Binding of the Peroxisomal Targeting Sequence SKL Is Specified by a Low-Affinity Site in Castor Bean Glyoxysomal Membranes

A Domain Next to the SKL Binds to a High-Affinity Site

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The carboxyl-terminal amino acid sequence serine-lysine-leucine (SKL) is the consensus peroxisomal targeting sequence 1 (PTS1) and is sufficient to direct a polypeptide to peroxisomes in vivo in plants, animals, and yeasts. However, there are two sites on alkali-stripped glyoxysomal membranes from castor bean (Ricinus communis) endosperm that bind the peptide YHKHLKPLQSKG (SKLp), the sequence of the last 12 amino acids of acyl-coenzyme A oxidase (N.E. Wolins, R.P. Donaldson [1994] Biol Chem 269: 1149–1153). It was hypothesized that one of these sites interacts with information other than the PTS1. To explore the sequence requirements for each SKLp binding site, we tested the peptides YHKHLKPLQSKG and YHKHLKPLQS and found that they bound to the high-affinity site, but not to the low-affinity site. When the high-affinity site was blocked with YHKHLKPLQSKG, SKLp bound to the low-affinity site with a dissociation constant (Kd) of 8.5 μM. In an attempt to disrupt high-affinity binding, two of the upstream, positively charged residues were replaced with negatively charged residues to make the peptide YHKETEPLQSKG. YHKETEPLQSKG did not bind to either site on the glyoxysomal membranes. These results indicate that the PTS1 binds to the low-affinity site and that the adjacent, positively charged domain binds to the high-affinity site.

Peroxisomal and glyoxysomal matrix proteins are synthesized on free ribosomes and then translocated through the peroxisomal membrane (Fujiki and Lazarow, 1985). There are a variety of amino acid sequences that target these matrix proteins to peroxisomes and there are different receptors for the different PTSs (McNew and Goodman, 1996). Because there are many steps required for a newly synthesized protein to be recognized, imported into peroxisomes, and processed into a functional enzyme, it is not surprising that many genes have been shown to be necessary for peroxisomal assembly. The proteins that are responsible for peroxisomal biogenesis are now designated "peroxins," and 12 peroxin genes found in yeast and human cells have been defined in a recent review as PEX

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through PEX 12 (Distel et al., 1996); we will use the nomenclature defined in this review.

The carboxyl-terminal sequence SKL has been shown to direct proteins to the peroxisomes of mammalian cells in vivo (Gould et al., 1989). Subsequently, this sequence has been found to be functional in yeast and plant peroxisomes as well as in trypanosomal glycosomes (Keller et al., 1991). Many mature peroxisomal proteins have the carboxyl-terminal amino acid sequence SKL or a similar sequence; this motif has been designated PTS1 (Gould et al., 1989). Additionally, a second peroxisomal-targeting sequence (PTS2) with the consensus sequence of RLXXXXXLH has been identified near the amino terminus of some peroxisomal proteins (Swinkels et al., 1991; Gietl et al., 1994). Proteins can also be imported as oligomeric complexes (Glover et al., 1994; McNew and Goodman, 1994; Walton et al., 1995), which can allow for polypeptides with no peroxisomal-targeting information to be imported into peroxisomes (McNew and Goodman, 1996). In spite of two well-defined PTSs and the identification of many PEX genes necessary for peroxisomal assembly, the interaction of these targeting sequences with the various peroxins and how they mediate the selective import of peroxisomal proteins remains to be demonstrated.

Glyoxysomes are specialized peroxisomes that catalyze many of the reactions that convert triglycerides into soluble, transportable carbohydrates in germinating oil seeds. Glyoxysomal matrix proteins share the same protein targeting information as the proteins in peroxisomes of protozoa and animals (Keller et al., 1991). We were interested in characterizing the components in the glyoxysomal membranes that recognize this targeting information. To this end we initiated an investigation of the binding interactions of PTSs with isolated castor bean (Ricinus communis) glyoxysomal membranes. Binding

Abbreviations: AHLHp, D-Tyr-PDVNQRIARISAHLH; ARMp, D-Tyr-AKARM; ETEp, D-Tyr-HKETEPLQSKL; Kd, dissociation constant; Ki, inhibitor constant; PEX, peroxin/peroxisome-biogenesis gene, Pex, peroxin protein; PTS, peroxisomal targeting sequence; SKLp, D-Tyr-HKHLKPLQSKL; SKGp, D-Tyr-HKHLKPLQSKG; S-p, D-Tyr-HKHLKPLQS.
studies have been used to identify the receptors for the targeting sequences in several subcellular compartments. For example, in the investigations of mitochondrial protein import, binding measurements defined two protein import receptors (Sollner et al., 1989). We developed an experimental system that would allow the characterization of the interactions of PTS peptides with PTS receptors, independent of other components in the import apparatus. Previously, we found that the peptide SKLP had the same sequence as the last 12 amino acids of rat acyl-CoA oxidase (p-Tyr-HKHLKPLQSKL) (Miyazawa et al., 1987), and bearing PTS1 with some nearby, positively charged residues, bound to two very abundant, saturable sites on the glyoxysomal membranes (Wolins and Donaldson, 1994).

PTS receptors in yeasts and humans, such as Pex5p and Pex7p (previously designated Pas8 and Pas7, respectively), are found in the cytosol, in peroxisomal membranes, and within the peroxisomal matrix (Distel et al., 1996; McNew and Goodman, 1996). A Pex5p-docking protein, Pex13p, has been identified in the membranes of Pichia pastoris peroxisomes (Gould et al., 1996) and other species (Distel et al., 1996). Pex5p, the peroxin product of the PEX5 gene, has been characterized as a PTS1 receptor. For example, P. pastoris PEX5 mutants do not import PTSI proteins but do import thiolase, a PTS2 protein (McCollum et al., 1993). Pex5p is a member of the tetratricopeptide-repetet family of proteins that includes the 72-kD mitochondrial import receptor (van der Liej et al., 1993). A PTS1 peptide very similar to our SKLP, including the positively charged residues near the PTS1, has been shown to bind to the isolated PpPex5 and to a protein in the peroxisomes of P. pastoris (McCollum et al., 1993; Terlecky et al., 1995). These authors concluded that Pex5p is the PTS1 receptor. However, Pex5p has only been shown to bind to one PTS1-containing peptide, and the demonstration of sequence specificity was limited to the deletion of the complete carboxyl-terminal SKL of the peptide.

There are indications that internal sequences, in addition to the SKL, are required for import. For example, in a mammalian system the deletion of 10 amino acids next to, but not including, the PTS1 abolishes import (Motley et al., 1995). Furthermore, the removal of the carboxyl-terminal from a yeast protein that is imported via the PTS1 receptor does not prevent import (Elgersma et al., 1995a). Finally, a recent report shows that many very different carboxyl triplets will function as a PTS1 (Elgersma et al., 1995b). Thus, it is likely that the recognition of a PTS1 protein for peroxisomal import involves more than the carboxyl three amino acids and more than one peroxin interaction.

We hypothesized that only one of the two SKLP binding sites in the glyoxysomal membrane is specific for the SKL (PTS1) sequence and that the second site recognizes other sequence information. To determine the sequence specificity for the two SKLP binding sites, we characterized the binding of the modified forms of SKLP and other peptides bearing peroxisomal targeting information to the glyoxysomal membrane. Here we present evidence that SKL binding is specified by the lower affinity site and that the high-affinity site interacts with other amino acid residues in SKLP.

MATERIALS AND METHODS

Peptides were synthesized by Chiron (San Diego, CA). Peptides SKLP, SKGP, S-p, ETEp, ARMp, and AHLHp were 93, 84, 77, 60, 84, and 95% pure, respectively, as determined by the manufacturer using reverse-phase HPLC. Radioiodination of all peptides was done as described previously (Wolins and Donaldson, 1994).

Binding was measured as described previously (Wolins and Donaldson, 1994) by incubating alkali-stripped glyoxysomal membranes with the 125I-peptide, then collecting the membranes on glass fiber and counting gamma emission from filters. The data were analyzed by the Macintosh version of the computer program LIGAND (Munson and Rodbard, 1980). Glyoxysomal membranes were isolated from castor bean (Ricinus communis L. var Hale) endosperm (Wolins and Donaldson, 1994).

RESULTS

Two SKLP binding sites were detected on the glyoxysomal membrane using a binding assay that employs a glass fiber filter to trap the glyoxysomal membrane and the 125I-SKLP bound to these membranes, allowing the free 125I-SKLP to pass through the filter (Wolins and Donaldson, 1994). Figure 1A shows data from the previous study (Wolins and Donaldson, 1994) that were reanalyzed using the computer program LIGAND (Munson and Rodbard, 1980). The data was best fit with two saturable binding sites: the high-affinity site had a $K_d$ of 79 nM and an abundance of 10.5 nmol mg$^{-1}$ protein and the low-affinity site had a $K_d$ of 6100 nM and an abundance 77 nmol mg$^{-1}$ protein. This fit was significantly (P = .057) better than fitting the data to one site and a nonsaturable site (background). A nonsaturable binding site would appear as a horizontal line on a Scatchard plot. In the present study derivatives of SKLP were used in the same assay system to determine the amino acid sequence specificity for each of these two binding sites. It has previously been shown that the carboxyl-terminal Leu was necessary for localization of luciferase to the peroxisomes of mammalian cells that had been transfected with a plasmid-expressing luciferase (Gould et al., 1989). Therefore, we hypothesized that elimination of the Leu hydrophobic side chain, by replacing the Leu with a Gly (SKGP), would change the interaction of the peptide with the glyoxysomal membrane. Figure 1B shows 125I-SKGP binding to the glyoxysomal membrane. LIGAND (Munson and Rodbard, 1980) analysis indicated that the data fit one binding site plus a nonsaturable, nonspecific binding site. This suggests that SKGP only binds to one site on the glyoxysomal membrane.

Removal of the Leu side chain is a minor change in the PTS1 and did not reduce the high-affinity binding of SKGP to the glyoxysomal membranes. Thus, what remained of the PTS1 in the SKGP mediated high-affinity binding to the glyoxysomal membrane. To further define the residues in SKLP that interact with the high-affinity site, we measured
Targeting Sequence Binding to Castor Bean Glyoxysomal Membranes

Figure 1. PTS1 is necessary for low-affinity binding, and high-affinity binding requires positively charged amino terminal residues. A to E, Scatchard transformations of $^{125}$I-peptides binding to glyoxysomal membranes. A, A line for each of the two sites we previously reported (Wolins and Donaldson, 1994) but reanalyzed by LIGAND (Munson and Rodbard, 1980). B, Binding of $^{125}$I-SKGp. C, Binding of $^{125}$I-S-p. D, Binding of $^{125}$I-ETEp. E, Binding of $^{125}$I-ARMp. Each peptide was labeled with $^{125}$I and the data in the Scatchard analysis were generated by calculating the amount of $^{125}$I-peptide that was bound to the glyoxysomal membrane at concentrations from 20 to 5000 nM. Each data point represents measurements made in triplicate with two separate membrane preparations. The data for SKGp and S-p were fit by LIGAND to a one-site model with a nonspecific binding site (Munson and Rodbard, 1980). The lines shown fit the specific binding; the best fit for the remaining points would be a horizontal line representing nonspecific binding. F, The same data as in A through E plotted as peptide binding versus peptide concentration.

the binding parameters of the peptide $\alpha$-Tyr-HKHLKPLQS (S-p), lacking the KL of PTS1. Figure 1C shows that S-p binds to one site in the glyoxysomal membrane. The points shown that do not fit the 300 nM $K_d$ line are best fit, in the LIGAND (Munson and Rodbard, 1980) analysis, to a horizontal line, which is nonspecific binding.

Because the high-affinity site was not totally dependent on either Lys or Leu of the PTS1, we hypothesized that upstream, positively charged amino acids were important for high-affinity binding. To test this hypothesis we replaced two positive residues in SKLp with two negative residues, resulting in peptide ETEp as opposed to SKLp, and we characterized the interaction of ETEp with the glyoxysomal membrane. Figure 1D shows that this peptide did not bind to the glyoxysomal membranes, demonstrating that the upstream sequences are important in SKLp binding to both the high- and low-affinity sites.

We tested the binding of another PTS1 peptide, ARMp, that lacked the upstream, positively charged residues. ARM is the carboxyl-terminal sequence of glyoxysomal isocitrate lyase from R. communis (Beeching and Northcote, 1987). The ARM sequence resembles PTS1, SKL, in the following ways: Ala is a functional substitute for Ser, Arg is a functional substitute for Lys (Gould et al., 1989), Met is a medium-sized hydrophobic amino acid like Leu, and this ARM sequence is carboxyl-terminal. Thus, we hypothesized that carboxyl-terminal ARM of isocitrate lyase is a functional PTS1 and should bind to the PTS1 receptor. However, Figure 1E shows virtually no binding of the $^{125}$I-ARMp to the glyoxysomal membrane under our assay conditions. Furthermore, 10 $\mu$M ARMp does not compete with $^{125}$I-SKLp binding to glyoxysomal membranes (data not shown). Again, this is indicative of the need for the upstream, positively charged residues found in the binding of the SKLp and SKGp.

SKGp and S-p were thought to bind to the same site as SKLp, and to directly demonstrate this possibility SKGp and S-p were used to compete with $^{125}$I-SKLp. Figure 2A
Figure 2. SKGp and S-p displace $^{125}$I-SKLp from the glyoxysomal membrane in a dose-dependent manner. Approximately 500 pm of $^{125}$I-SKLp, with a specific activity of about 200 Ci/mmol, was added to each sample. Then varying amounts of unlabeled SKGp (A) or S-p (B) were added to the sample and the tubes were incubated at 4°C for 2 h. The membranes and the $^{125}$I-SKLp bound to the membranes were trapped on filters and counted as described in “Materials and Methods.”

Table I is a summary of the various peptides that were used in this investigation and indicates the ability of each peptide to bind to the high- and low-affinity sites. SKLp, SKGp, and S-p bind the high-affinity site, but only SKLp binds to the low-affinity site. ETEp, ARMp, and AMLHp do not bind to either site on the glyoxysomal membrane. This suggests that many of the amino acids of SKLp, except the carboxyl-terminal Leu, are required for binding to the glyoxysomal membrane and that the carboxyl-terminal Leu is necessary for binding to the low-affinity site. In vivo studies show that the first amino acid in PTS1 can be Ala, Ser, Cys, Lys, or Arg; the second amino acid can be Lys, Arg, His, or Ser; and the last amino acid must be Leu (Gould et al., 1989; Keller et al., 1991; Motley et al., 1995). It seems doubtful that such divergent three amino acid sequences could mediate an interaction with sufficient affinity and specificity to effectively recruit peroxisomal matrix proteins to the peroxisomal import apparatus. It is possible that there are two levels of selection for peroxisomal import: the highest affinity binding would initially bind the peroxisomal protein to a receptor and then the PTS1 would serve to direct the protein into the peroxisomal import.
apparatus. The high-affinity site would concentrate proteins from the cytosol and the low-affinity site would be more stringent in selecting proteins for transfer to the import channel. If the protein concentration is high enough, there would be a direct interaction of PTSl proteins from the cytosol and the low-affinity site would be bypassed. The high-affinity site would concentrate proteins with the low-affinity site of the import apparatus, bypassing the import channel. If the protein concentration is high, it is likely that there are interactions between peroxisomal proteins and the peroxisomal import apparatus that have not been elucidated. Indeed, an investigation of the import of a peroxisomal protein in mammalian cells showed that the deletion of 10 amino acids immediately upstream of the PTS1 caused the protein not to be imported, although the PTS1 was present (Motley et al., 1995). We have demonstrated that the low-affinity site is PTS1-specific and is probably the entity that confers PTS1 specificity to import in R. communis. The affinity of about 10⁶ M⁻¹ is reasonable for an interaction mediated by a degenerate three amino acid sequence, but is at least 100-fold less than that reported for the nuclear import receptor (Silver et al., 1989), the mitochondrial import receptors (Söllner et al., 1989), and the ER retention receptor (Wilson et al., 1993). Furthermore, glyoxysomal matrix proteins bind to the glyoxysomal membrane with an affinity of at least 1000-fold greater than the PTS1 binding (Wolins and Donaldson, 1994). Thus, it is unlikely that PTS1 binding (the low-affinity site) alone concentrates glyoxysomal proteins on the glyoxysomal membrane for import. Nevertheless, interactions having this low-affinity level have been found to be important. For example, during sorting in the secretory pathway adaptins and the Tyr-based, four-amino acid-sorting signal interact with an affinity in the range of a few micromoles (Ohno et al., 1995). The PTS1 and PTS2 receptors Pex5p and Pex7p were initially thought to be in the membranes of peroxisomes. Recently, it has become apparent that the PTS receptor proteins are mostly cytoplasmic in yeasts and humans, although small amounts of the receptors are associated with peroxisomes (Gould et al., 1996). The PTS1 receptor protein has been shown to interact with a docking protein, Pex13p, in the membrane. Additional membrane proteins may be involved in the translocation steps of import. A further degree of complexity is in the import of multisubunit aggregates, oligomeric folded proteins, into peroxisomes (Glover et al., 1994; Walton et al., 1995; McNew and Goodman, 1996). Thus, a protein lacking a PTS can be imported in association with a protein possessing a PTS. It is not clear to what extent these principles apply to plant peroxisomal import systems, as well as how the PTS information interacts with the various components of the import apparatus, the cytosolic PTS receptors, the docking protein, and translocators. Thus, the binding of PTS1, which we observed with isolated glyoxysomal membranes, could represent interactions with residual PTS receptors in the membrane or interactions of the PTS with other components of the docking and translocation apparatus in the membranes.

Table 1. Peptide binding to the high- and low-affinity sites is determined by positive amino acid residues and the C-terminal SKL.
The first five peptides are based on the C-terminal sequence of acyl-CoA oxidase (Miyazawa et al., 1987). The last peptide is the glyoxysomal-targeting domain from near the N terminus of malate dehydrogenase (Gietl et al., 1994).

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Amino Acid</th>
<th>Origin</th>
<th>High-Affinity Binding</th>
<th>Low-Affinity Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKLp</td>
<td>YHKLKLPLLQSKL</td>
<td>Acyl-CoA oxidase</td>
<td>+ + a</td>
<td>4 b</td>
</tr>
<tr>
<td>SKGp</td>
<td>YHKLKLPLLQSG</td>
<td>Acyl-CoA oxidase</td>
<td>+ +</td>
<td>- c</td>
</tr>
<tr>
<td>S-p</td>
<td>YHKLKLPLLQ</td>
<td>Acyl-CoA oxidase</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ETEp</td>
<td>YKTETEPLQSKL</td>
<td>Acyl-CoA oxidase</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ARMp</td>
<td>YAKARM</td>
<td>Isocitrate lyase</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AHHlp</td>
<td>YPDYNQRIARISAH</td>
<td>Malate dehydrogenase</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a + + , K_d < 200 nM, b + , K_d > 200 nM, c - , No binding.
The high-affinity site does not bind to PTSL, but has many characteristics of a glyoxysomal import receptor. First, it is specifically located in glyoxysomal membranes; SKLp does not bind to the ER or mitochondrial membranes (Wolins and Donaldson, 1994). Second, this site binds multiple glyoxysomal proteins (data not shown) and the binding to this site is specific for glyoxysomal proteins (Wolins and Donaldson, 1994). Third, binding to the high-affinity site is pH-dependent, with a pH optimum close to a cytosolic pH (Wolins and Donaldson, 1994). Peptide binding to this site requires positive charges in the peptide (Table I).

The underlined positive charges within the SKLp, HKHLKPLQSKL, may be necessary for binding to both the low- and high-affinity sites, because ETEp and ARMp, which both lack the positive charges, did not bind. In the case of ETEp the negative charges introduced may prevent the peptide from approaching either binding site. The six amino acid peptide, YAKARM (ARMp), which has the sequence of the carboxyl terminus of R. communis isocitrate lyase, did not compete with the binding of the SKLp to glyoxysomal membranes. The ARM sequence functions as a PTS1 in some cases and in our case this sequence was not sufficient for binding to either binding site. However, the ARMp is much shorter than the SKLp and lacks the several positively charged residues that seemed to be associated with high-affinity binding (Table I). The ARM sequence is functionally similar to SKL and, for example, confers the ability to import of cottonseed isocitrate lyase into mammalian cell peroxisomes (Trelease et al., 1994). However, the removal of the ARM sequence from R. communis isocitrate lyase does not interfere with in vitro import of the protein (Behari and Baker, 1993). Thus, R. communis isocitrate lyase may contain targeting information in addition to PTS1. We also tested the possibility that a PTSL-containing peptide, AHLHp, might interact with either the low- or high-affinity SKLp binding site in R. communis glyoxysomal membranes. We observed that 1600 nM AHLHp did not displace any 125I-SKLp, suggesting that the high-affinity site is not the PTS2 receptor. Binding sites for PTS2 peptides would be the subject of another investigation; our focus here was on PTS1 binding. Overall, the targeting of proteins to peroxisomes and glyoxysomes may involve a variety of nonspecific and specific targeting sequence domains that interact with several receptor sites.

The events that are necessary for a newly synthesized protein to be assembled into a peroxisome include being recruited from the cytosol, passing through the membrane, folding into a functional conformation, and assembling with other subunits. Some of the 12 different PEX genes (Distel et al., 1996) probably code for the various proteins that are responsible for this series of activities. However, investigations of the yeast secretory (SEC) mutants required the development of specific assays to determine the functions of the each of the sec genes. The experimental system described here allows the interaction of the targeting information with the receptor and the organelle membrane to be measured independently from the other events in peroxisomal assembly, and may be useful in the elucidation of the functions of one or more of the peroxins.

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