{max} β-oxidation activities of these proteins are shown to be several fold higher, than the 17β-hydroxysteroid dehydrogenase activity [Dieuaide-Noubhani et al., 1996; Leenders et al., 1996; Novikov et al., 1997; Qin et al., 1997], it is questionable whether 17β-estradiol is a physiological substrate. On the other hand, the enzyme has 0.2 μM K_m towards steroids, is abundant in steroid-responsive tissues [Adamski et al., 1995; Normand et al., 1995] and evidence is reported that the enzyme, and its processing to a 32 kDa dehydrogenase, is regulated by steroids such as progesterone in kidney and uterus [Kaufmann et al., 1995]. Until physiological substrate issue is clarified, we will refer to the 80 kDa protein as 17β-hydroxysteroid dehydrogenase IV/D-specific multifunctional protein 2 (17β-HSDIV/MFP-2).

Peroxisomal Targeting Signal PTS1

Most peroxisomal matrix proteins contain at their C-termini a topogenic signal, called PTS1 (peroxisome targeting signal 1) that is recognized by a PTS1-binding protein Pex5p (peroxin protein 5) [Distel et al., 1996], and that will mediate protein import into peroxisomes [see ref. Subramani, 1993, 1996 for a review]. PTS1 was first defined as the tripeptide SKL [Gould et al., 1987], but subsequent studies have shown some degeneracy. In mammals, this has lead to the consensus **S**/A/C - **K**/R/H - **L** [Gould et al., 1989]. Whereas the C-terminus of rat [Corton et al., 1996; Dieuaide-Noubhani et al., 1996; Qin et al., 1997], mouse [Normand et al., 1995], and human [Adamski et al., 1995] 17β-HSDIV/MFP-2 corresponds to AKL, the porcine protein ends with AKI [Leenders et al., 1994a]. The latter combination has hitherto not been proven to be functionally active in mammals. Moreover, a terminal isoleucine in the target motif seems to result in a weak PTS1, since substitution of I for L in the SKL-motif of luciferase diminished (or abolished) its import in mammalian CV1-cells [Gould et al., 1989] and the predominant cytosolic localization of epoxide hydrolase in mouse and rat liver has been ascribed to the presence of SKI at its C-terminus [Arand et al., 1991; Grant et al., 1993]. On the other hand, AKI fits to the more relaxed consensus found for glycosomal protein targeting , being **S**/A/C/G/H/N/P - **K**/H/M/N/R/S - **L**/I/M/Y [Sommer et al., 1992], and in yeast, this tripeptide has been reported to be essential for targeting the *Candida tropicalis* trifunctional protein (the fungal counterpart of 17β-HSDIV/MFP-2) to the peroxisomes [Aitchison et al., 1991]. PTS1 functionality might be context specific however, since AKI fused to chloramphenicol acetyltransferase did not target the enzyme to the peroxisomes in yeast [Fransen et al., 1995].

In this work we have investigated the role of AKI as peroxisome targeting signal 1 by means of direct measurements of the interactions between recombinant porcine 17β-HSD IV/MFP-2 and recombinant human Pex5p and by transfection studies followed by immunohistochemistry.

MATERIALS AND METHODS

Construction of Mutants

A DNA fragment containing the coding sequence for the amino acids 606–738 (the C-terminal domain similar to sterol carrier protein 2 abbreviated as pSCP2) of the porcine 17β-HSDIV/MFP2 obtained from the cDNA [Leenders et al., 1994a] was used as a template for PCR amplification of mutant DNA. Appropriate oligonucleotide primer pairs adding *Bam*HI and *Kpn*I restriction sites at the 5′- and 3′-ends were applied to amplify constructs coding for wild type protein and deletion mutants lacking the last 3, 6, or 14 amino acids (Table I). The last two constructs were chosen to explore if the internal sequences QMIL, QKL, and NIML (Fig. 1, upper panel) similar to that of plant and human catalase [Korneluk et al., 1984; Mullen et al., 1997a,b] could be also recognized by Pex5p. The PCR amplified DNA was digested with *Bam*HI and *Kpn*I and cloned directionally into the *BamH*I and *Kpn*I restriction sites of the vector pRep10 (Invitrogen, Leek, The Netherlands) or into the vector pGex 2T PL2, a modified pGex 2T vector (Pharmacia, Freiburg, Germany), with an additional *KpnI* site [Leenders et al., 1994a]. The constructs were verified by restriction analysis and full length sequencing.

TABLE I. PCR Amplification of *17β-HSDIV/MFP2* Mutants[a]

Primer	Sequence 5′-3′	Position in the *HSD17B4* cDNA	C-terminal amino acid sequence	Abbreviation
forward	ttttggatccATGACTGTCATTTCAAATGCATACGTG	1858–1881		
reverse	ttttggtaccTCAAATCTTGGCATAGTCTTTAAG	2260–2280	AKI	wt
reverse	ttttggtaccTCAATAGTCTTTAAGAATCATCTGAAG	2248–2271	KDY	Δ3
reverse	ttttggtaccTCAAAGAATCATCTGAAGCTTCTGGCTC	2238–2262	MIL	Δ6
reverse	ttttggtaccTCACAGCATGATGTTCCCTCTG	2220–2238	IML	Δ14

[a]PCR parameters: 2 min. 94°C, (30 s 94°C, 30 sec 62°C, 2 min 72°C) × 30 cycles, 7 min 72°C, Pfu polymerase (Stratagene, Heidelberg, Germany) was used. Mismatches introducing restriction sites *BamHI* (forward) and *Kpn* I (reverse) are in lower case, start and stop codons are underlined.

Transfection and Immunofluorescence Analysis of Cells

Human embryonal kidney HEK 293 Ebna cells (Invitrogen) were grown on glass coverslips in 3 cm dishes in RPMI Medium supplemented with 10% FCS, L-glutamine (200 µg/ml), streptomycin (100 U/ml), and penicillin (100 µg/ml). The cells were transfected at 40% confluence by the calcium phosphate coprecipitation method [Wigler et al., 1977] with 4 µg of the plasmids pRep10-wt, pRep10-Δ3, pRep10-Δ6 and pRep10-Δ14 (see Table 1 and Fig. 1 for abbreviations), respectively. The coverslips were collected 72 h after transfection, fixed in methanol/acetone at −20°C, blocked with 3% BSA in PBS and labeled with polyclonal rabbit antiserum KS (dilution 1:800) against the porcine SCP2 domain of 17β-HSDIV/MFP2 (pSCP2), followed by a secondary goat anti-rabbit antibody (dilution 1:500) decorated with Cy3 (Dianova, Hamburg, Germany) as described [Husen et al., 1994]. Controls by staining of non-transfected cells with primary and secondary antibody or by staining of pRep10-wt transfected cells with secondary antibody only, showed no labeling. The porcine kidney AM C6C9 cell line constitutively expressing 17β-HSDIV/MFP2 was used a positive control. Counterstaining of nuclei was performed by DAPI (Boehringer Mannheim, Germany). The coverslips were viewed on a Zeiss Axiophot (Zeiss, Oberkochen, Germany), fluorescence signals were digitized by a CCD camera and blue and red immunofluorescence superimposed with ISIS software (MetaSystems, Altlussheim, Germany).

Expression of the SCP2 Domain in *E. coli*

The *E. coli* strain JM107 containing the pGex-wt, pGex-Δ3, pGex-Δ6, or pGex-Δ14 plasmids were grown in LB media containing 50 µg/ml Ampicillin. The cells were grown in a rotary shaker at 37°C until absorbency at 600 nm reached 0.6. Protein expression was induced by 0.2 mM (final) isopropyl-D-thiogalactopyranoside and incubation for additional 3 h. Cell lysis, purification of the glutathione-S-transferase (GST) fusion proteins, cleavage with thrombin and SDS-PAGE analysis of purified proteins were performed as described [Adamski et al., 1992a; Leenders et al., 1996].

In Vitro Binding Assays With PEX5p

Recombinant proteins were subjected to SDS-PAGE, followed by electroblotting to nitrocellulose. After blocking, blots were stained or incubated with partially purified bacterially expressed and biotinylated Pex5p fusion protein and formed complexes were visualized by streptavidine-alkaline phosphatase as described before [Fransen et al., 1995, 1996].

Recombinant proteins were also coated in wells of a 96-well microtiter plate followed by incubation with the biotinylated Pex5p fusion protein as described before [Fransen et al., 1995] with some modifications to improve the stability of alkaline phosphatase. For competition experiments, increasing amounts of peptides P1 or P2, corresponding to C-terminus of the rat acyl-CoA oxidase, were added during the incubation with Pex5p. P1 ends in SKL, whereas this tripeptide is deleted in P2 [Fransen et al., 1995].

RESULTS

In Vitro Binding of Porcine17β-HSD IV/MFP-2 to Pex5p

The purity of the isolated wild-type and deletion mutants of the SCP2 domain of 17β-HSD/MFP-2 was checked by SDS-PAGE (Fig. 1). The

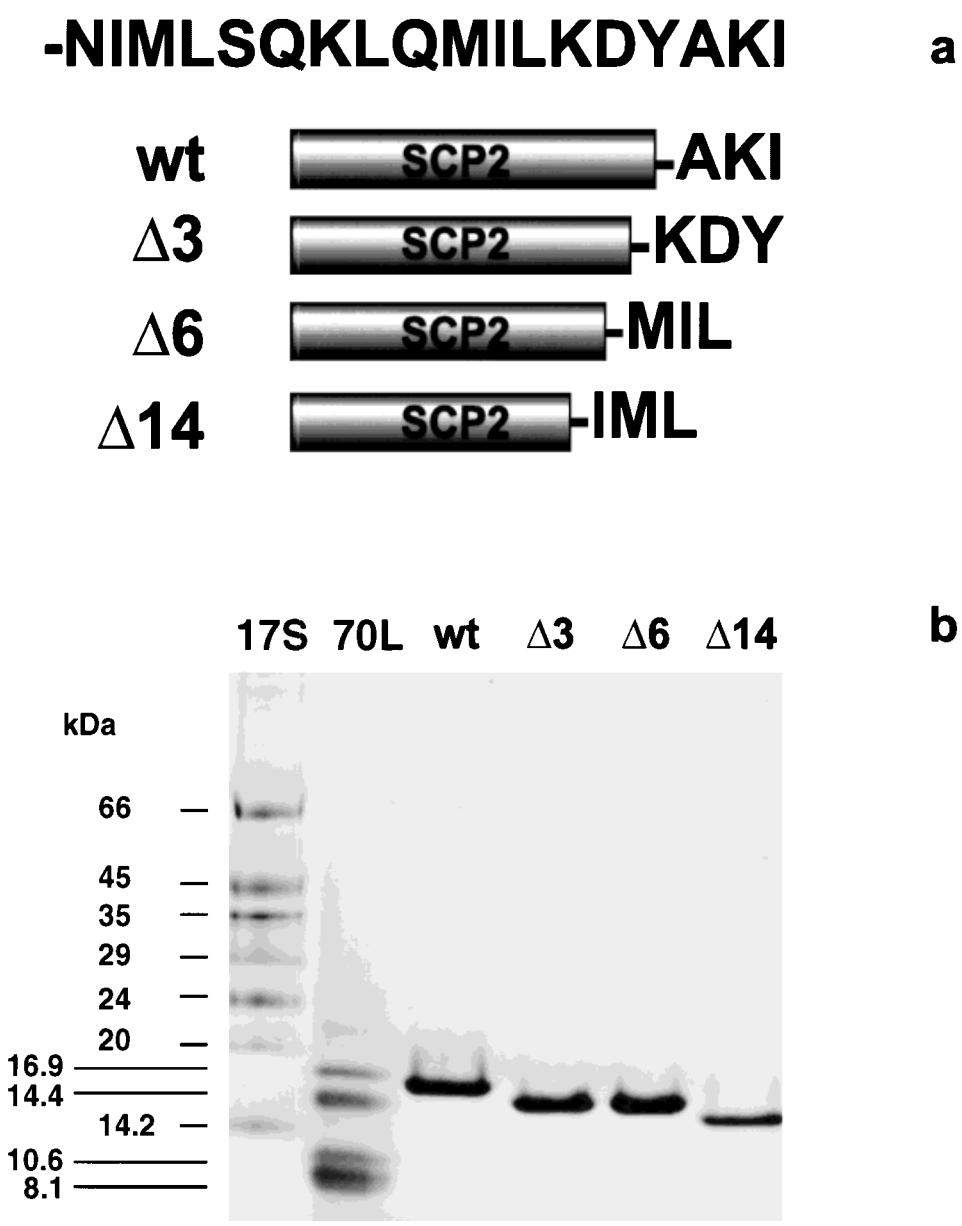

Fig. 1. Mutants of SCP2 domain of porcine 17β-hydroxysteroid dehydrogenase IV/D-specific multifunctional protein 2. **a**: Schematic presentation of wild type protein and mutants. Amino acid sequence from position 720 to 737 is given at the top, abbreviations are given on the left, C-terminal amino acids on the right. **b**: SDS-PAGE of purified proteins from *E. coli*. Molecular mass standards 17S and 70L (Sigma, Deisenhofen, Germany) are given on the left. Designations of proteins: wt, wild-type SCP2 domain of *HSD17B4* , Δ3, Δ6, and Δ14 represent the deletion mutants (see Table I for details). Five µg of purified proteins were applied per lane, resolved by 10% SDS-PAGE and stained with Coomassie Brilliant Blue.

theoretical molecular mass of the wild type SCP2 domain is 15 kDa. Interestingly, although the wt, Δ3 and Δ6 proteins differ sequentially by three amino acids in length (verified by DNA-sequencing of pGEX inserts) there is very little mobility change between Δ3 and Δ6 but a clearly visible one between wt and Δ3. The purified proteins were used for in vitro binding to Pex5p. As reported before [Fransen et al., 1996], the interaction between a PTS1-containing protein and (human) PEX5p is not affected by SDS-PAGE and blotting of the PTS1-containing protein. The fragment corresponding to the wt-SCP2-domain interacted with PEX5p, but no interactions were observed with the Δ3, Δ6, Δ14 recombinant proteins, neither as GST-fusions nor after removal of the tag (data not shown).

Similarly, in the microtiter binding assay, interactions could only be demonstrated when the intact C-terminus was present (Fig. 2). Addition of an SKL-containing rat peptide P1 competitively diminished the binding of Pex5p to the complete SCP2-like domain whereas the P2 peptide without SKL signal was not competitive. Compared to the binding of Pex5p to human serum albumin, covalently linked to an SKL-ending peptide, the interaction with the SCP2-domain of porcine 17β-HSDIV/MFP-2 is less strong.

Targeting of 17β-HSD/MFP-2 to Peroxisomes In Vivo

We have previously shown by various different approaches such as immunogold electron microscopy and density gradient analyses that the wild-type 17β-HSDIV/MFP-2 localizes solely in peroxisomes [Markus et al., 1995]. The porcine AM C6C9 cell line constitutively expresses the 17β-HSD IV/MFP-2 [Carstensen et al., 1996]. These cells were employed to prove that the cell culture conditions and the immunofluorescence analysis used in this study were suitable for detecting native enzyme in peroxisomes (Fig. 3). The human embryonal kidney 293 Ebna cell line is devoid of activity of this enzyme [Carstensen et al., 1996] and was applied to check the targeting of porcine 17β-HSDIV/MFP2. When transiently transfected with the pRep10 vector coding for the wild type SCP2 domain carrying the AKI signal at its C-terminus the immunofluorescence gave the same spotted pattern as in AM C6C9 cells, which is characteristic for peroxisomes. In some cells, we observed a weak cytoplasmic background. In contrast, transfections with pRep10 coding for Δ3, Δ6, and Δ14 deletion mutants revealed diffuse staining, proving that the mutant proteins were not directed into the peroxisomes. The specificity of the staining with the anti-17β-HSDIV/MFP2 antibody is illustrated by the lack of peroxisomal-located immunofluorescence in nontransfected HEK 293 Ebna cells.

DISCUSSION

AKI is a Rare Signal

The sequence of porcine 17β-HSDIV/MFP2 ends with AKI. This motif indeed is similar to a proposed consensus for PTS1 [Sommer et al., 1992], but this consensus was defined for glycosomal import and is more relaxed than that seen in mammalian systems in which AKI has not yet been shown to be functional. While mammalian peroxisomal proteins carrying a C-terminal SKL-motif are found frequently, up to now the porcine 17β-HSDIV/MFP2 is the only representative of corresponding proteins ending in AKI. In other organisms the AKI is also not often present at the C-terminus of peroxisomal proteins. The sole known example is the multifunctional β-oxidation protein of *Candida tropicalis* [Nuttley et al., 1988]. Another candidate might be Cu,Zn-superoxide dismutase in *Drosophila sp.* [Kwiatowski et al., 1994], which has been described in man [Keller et al., 1991] and rat [Dhaunsi et al., 1992; Wanders and Denis, 1992].

Not Every Protein Presenting C-Terminal Signal AKI is Peroxisomal

The C-terminal motif AKI/L seems not to suffice for the targeting of proteins exclusively into peroxisomes, as is illustrated by the following examples. Human proteins showing this motif may be nuclear receptors (DAX-1, Swiss-Prot: P79386), or be localized at the plasma membrane (OX40) [Latza et al., 1994], may reveal mixed localization in peroxisomes, mitochondria, and endoplasmic reticulum (carnitine O-acetyltransferase) [Corti et al., 1994] or indeed reside solely in peroxisomes (insulin-degrading enzyme) [Authier et al., 1995]. Furthermore, it was shown that glycosomal glyceraldehyde phosphate dehydrogenase from *Trypanosoma brucei*, featuring AKL at its C-terminus, switched to cytosolic localization when transfected into human CV-1 cells [Borst, 1989]. In the case of plant peroxisomal catalase, combined C-terminal motifs were found to be responsible for the direction of the protein [Mullen et al., 1997a; Mullen et al., 1997b], i.e., an internal SHL sequence located −8 amino acids from the C-terminus requires in addition the C-terminal tripeptide PSI for peroxisomal targeting. Interestingly, the engineered C-terminal motifs KANL or KSLL alone were sufficient for the peroxisomal localization. It is likely, that the direction of human catalase to peroxisomes is driven by its C-terminal tetrapeptide KANL [Korneluk et al., 1984]. A closer look at the sequence of the porcine 17β-HSDIV/MFP2 reveals a putative targeting sequence QKL at position −10 from the C-terminus (Fig. 1) which

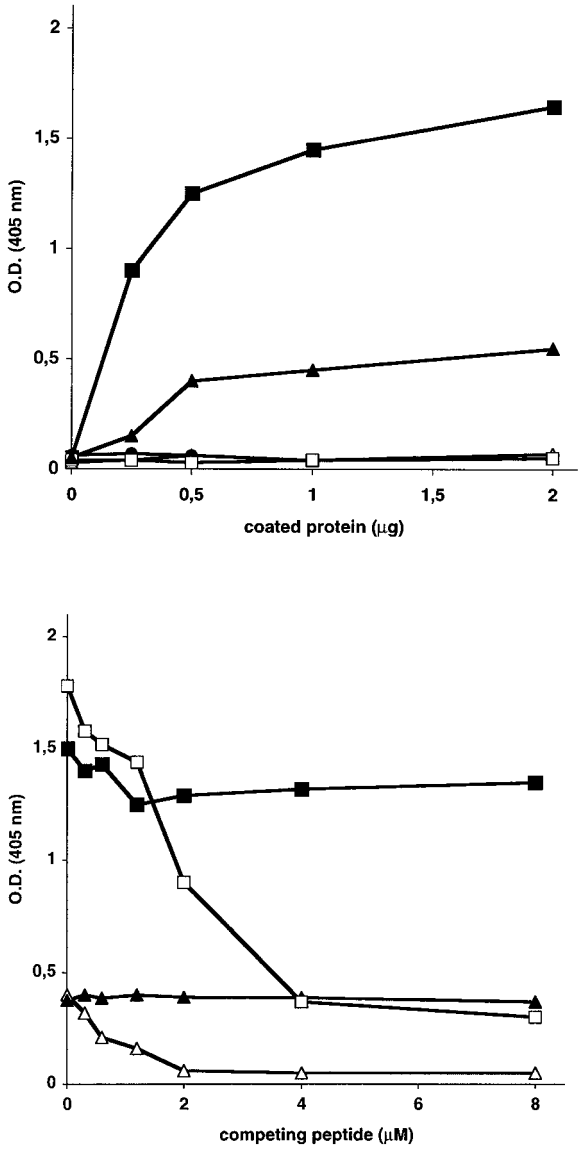

Fig. 2. In vitro interaction of the SCP2 domain of 17β-hydroxysteroid dehydrogenase IV/D-specific multifunctional protein 2 with Pex5p. **A**: Microtiter wells were coated with increasing amounts of bacterially expressed wild-type and deletion mutants of the SCP2 domain, freed from the GST tag by thrombin digestion, followed by incubation with biotinylated PEX5p, streptavidin-alkaline phosphatase and detection of the formed complexes after 120 min of incubation with p-nitrophenyl phosphate [Fransen et al., 1995]. Closed triangles, SCP wild-type; closed circles, SCP2-Δ3; open triangles, SCP2-Δ6; open squares, SCP2-Δ14. For comparison, the interaction with human albumin crosslinked to a peptide ending in SKL (closed squares) was analyzed. Complexes were measured after 90 min of incubation with p-nitrophenyl phosphate. In cases where values overlap only open symbols are shown. **B**: Microtiter plate wells were coated with 1 μg of bacterially expressed wild-type SCP2, freed fom the GST tag (triangles) or human albumin crosslinked to a SKL-peptide (squares) and incubated with biotynilated PEX5p and the formed complexes were detected as described above. During the incubation with PEX5p, increasing amounts of P1 peptide, ending in SKL (open symbols), and P2 peptide (closed symbols) were present.

could perhaps also be necessary for successful peroxisomal import.

Pex5p Protein Binds Specifically AKI Tripeptide

Recent cloning and in vitro expression of the PTS1 receptor protein Pex5p, [Fransen et al., 1995] allowed for a detailed study of the targeting mechanism of the 17β-HSDIV/MFP2. We prepared several mutant proteins lacking the C-terminal tripeptide including one lacking also the putative internal targeting motif. Our in vitro and in vivo studies are consistent and show that the deletion of AKI drives the protein to the cytosol caused by the loss of binding to Pex5p. Although the Δ6 mutant still contains both the internal tripeptide QKL as well as the C-terminal motif QMIL (functioning as a targeting signals in other organisms) [Korneluk et al., 1984; Mullen et al., 1997a,b] this construct remains also cytosolic. The same is observed for the Δ14 mutant lacking all above motifs. This proves that the C-terminal tripeptide AKI is a functional PTS1 signal in mammals and that the peroxisome targeting of the 17β-HSDIV/MFP2 is mediated by Pex5p.

As pointed out by Arand et al. [1991], some PTS1, such as SKI, may function less efficiently, explaining the predominant cytosolic localization of epoxide hydrolase in rat and mouse liver. Also the interaction of the functional C-terminus of the porcine 17β-HSDIV/MFP2 with Pex5p seems much weaker than that seen with SKL- or AKL-containing proteins. This might explain the weak cytoplasmic background staining of the HEK 293 cells transfected with the wild-type SCP2 domain of 17β-HSDIV/MFP2 sometimes observed. Alternatively, the level of expressed protein might have been too high to be completely imported into the peroxisomes. However, such weak interaction does not result in a partly cytosolic distribution of 17β-HSDIV/MFP-2 in porcine tissues. Based on immunogold electron microscopy data [Markus et al., 1995] and density gradient analysis [Adamski, 1991] the 17β-HSDIV/MFP2 appears to be solely organelle associated.

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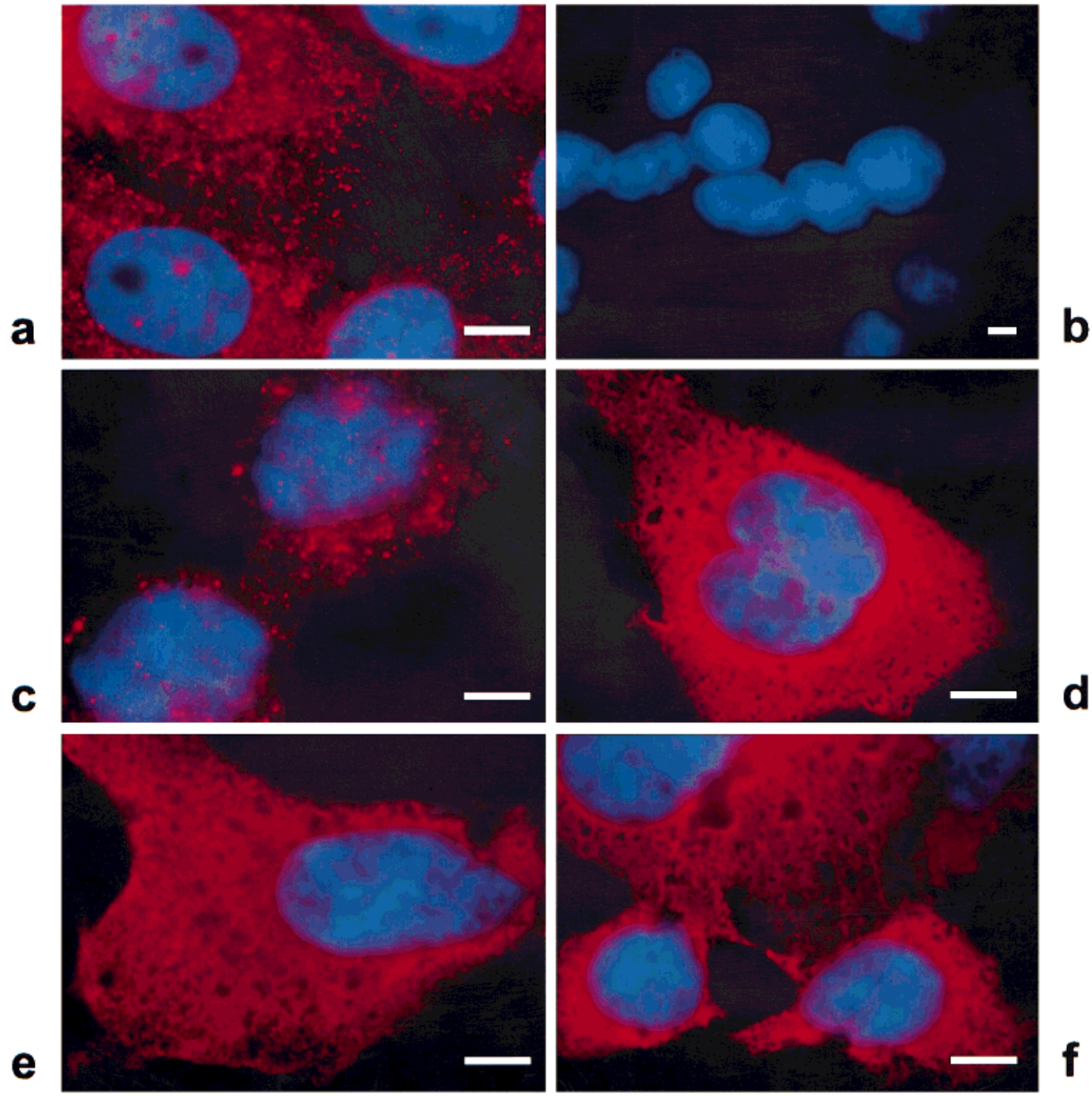

Fig. 3. Targeting of 17β-hydroxysteroid dehydrogenase IV/D-specific multifunctional protein 2 in vivo. Cells were grown on glass coverslips and fixed as described in Materials and Methods. All cells were stained with rabbit anti-porcine SCP2-domain antiserum (KS) followed by Cy3 conjugated goat anti-rabbit antibody, counterstaining of nuclei was done with DAPI; **a**: positive control of native enzyme in AM C6C9 cells; **b**: HEK 293 Ebna nontransfected cells; **c**: HEK 293 Ebna cells transfected with the wild-type SCP2-domain of 17β-HSDIV/MFP2; **d–f**: HEK 293 Ebna cells transfected with deletion mutants Δ3, Δ6, and Δ14 of the SCP2-domain, respectively. Scale bars = 10 μm.

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REFERENCES

Adamski J. 1991. Isolation of vesicles mediating the conversion of 17β-estradiol to estrone. Eur J Cell Biol 54:166–170.

Adamski J, Husen B, Marks F, Jungblut PW. 1992a. The membrane-bound 17β-estradiol dehydrogenase of porcine endometrial cells: Purification, characterization and subcellular localization. J Ster Biochem Mol Biol 43:1089–1093.

Adamski J, Husen B, Marks F, Jungblut PW. 1992b. Purification and properties of oestradiol 17β-dehydrogenase extracted from cytoplasmic vesicles of porcine endometrial cells. Biochem J 288:375–381.

Adamski J, Normand T, Leenders F, Monte D, Begue A, Stehelin D, Jungblut PW, de Launoit Y. 1995. Molecular cloning of a novel widely expressed human 80 kDa 17β-hydroxysteroid dehydrogenase IV. Biochem J 311:437–443.

Aitchison JD, Murray WW, Rachubinski RA. 1991. The carboxyl-terminal tripeptide Ala-Lys-Ile is essential for targeting Candida tropicalis trifunctional enzyme to yeast peroxisomes. J Biol Chem 266:23197–203.

Arand M, Knehr M, Thomas H, Zeller HD, Oesch F. 1991. An impaired peroxisomal targeting sequence leading to an unusual bicompartmental distribution of cytosolic epoxide hydrolase. FEBS Lett 294:19–22.

Authier F, Bergeron JJ, Ou WJ, Rachubinski RA, Posner BI, Walton PA. 1995. Degradation of the cleaved leader peptide of thiolase by a peroxisomal proteinase. Proc Natl Acad Sci USA 92:3859–3863.

Borst P. 1989. Peroxisome biogenesis revisited. Biochim Biophys Acta 1008:1–13.

Carstensen JF, Tesdorpf JG, Kaufmann M, Markus MM, Husen B, Leenders F, Jakob F, de Launoit Y, Adamski J. 1996. Characterization of 17β-hydroxysteroid dehydrogenase IV. J Endocrinol 150:3–12.

Corti O, Finocchiaro G, Rossi E, Zuffardi O, DiDonato S. 1994. Molecular cloning of cDNAs encoding human carnitine acetyltransferase and mapping of the corresponding gene to chromosome 9q34.1. Genomics 23:94–99.

Corton JC, Bocos C, Moreno ES, Merritt A, Marsman DS, Sausen PJ, Cattley RC, Gustafsson JÅ. 1996. The rat 17β-hydroxysteroid dehydrogenase type IV is a novel peroxisome-proliferator-inducible gene. Mol Pharmacol 50:1157–1166.

Dhaunsi GS, Gulati S, Singh AK, Orak JK, Asayama K, Singh I. 1992. Demonstration of Cu-Zn superoxide dismutase in rat liver peroxisomes. Biochemical and immunochemical evidence. J Biol Chem 267:6870–6873.

Dieuaide-Noubhani M, Asselberghs S, Mannaerts GP, Van Veldhoven PP. 1997a. Evidence that multifunctional protein 2, and not multifunctional protein 1, is involved in the peroxisomal beta-oxidation of pristanic acid. Biochem J 325:367–373.

Dieuaide-Noubhani M, Novikov D, Baumgart E, Vanhooren JC, Fransen M, Goethals M, Vandekerckhove J, Van Veldhoven PP, Mannaerts GP. 1996. Further characterization of the peroxisomal 3-hydroxyacyl-CoA dehydrogenases from rat liver. Relationship between the different dehydrogenases and evidence that fatty acids and the C27 bile acids di- and tri-hydroxycoprostanic acids are metabolized by separate multifunctional proteins. Eur J Biochem 240:660–666.

Dieuaide-Noubhani M, Novikov D, Vandekerckhove J, Veldhoven PPV, Mannaerts GP. 1997b. Identification and characterization of the 2-enoyl-CoA hydratases involved in peroxisomal beta-oxidation in rat liver. Biochem J 321:253–259.

Distel B, Erdmann R, Gould SJ, Blobel G, Crane DI, Cregg JM, Dodt G, Fujiki Y, Goodman JM, Just WW, Kiel JA, Kunau WH, Lazarow PB, Mannaerts GP, Moser HW, Osumi T, Rachubinski RA, Roscher A, Subramani S, Tabak HF, Tsukamoto T, Valle D, van der Klei I, van Veldhoven PP, Veenhuis M. 1996. A unified nomenclature for peroxisome biogenesis factors. J Cell Biol 135:1–3.

Fransen M, Brees C, Baumgart E, Vanhooren JCT, Baes M, Mannaerts GP, Van Veldhoven PP. 1995. Identification and characterisation of the putative human peroxisomal C-terminal targeting signal import receptor. J Biol Chem 270:7731–7736.

Fransen M, Brees C, Van Veldhoven PP, Mannaerts GP. 1996. The visualization of peroxisomal proteins containing a C-terminal targeting sequence on western blot by using the biotinylated PTS1- receptor. Anal Biochem 242:26–30.

Gould SG, Keller GA, Subramani S. 1987. Identification of a peroxisomal targeting signal at the carboxy terminus of firefly luciferase. J Cell Biol 105:2923–2931.

Gould SJ, Keller GA, Hosken N, Wilkinson J, Subramani S. 1989. A conserved tripeptide sorts proteins to peroxisomes. J Cell Biol 108:1657–1664.

Grant DF, Storms DH, Hammock BD. 1993. Molecular cloning and expression of murine liver soluble epoxide hydrolase. J Biol Chem 268:17628–17633.

Husen B, Adamski J, Szendro PI, Jungblut PW. 1994. Alterations in the subcellular distribution of 17β-estradiol dehydrogenase in porcine endometrial cells over the course of the estrous cycle. Cell Tissue Res 278:227–233.

Jiang LL, Kobayashi A, Matsuura H, Fukishima H, Hashimoto T. 1996. Purification and properties of human D-3-hydoxyacyl-CoA dehydratase: Medium chain Enoyl-CoA hydratase is D-3-hydoxyacyl-CoA dehydratase. J. Biochem 120:624–632.

Jiang LL, Kurosawa T, Sato M, Suzuki Y, Hashimoto T. 1997. Physiological role of D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein. J Biochem (Tokyo) 121:506–513.

Kaufmann M, Carstensen J, Husen B, Adamski J. 1995. The tissue distribution of porcine 17β-estradiol dehydrogenase and its induction by progesterone. J Ster Biochem Mol Biol 55:535–539.

Keller GA, Warner TG, Steimer KS, Hallewell RA. 1991. Cu,Zn superoxide dismutase is a peroxisomal enzyme in human fibroblasts and hepatoma cells. Proc Natl Acad Sci USA 88:7381–7385.

Korneluk RG, Quan F, Lewis WH, Guise KS, Willard HF, Holmes MT, Gravel RA. 1984. Isolation of human fibroblast catalase cDNA clones. Sequence of clones derived from spliced and unspliced mRNA. J Biol Chem 259: 13819–13823.

Kwiatowski J, Skarecky D, Bailey K, Ayala FJ. 1994. Phylogeny of Drosophila and related genera inferred from the nucleotide sequence of the Cu,Zn Sod gene. J Mol Evol 38:443–454.

Latza U, Durkop H, Schnittger S, Ringeling J, Eitelbach F, Hummel M, Fonatsch C, Stein H. 1994. The human OX40 homolog: cDNA structure, expression and chromosomal assignment of the ACT35 antigen. Eur J Immunol 24:677–683.

Leenders F, Adamski J, Husen B, Thole HH, Jungblut PW. 1994a. Molecular cloning and amino acid sequence of the porcine 17β-estradiol dehydrogenase. Eur J Biochem 222: 221–227.

Leenders F, Husen B, Thole HH, Adamski J. 1994b. The sequence of porcine 80 kDa 17β-estradiol dehydrogenase reveals similarities to the short chain alcohol dehydrogenase family, to actin binding motifs and to sterol carrier protein 2. Mol Cell Endocrinol 104:127–131.

Leenders F, Tesdorpf JG, Markus M, Engel T, Seedorf U, Adamski J. 1996. Porcine 80 kDa protein reveals intrinsic 17β-hydroxysteroid dehydrogenase, fatty acyl-CoA-hydratase/dehydrogenase and sterol transfer activities. J Biol Chem 271:5438–5442.

Markus M, Husen B, Leenders F, Jungblut PW, Hall PF, Adamski J. 1995. The organelles containing porcine 17β-hydroxysteroid dehydrogenase are peroxisomes. Eur J Cell Biol 68:263–267.

Mullen RT, Lee MS, Flynn CR, Trelease RN. 1997a. Diverse amino acid residues function within the type 1 peroxisomal targeting signal. Implications for the role of accessory residues upstream of the type 1 peroxisomal targeting signal. Plant Physiol 115:881–889.

Mullen RT, Lee MS, Trelease RN. 1997b. Identification of the peroxisomal targeting signal for cottonseed catalase. Plant J 12:313–322.

Normand T, Husen B, Leenders F, Pelczar H, Baert JL, Begue A, Flourens AC, Adamski J, de Launoit Y. 1995. Molecular characterization of mouse 17 beta-hydroxysteroid dehydrogenase IV. J Ster Biochem Mol Biol 55:541–548.

Novikov D, Dieuaide-Noubhani M, Vermeesch JR, Fournier B, Mannaerts GP, Van Veldhoven PP. 1997. The human peroxisomal multifunctional protein involved in bile acid synthesis: Activity measurement, deficiency in Zellweger syndrome and chromosome mapping. Biochim Biophys Acta 1360:229–240.

Novikov DK, Vanhove G, Carchon H, Asselberghs S, Eyssen HJ, Van Veldhoven PP, Mannaerts GP. 1994. Peroxisomal β-oxidation. Purification of four novel 3-hydroxyacyl-CoA dehydrogenases from rat liver peroxisomes. J Biol Chem 269:27125–27135.

Nuttley WM, Aitchison JD, Rachubinski RA. 1988. cDNA cloning and primary structure determination of the peroxisomal trifunctional enzyme hydratase-dehydrogenase-epimerase from the Yeast *Candida tropicalis* pK233. Gene 69:171–180.

Qin YM, Poutanen MH, Helander HM, Kvist AP, Siivari KM, Schmitz W, Conzelmann E, Hellman U, Hiltunen JK. 1997. Peroxisomal multifunctional enzyme of beta-oxidation metabolizing D-3-hydroxyacyl-CoA esters in rat liver: Molecular cloning, expression and characterization. Biochem J 321:21–28.

Sommer JM, Cheng QL, Keller GA, Wang CC. 1992. In vivo import of firefly luciferase into the glycosomes of Trypanosoma brucei and mutational analysis of the C-terminal targeting signal. Mol Biol Cell 3:749–759.

Subramani S. 1993. Protein import into peroxisomes and biogenesis of the organelle. Annu Rev Cell Biol 9:445–478.

Subramani S. 1996. Protein translocation into peroxisomes. J Biol Chem 271:32483–32486.

Wanders RJ, Denis S. 1992. Identification of superoxide dismutase in rat liver peroxisomes. Biochim Biophys Acta 1115:259–262.

Wigler M, Silverstein S, Lee LS, Pellicer A, Cheng VC, Axel R. 1977. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell 11:223–232.