C-terminal tripeptide Ser-Asn-Leu (SNL) of human D-aspartate oxidase is a functional peroxisome-targeting signal

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The functionality of the C-terminus (Ser-Asn-Leu; SNL) of human D-aspartate oxidase, an enzyme proposed to have a role in the inactivation of synaptically released D-aspartate, as a peroxisome-targeting signal (PTS1) was investigated *in vivo* and *in vitro*. Bacterially expressed human D-aspartate oxidase was shown to interact with the human PTS1-binding protein, peroxin protein 5 (PEX5p). Binding was gradually abolished by carboxypeptidase treatment of the oxidase and competitively inhibited by a Ser-Lys-Leu (SKL)-containing peptide. After transfection of mouse fibroblasts with a plasmid encoding green fluorescent protein (GFP) extended by PKSNL (the C-terminal pentapeptide of the oxidase), a punctate fluorescent pattern was evident. The modified GFP co-localized with peroxisomal thiolase as shown by indirect immunofluorescence. On transfection in fibroblasts lacking PEX5p receptor, GFP–PKSNL staining was cytosolic. Peroxisomal import of GFP extended by PGSNL (replacement of the positively charged fourth-last amino acid by glycine) seemed to be slower than that of GFP–PKSNL, whereas extension by PKSNG abolished the import of the modified GFP. Taken together, these results indicate that SNL, a tripeptide not fitting the PTS1 consensus currently defined in mammalian systems, acts as a functional PTS1 in mammalian systems, and that the consensus sequence, based on this work and that of other groups, has to be broadened to (**S**/A/C/K/N)-(**K**/R/H/Q/N/S)-**L**.

INTRODUCTION

Only recently has the presence of free D-amino acids in mammalian tissues become accepted. Neuronal tissues in particular seem to be enriched in such amino acids, as reported for D-serine [1] and D-aspartate [2–7]. Moreover the levels of D-aspartate vary according to age, being highest in the neonatal stage where millimolar concentrations are reached [2–6]. On the basis of the discrete neuronal localizations of D-aspartate, this amino acid being concentrated in the external plexiform layer of the olfactory bulb, hypothalamic supraoptic and paraventricular nuclei, the medial habenula and certain brainstem nuclei in rat [7], and the known activation of the *N*-methyl-D-aspartate receptor by D-aspartate [8], Snyder and co-workers [7] suggested that D-aspartate might function as a neurotransmitter. The high concentrations of D-aspartate in various endocrine glands such as adrenals, posterior pituitary, and pineal gland [2,7] could imply a role in neuroendocrine modulation. The regulation of sex steroid production by D-aspartate was recently reported [9].

Although the origin of D-aspartate is not known, its removal is thought to be mediated by D-aspartate oxidase (DDO). This idea is based on the inverse relationship between D-aspartate levels and the oxidase activity during ontogenic development [2,10] and the reciprocal localizations of DDO and D-aspartate in brain [7]. DDO was discovered approx. 45 years ago in rabbit kidney [11]. The enzyme catalyses the oxidative deamination of dicarboxylic D-amino acids such as D-glutamate and D-aminoadipate, but D-aspartate and *N*-methyl-D-aspartate are preferred substrates [10,12]. Highest activities are found in kidney, followed by liver and brain [10,13] and the enzyme activity was shown to be localized in peroxisomes in rat and human liver [10] and in rat, bovine and sheep kidney [12].

Peroxisomal proteins are synthesized on free polyribosomes. Most peroxisomal matrix proteins contain at their C-terminus a topogenic signal [called peroxisome-targeting signal 1 (PTS1)] that is recognized by a PTS1-binding protein, renamed peroxin protein 5 (PEX5p) [14], which mediates their import into peroxisomes (reviewed in [15]). PTS1 was first defined as the tripeptide Ser-Lys-Leu (SKL) [16] but subsequent studies have shown some degeneracy. In mammals, this has led to the consensus (**S**/A/C)-(**K**/R/H)-**L** [17]. Whereas the C-terminus of bovine DDO corresponds to SKL [18], the human enzyme was reported to end in Ser-Asn-Leu (SNL) [19]. The latter combination has hitherto not been proved to be functionally active in mammals. In fact, engineered chloramphenicol acetyltransferase (CAT) ending in SNL was shown to be cytosolic in transfected monkey kidney CV-1 cells [20]. The tripeptide SNL, however, fits the consensus proposed for glycosomal protein targeting, which is (**S**/A/C/G/H/N/P)-(**K**/R/S/H/M/N/S)-(**L**/I/M/Y), and is active in *Trypanosoma brucei* [21]. To investigate the role of SNL in the targeting of human DDO in more detail, the interactions between the C-terminus of this protein and PEX5p were analysed *in vitro* and *in vivo*.

MATERIALS AND METHODS

Binding to PEX5p *in vitro*

Human DDO was expressed in *Escherichia coli* and purified as described previously [19]. Recombinant proteins were subjected to SDS/PAGE, followed by staining of the gels or electroblotting to nitrocellulose. Blots were transiently stained with Ponceau Red and, after blocking, were incubated with a bacterially

Abbreviations used: CAT, chloramphenicol acetyltransferase; DDO, D-aspartate oxidase; (E)GFP, (enhanced) green fluorescent protein; PEX5p, peroxin protein 5; PTS1, peroxisome-targeting signal 1.

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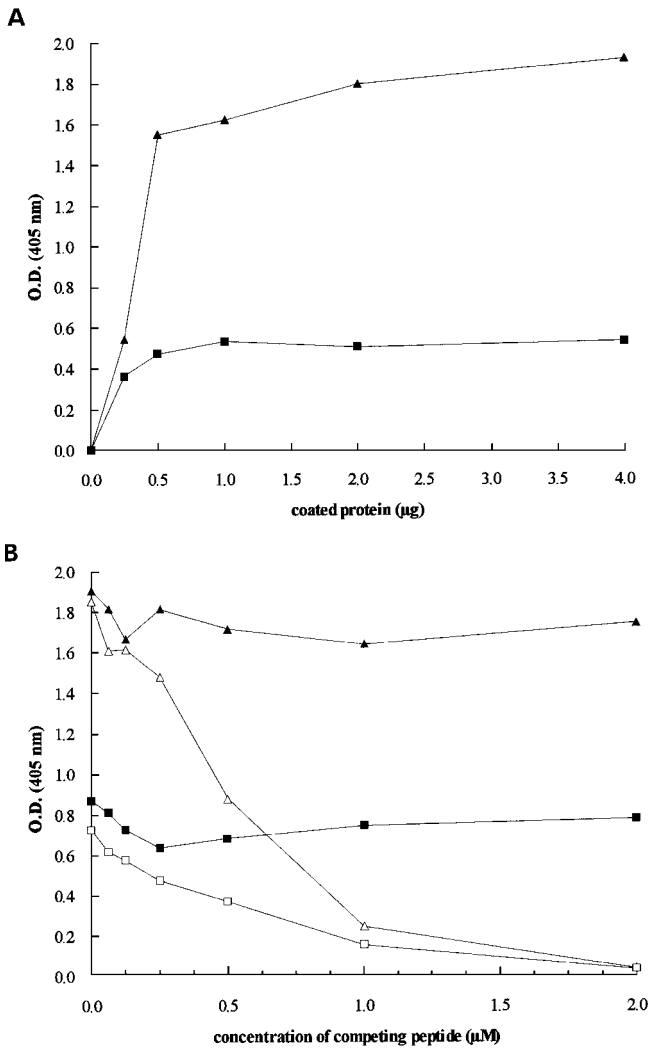

Figure 1 Interaction of human PEX5p with human DDO

(**A**) The indicated amounts of bacterially expressed and purified human DDO (▲) were coated in microtitre wells, followed by incubation with human biotinylated PEX5p and detection of the complexes formed after 60 min, as described previously [22]. For comparison, the interaction with human albumin cross-linked to peptide P1 ending in SKL (HSA-SKL) (■) was analysed. On average, ten P1 peptides were coupled to one albumin molecule. (**B**) Wells were coated with 1 μg of DDO (△,▲) or HSA-SKL (□,■) and during the incubation with PEX5p increasing amounts of competing peptides P1 (△,□) or P2 (▲,■) were added. The P2 peptide resembles P1 but does not contain SKL [22].

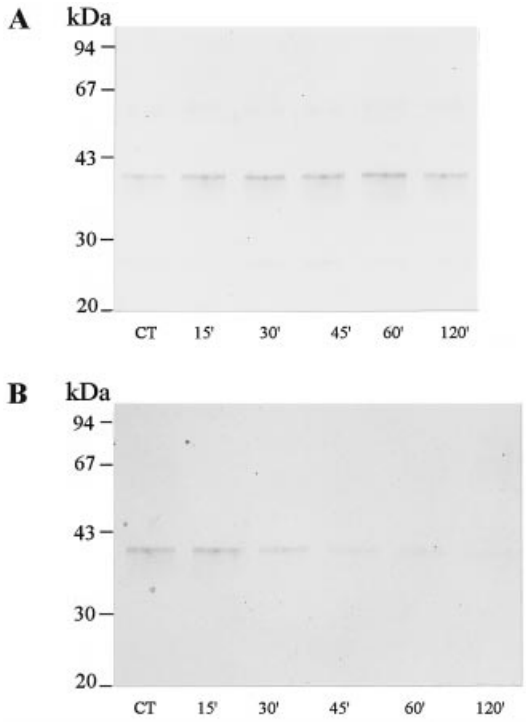

Figure 2 Interaction of human PEX5p with human DDO treated with carboxypeptidase

Bacterially expressed human DDO (3 μg) was dissolved in 60 μl of 100 mM Tris/HCl buffer (pH 7.5)/0.04% (w/v) Tween 20 containing 75 ng of carboxypeptidase A (Sigma) and incubated at room temperature. At the indicated time points (15–120 min), duplicate aliquots of 6 μl were removed and precipitated with trichloroacetic acid. The pellets were subjected to SDS/PAGE followed by silver staining (**A**) or by blotting to nitrocellulose and incubation with biotinylated PEX5p [22,23] (**B**). Abbreviation: CT, control (no carboxypeptidase A added).

expressed and biotinylated human PEX5p fusion protein [22,23]; complexes formed were detected by streptavidin–alkaline phosphatase as described previously [23].

For competition experiments, recombinant protein was coated in wells of a 96-well microtitre plate followed by incubation with the biotinylated PEX5p fusion protein [23]. During the incubation with PEX5p, increasing amounts of peptides P1 or P2, corresponding to the C-terminus of rat acyl-CoA oxidase, were added. P1 ended in SKL, whereas this tripeptide was deleted from P2 [23].

Transfection studies

To generate a green fluorescent protein (GFP) fusion product containing the C-terminus of human DDO, the enhanced GFP (EGFP)–N1 vector (Clontech) was cleaved by *Bsr*GI and *Not*I, thereby removing the last 10 coding bases, including the stop codon and four 3′ non-coding bases. Two 24-mers (5′-GTAC-ATTCCCAAGTCAAACCTGTA-3′ and 5′-GGCCTACAGG-TTTGACTTGGGAAT-3′) were allowed to hybridize to generate a DNA insert with the appropiate overhangs and ligated to the cleaved EGFP–N1. In the resulting plasmid, the codons for L and Y of GFP were restored, the codon for the last amino acid (K) of GFP was replaced by a sequence coding for PKSNL, and the *Not*I site was destroyed. Two other GFP fusion products were generated similarly: GFP–PGSNL with the 24-mers 5′-GTACATTCCCGGTTCAAACCTGTA-3′ and 5′-GGCCTA-CAGGTTTGAACCGGGAAT-3′, and GFP–PGSNG with the 24-mers 5′-GTACATTCCCAAGTCAAACGGTTA-3′ and 5′-

Figure 3 Subcellular localization of GFP ending in SNL in cultured mouse fibroblasts

Primary mouse PEX5$^{+/+}$ (**A**), primary mouse PEX5$^{-/-}$ (**B**) and immortalized mouse PEX5$^{+/+}$ (**C**, **D**) were transfected with the modified EGFP–N1 plasmid encoding GFP ending in YPKSNL, and analysed after 26 h (**A**, **B**) or 48 h (**C**, **D**) by fluorescence microscopy either directly (**A**, **B**, **C**) or after fixation and immunostaining for peroxisomal thiolase (**D**). (**E**–**H**) Fluorescence microscopy was performed at different time intervals after transfection of immortalized mouse PEX5$^{+/+}$ fibroblasts with the modified EGFP–N1 plasmids coding for GFP ending in PKSNL (**E**, **F**) or in PGSNL (**G**, **H**). Representative results are shown for pictures after 8 h (**E**, **G**) and after 38 h (**F**, **H**). Scale bars: 10 μm.

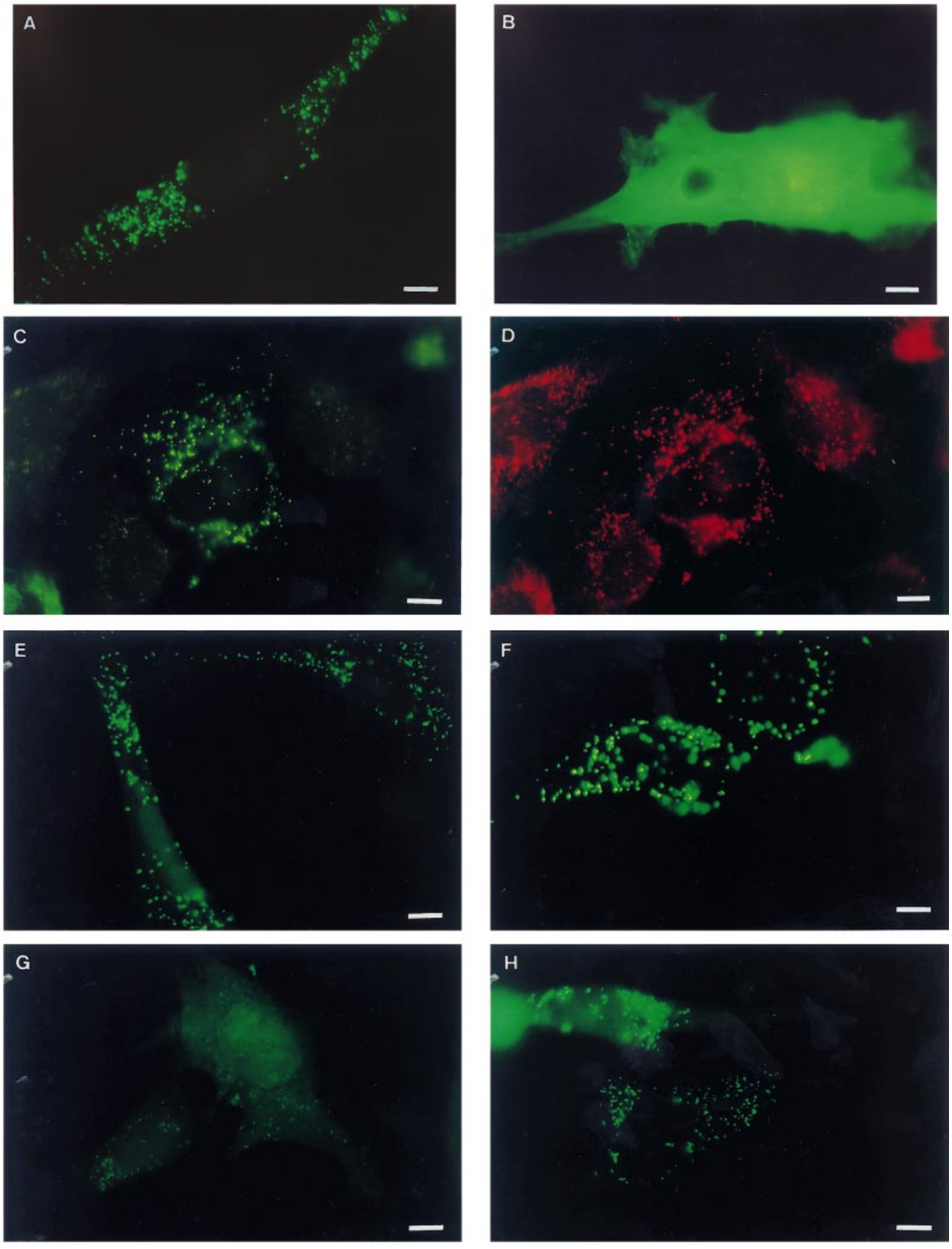

Figure 3 For legend see opposite page

GGCCTAACCGTTTGACTTGGGAAT-3′. Positive clones were verified by base sequencing (ALF-DNA sequencer, Pharmacia) using a primer corresponding to bases 721–746 of EGFP–N1 (5′-AGCAAAGACCCCAACGAGAAGCGCG-3′).

Primary fibroblasts were derived from embryonal wild-type mice and from PEX5 knock-out mice [24] and immortalized by introduction of the SV40 large T antigen with an adenoviral vector [25]. The cells were transfected with the plasmids encoding the GFP fusion proteins by using Lipofectamine Plus reagent (Gibco–BRL); the subcellular localization of these proteins was monitored 8–74 h later by fluorescence microscopy.

For immunostaining, monolayers were fixed with 4% (v/v) paraformaldehyde for 20 min and incubated sequentially with 0.2% (w/v) Triton X-100 for 10 min, 1% (w/v) glycine for 10 min, preimmune pig serum (diluted 5-fold) (Dako) for 30 min, polyclonal rabbit anti-(rat peroxisomal thiolase) [26] (diluted 1:100) for 1 h at 37 °C, biotinylated pig anti-(rabbit antibodies) (diluted 1:400) (Dako) for 45 min and CY3–streptavidin (diluted 1:50) (Zymed) for 1 h. All dilutions were made in PBS containing 5% (w/v) BSA and cells were washed three times with PBS between each incubation. Fluorescence was observed under a Leica microscope with standard FITC filters for EGFP, and rhodamine B isothiocyanate (RITC) filters for CY3.

RESULTS AND DISCUSSION

To evaluate the role of the C-terminal tripeptide, SNL, of human DDO [19] in the peroxisome targeting of this oxidase, the interaction of recombinant human DDO with human PEX5p was analysed. As shown in Figure 1, a fusion product containing the tetratricopeptide repeat domain of human PEX5p, known to be required for the interaction with PTS1 [27], was able to bind to DDO that was coated in microtitre wells. Moreover the interaction was competitively inhibited in the presence of peptide ending in SKL. Also after electrophoresis and blotting, interaction between DDO and PEX5p was apparent (Figure 2). By subjecting DDO to proteolysis with carboxypeptidase A, a time-dependent loss of interaction with PEX5p was observed (Figure 2), indicating that the C-terminus is responsible for PEX5p binding.

To demonstrate that SNL can also mediate import, a vector was made that expressed GFP ending in LYPKSNL instead of LYK. The last five residues of the modified GFP are identical with those of human DDO. After transfection of HepG2 cells, a human hepatoma cell line (results not shown), or mouse fibroblasts, either primary cultures or immortalized cells, with the modified plasmid a punctate fluorescence pattern was observed (Figures 3A and 3C). Such staining is reminiscent of that seen with a GFP fusion protein ending in SKL [24] and, on the basis of the co-localization to peroxisomal thiolase, these fluorescent organelles were identified as peroxisomes (Figures 3C and 3D). Moreover the same construct, when transfected in mouse fibroblasts lacking a functional PEX5p [24], resulted in a cytosolic fluorescence (Figure 3B).

On the basis of the initial studies by Subramani and co-workers [17], (**S**/A/C)-(**K**/R/H)-**L** has been proposed as a consensus for PTS1 in mammals. Meanwhile it has become clear that this consensus is prone to more degeneracy. Examples of new and functional mammalian PTS1s include SQL, present in rabbit alanine:glyoxylate aminotransferase [28,29] and rat pristanoyl-CoA oxidase [30], and ANL, present in human catalase [31]. Other mammalian PTS1s, on the basis of the targeting of engineered reporter proteins, seem to be KKL, SSL and NKL [29]. Our own experiments and import studies *in vitro*

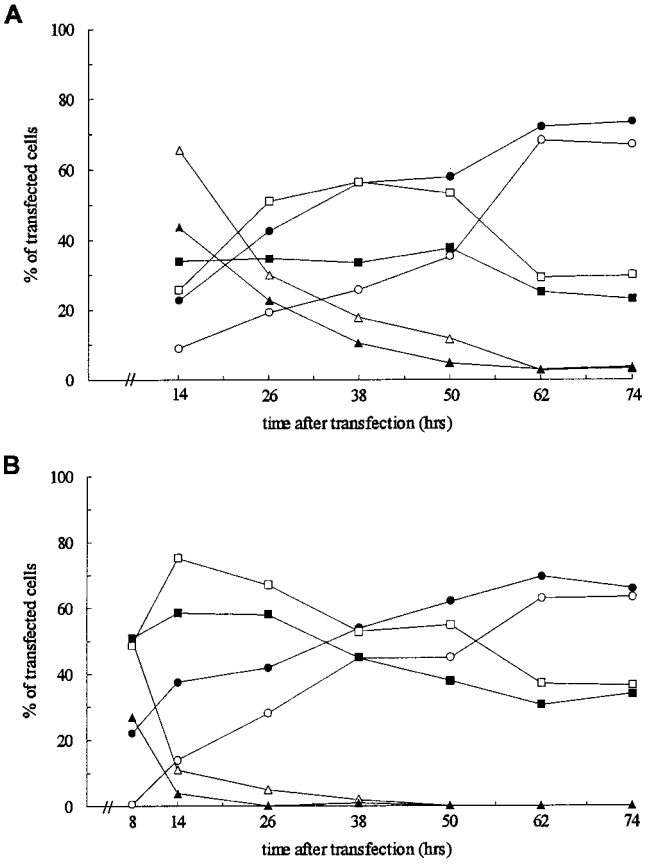

Figure 4 Analysis of fluorescent patterns in mouse fibroblasts expressing GFP ending in PKSNL or PGSNL

Primary (**A**) (results derived from one of two separate experiments) and immortalized (**B**) (results are averages of duplicate transfections) mouse PEX5$^{+/+}$ fibroblasts were transfected with the modified EGFP–N1 plasmids coding for GFP ending in PKSNL (●,▲,■) or PGSNL (○,△,□). The fluorescent patterns in 100–400 cells (**A**) or 400 cells (**B**) were analysed at the indicated time points after transfection so as to obtain the percentages of cells with a peroxisomal (○,●), a cytosolic (△,▲) or a cytosolic plus peroxisomal fluorescence (□,■). In cases where values overlap, only the open symbols are shown.

presented here convincingly indicate that SNL, in contrast with previous results [20], has to be added to the list of functional PTS1 variants in mammals. Therefore the consensus sequence can be broadened to (**S**/A/C/K/N)-(**K**/R/H/Q/N/S)-**L**. In particular the degeneracy at the penultimate position starts to resemble that observed for PTS1 in glycosomal import [21]. One should keep in mind, however, that besides the C-terminal tripeptide other residues might affect the interactions. To be effective, the tripeptide ANL, present in human catalase and resembling the C-terminus of DDO, has to be preceded by a positively charged residue, Arg or Lys [31]. Interestingly, also in human DDO, the fourth-last residue is Lys [19]. To demonstrate the possible importance of a positive charge at the fourth-last residue, we analysed the subcellular localization of GFP extended by PGSNL. This fusion protein was also imported into peroxisomes but at a slower rate than GFP–PKSNL (Figures 3E–3H and 4). Hence, compared with ANL [31], the charge of the amino acid preceding SNL is less critical but a positive charge seems to enhance the import rate. A similar ameliorating effect of Lys, one residue upstream of PTS1, was seen on the glyoxysomal import of CAT ending in ANL [32]. Perhaps these observations can

explain the apparent conflict of our data with a report by Gould et al. [20], who concluded that SNL, on the basis of the cytosolic localization of CAT ending in GSNL, in CV-1 monkey kidney cells, was not functional as PTS1 in mammals. If immunolocalization of this reporter was done rather early after transfection, a cytosolic staining might indeed have been seen, as occurred in some of our experiments with GFP–PGSNL (Figure 4). In this context it is worth noting that the firefly luciferase construct ending in SNL and shown to be targeted to glycosomes [21] is also preceded by a Lys residue. Changes, although small, at the last position of SNL as a PTS1 are not tolerated or are less well tolerated. Replacement of the C-terminal leucine residue of GFP–PKSNL by glycine resulted in a completely cytosolic staining in control mouse fibroblasts, even when analysed 74 h after transfection (results not shown).

Finally, with respect to SKL, the number of peroxisomal matrix proteins ending in SNL is very small. In mammals, SNL is found and is preceded by Arg in the recently cloned trihydroxycoprostanoyl-CoA oxidase from rabbit [33]. Whereas the human counterpart of this oxidase contains the classical SKL motif [34], the C-terminus of the rat enzyme contains HKM [35]. Although the latter sequence fits the consensus for PTS1 described for glycosomal import [22], it does not seem to be functional [35]. A search in the databases did not reveal other (putative) peroxisomal SNL-containing mammalian proteins (L. Amery and P. P. Van Veldhoven, unpublished work). In addition, in other kingdoms SNL does not seem to be abundant. One possible candidate is hydroxymethylglutaryl-CoA synthase from *Dictyostelium discoideum* (C-terminus QSNL; accession number 54872). This enzyme catalyses one of the first steps in the synthesis of isoprenoids, a pathway that is at least partly peroxisomal in mammals [36,37]. Another candidate is quinate 5′-dehydrogenase, ending in KSNL, of *Aspergillus nidulans* [38].

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