# 4. Discussion

Peroxisomes were first observed as an unknown organelle several decades ago; since then more and more scientists have done excellent works on this topic. The most exciting discoveries have been made in recent years, especially with yeast *S.cerevisiae* as a model organism. Taking yeast *S.c.* as the research model has at least three advantages: first its life circle is very short; secondly yeast peroxisomes with particular characters could readily be induced by growing yeast in different conditions such as in oleate or methanol medium, considering peroxisomes in yeast are inducible. This allowed to investigate conditions in which peroxisomes in yeast (mutants) are defective and nonfunctional in a short period, even to isolate the intact peroxisomes for research; finally the complete genome of *S.c.* has been sequenced, which makes it feasible to handle all known genes for peroxisomal biogenesis, including to isolate, mutate and knockout genes, and to discover new genes for peroxisomal biogenesis.

Peroxisomal matrix proteins are nuclear encoded, synthesized on free polyribosomes and are released into the cytoplasm before they are imported into peroxisomes. The newly synthesized peroxisomal proteins are directed by either of the two peroxisomal protein receptors (Pex5p for PTS1 and Pex7p for PTS2) onto the docking complex on the peroxisomal membrane, and are further translocated into peroxisomes by the transport machinery.

Pex13p is one of the pivotal components of the protein transport machinery. In fact, Pex13p and Pex14p constitute the docking machinery on the peroxisomal membrane for import of the peroxisomal matrix proteins. This work has illustrated the tertiary structure of the Pex13p SH3 domain located at the C-terminus of the Pex13p protein and manifested the interaction among Pex13p, Pex14p and the peroxisomal matrix protein receptor type I – Pex5p. These studies give an insight of the mechanistics of transporting peroxisomal matrix protein into the peroxisome.

# 4.1 Structure of Pex13p SH3 Domain

## 4.1.1 SH3 Domain in General

The SH3 (Src homology 3) domain was first described in the Src cytoplasmic tyrosine kinase (Anderson SK, 1985; Tanaka A, 1987). SH3 domains are small protein modules consisting of approximately 50 amino acids. They are found in a great variety of intracellular or membrane-associated proteins (Musacchio et al., 1992; Pawson, 1995). SH3 domain proteins comprise a variety of proteins with enzymatic activity, adaptor proteins that lack catalytic sequences and cytoskeletal proteins, such as fodrin and the yeast actin binding protein ABP-1.

The SH3 domain has a characteristic fold which consists of five or six beta-strands arranged as two tightly packed anti-parallel beta sheets. The linker regions may contain short helices. Although usually found as single copies, two to four copies of the domain can be present in a single polypeptide, particularly in the adaptor proteins (Mayer and Eck, 1995; Morton and Campbell, 1994; Musacchio et al., 1992). SH3 domains are commonly found in proteins that also contain SH2 (src Homology-2) domains, suggesting that their functions are inter-related. The function of the SH3 domain is not well understood but they may mediate assembly of specific protein complexes via binding to proline-rich peptides (Morton and Campbell, 1994) and some may be involved in linking signals transmitted from the cell surface by protein tyrosine kinases to "downstream effector proteins".

## 4.1.2 ScPex13p SH3 Domain Structure

The ScPex13p SH3 domain is not involved in the signal transmission but in protein-protein interaction. In fact ScPex13p binds to ScPex14p through its C-terminal SH3 domain, these two proteins seems to build the key skeleton of the peroxisomal protein import machinery (Holroyd and Erdmann, 2001). In contrast to other SH3 domains the Pex13p SH3 domains of different species have a unique long n-Src loop, which is in proximity of the PxxP binding pocket (W349) providing an extra binding area for the PxxP ligand of Pex14p – at least involved in the binding to the Pex14p ligand (see Fig. 50). The long n-Src loop appears in the all know Pex13p homologous proteins (see Fig. 7).

Since the long n-Src loops appear only in Pex13p proteins whose structures only studied recently, the computer modeling of such particular SH3 domain proteins was not satisfying (see Fig. 25, 31), since it could not explain the correction conformation of the long n-Src loops.

It is the first time that the structure of a group of SH3 domains having a long n-Src loop such as that of ScPex13p has been illustrated by X-ray crystallography (Douangamath et al., 2002) or NMR spectroscopy (this work, see Fig. 30). Such work gives us a deep insight into the binding mechanism of the PxxP motif of the Pex14p proteins on peroxisome membrane and that of the non PxxP motif of the Pex5p. The SH3 domain of Pex13p proteins comprises a new group which has two distinguishable binding sites for two different ligands.

The structure data provided by NMR spectroscopy in this work show that these two different ligands could bind spatially different und independently on the ScPex13p SH3, and might. Furthermore, structure might reveal insight to the function of this protein in peroxisomal protein import, especially as the SH3 domain can bind two of the component of the peroxisomal protein import machinery.

## *4.2 Protein-Protein Interactions Related to Protein Import into Peroxisomes*

Peroxisomal matrix proteins harboring PTS1 or PTS2 targeting signals are captured by the peroxisomal protein receptor Pex5p (for PTS1 proteins) or Pex7p (for PTS2 proteins), these cargo-receptor complexes land then on the docking complex on the peroxisomal membrane. The docking complex consists at least of Pex14p and Pex13p (Erdmann and Blobel, 1996; Erdmann et al., 1997; Holroyd and Erdmann, 2001). As elucidated by the structure data of the ScPex13p SH3 domain these two proteins ScPex14p and ScPex13p in yeast binds to each other on the peroxisomal membrane through the interaction of the PxxP motif of ScPex14p and the SH3 domain at the C-terminus of ScPex13p extending to cytosol (also see Fig. 32, 33, 34, 50). Docking of the cargo-receptor complex proceeds by steps of ScPex5p or ScPex7p interacting with ScPex14p (Otera et al., 2002; Urquhart et al., 2000; Will et al., 1999), ScPex7p

interacting with the N-terminus of ScPex13p (Girzalsky et al., 1999; Stein et al., 2002) or ScPex5p interacting with the SH3 domain of ScPex13p (Bottger et al., 2000; Otera et al., 2002; Urquhart et al., 2000). In this scenario the ScPex13p SH3 can bind to two different ligands, ScPex14p and ScPex5p, where the interaction between ScPex14p and the ScPex13p SH3 domain is a classic one between proline rich motifs (PxxP) and SH3 domains (Erdmann and Blobel, 1996), while that between ScPex5p and the ScPex13p SH3 represents a novel non canonical binding of SH3 domains (Bottger et al., 2000; Douangamath et al., 2002). Many studies on the interaction of PxxP motifs and SH3 domains have been done in recent years (Tong et al., 2002), however the particular interaction of ScPex13p and ScPex14p was first studied on the molecular structure level in this work (the similar work was done at same time by Ben Distel's research group in the Netherlands (Douangamath et al., 2002)). The novel binding capability of ScPex13p SH3 to ScPex5p was also illustrated by determination of the stereo structure of the ScPex13p SH3 domain.

### 4.2.1 Binding Sites of ScPex5p / ScPex14p for ScPex13p SH3 Domain

The spot blot technology, which immobilize the peptide library on the cellulose membrane (Reineke et al., 2001), developed in the research group of J. Schneider-Mergener, provides a very helpful method besides yeast two-hybrid to locate a binding site in a protein. Scanning the 12 residues peptide library covering the complete ScPex14p protein sequence in this way, the binding motif of ScPex14p for ScPex13p SH3 domain has been mapped to the peptide (MPPTLPHRDW) representing a classic type II ligand for SH3 domains (Fig. 32). Further in vitro binding experiments by NMR spectrum confirmed this result. This is consistent with the earlier studies(Albertini et al., 1997; Girzalsky et al., 1999), the ScPex14p containing the double point mutants P87A and P90A (MPPTL<u>P</u>HRDW), which appear in the binding motif for the ScPex13p SH3 domain, failed to binding the ScPex13p SH3 domain in the yeast two-hybrid assay.

The binding motif of ScPex5p for ScPex13p SH3 domain was also located to the novel peptide from 202 to 215 amino acids by combination of yeast two-hybrid and scanning peptide library (Fig. 35, 36, 37, 38). Further analyses including substitution

and NMR titration experiments are consistent with this peptide scanning results, suggesting that the flank of several amino acids around this peptide are necessary for its formation of an α-helix conformation while bounded to the ScPex13p SH3 domain. Substitution and truncation experiments carried out with the peptide libraries based on the sequence VNEQEQQPWTDQFEKLEKEVSENLDI (26 aa), which represents amino acids 195 to 221 of ScPex5p, revealed that the first eight amino acids of the peptide can be replaced by any other amino acid without significant loss of binding capacity while Trp204 and Phe208 are indispensable for binding (WxxxF). Furthermore, no amino acid of the central region of 13 amino acids (WTDQFEKLEKEVS) could be functionally replaced by proline, which would interfere with the proposed alpha-helical conformation of this region. Also glycine cannot substitute for any of the amino acids of the central region probably for the same reason. Shorter peptides truncated from right or left were still functional as long as the central region was not affected.

The shortest peptide which still did interact with the ScPex13p SH3 domain consists of 16 amino acids covering the central region (QQPWTDQFEKLEKEVS; 16aa). Binding to the SH3 domain of the short (QQPWTDQFEKLEKEVS; 16aa) and a long peptide (QQPWTDQFEKLEKEVSENLDI; 21aa) were further investigated by NMR studies, and the results confirmed the results gained by scanning the ScPex5p peptide libraries.

## 4.2.2 Binding Sites of the ScPex13p SH3 Domain for Pex5p and Pex14p

After the stereo structure of ScPex13p SH3 domain was calculated based on the NMR spectrum data, the binding sites on the SH3 domain were mapped by observation of the chemical shift ($^{15}$N HSQC signal) of every residue of the SH3 domain upon addition of its binding ligand peptide of ScPex14p or ScPex5p (Fig. 50).

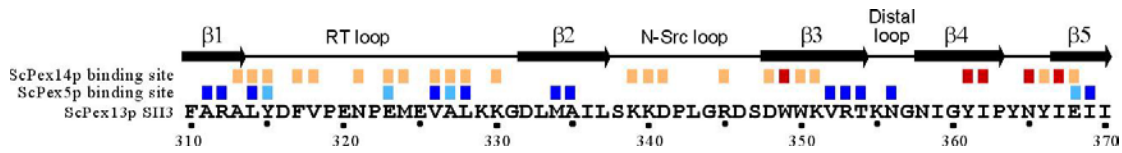


**Fig. 50 Diagram of the ScPex13p SH3 domain structure and the binding sites for ScPex5p and ScPex14p**. The binding sites of the SH3 domain for ScPex5p and

ScPex14p were mapped according to the chemical shift changes acquired in the NMR studies. The residues showing a strong binding to Pex14p are indicated with a red rectangle, the moderate ones are shown in yellow; the residues showing strong binding to Pex5p are indicated by the dark-blue rectangles and moderate ones by the light-blue rectangles. The structure of the ScPex13p SH3 domain is also illustrated by indicating its structural segments.

The ScPex14p binding site lies on the β3 and β4 sheet, confirming the pivotal role of W349 for the binding of Pex14p on the SH3 domain, while the amino acids nearby of RT loop and n-Src loop may influence the binding (Fig. 33, 34, 50). We showed for the first time that the unique long n-Src loop in ScPex13p SH3 domains eventually also contributes to the interaction with the type II PxxP proline rich motif of ScPex14p. The earlier studies showed a point mutation of E320K (Girzalsky et al., 1999) could destroy the interaction between Pex14p and the SH3 domain in the yeast two-hybrid system. However, our NMR data show that the residue E320 on the SH3 domain is not part of the direct binding sites for Pex14p. Thus the point mutation of the glutamic acid (320) may affect the space conformation of the nearby amino acids including N321 and V318 (possibly), which are two binding residues for Pex14p (see Fig. 50).

Exclusively shifted by the ScPex5p peptide are: A311, R312, M334, A335, V352, R353, T354, N356 and I369. These residues are located at the N terminus on the opposite side of the SH3 domain, forming an exposed hydrophobic region together with F310 and L333, which include strands β1 and β2, the distal loop, and the RT loop. In agreement with their functional role, these amino acids are partially conserved in SH3 domains of Pex13p (Fig. 7).

Our data indicate the existence of two distinct Pex13p binding sites for the Pex5p and Pex14p peptides, which is also supported by a competition assay, demonstrating that both peptides can bind simultaneously to the SH3-domain. These results are in agreement with a previous study (Barnett et al., 2000), in which the amino acids R353 and K355 from the distal loop, and N321 and E323 from the RT loop were identified by analysis of SH3 suppressor mutants as being important for the binding of Pex5p.

## 4.3 Binding Constants of the Bindings among ScPex5p, ScPex13p and ScPex14p

All the bindings among ScPex5p, ScPex13p and ScPex14, including the binding pairs: ScPex5p and ScPex13p (SH3), ScPex5p and ScPex14p, ScPex13p (SH3) and ScPex14p, were quantitatively determined by one or other method. The strongest binding appears between ScPex5p and ScPex14p, the dissociation constant of which is 33.5 pM (BIAcore, see 3.12.1.2; Fig. 47); the weakest binding is between ScPex5p and ScPex13p with a dissociation constant of 55.4 μM (BIAcore, see 3.12.2; Fig. 48) or 640 μM (NMR, see 3.12.2); while the dissociation constant of the binding between ScPex14 (peptide) and ScPex13p (SH3) was determined of 50 μM (NMR see 3.12.3) in this work, and similar result 44 μM was reached by the research group of Ben Distel (Douangamath et al., 2002).

It should be pointed out that in all these experiments the SH3 domain with flank at both sides, which might contains the second binding site for ScPex5p, in stead of the full length ScPex13p was used. The difference between the dissociation constants obtained by BIAcore studies and by NMR studies is due to that the full length of ScPex5p was used in BIAcore studies while only the binding peptides of ScPex5p were used in NMR studies. In the case of NMR studies of ScPex5p and ScPex13p (SH3), which would give different dissociation constants: 640 μM with the long ScPex5p peptide, 2 mM with the short peptide, and 36 μM determined by the research group of Ben Distel (Douangamath et al., 2002); this is due to other amino acids besides the key amino acid (WxxxF) of ScPex5 peptide are needed to fold properly an a-helix upon binding on the ScPex13p SH3 domain.

These data about the bindings among peroxins ScPex5p, ScPex13p and ScPex14p provide a clue to understand the competition between ScPex5p and ScPex14p for the binding on the ScPex13p SH3 domain, also to understand transient binding processes during import of the PTS1matrix proteins, which are imported into peroxisomes via Pex5p.

## 4.3 Competition between Pex5p and Pex14p for Binding on the SH3 Domain

Both binding sites on the ScPex13p SH3 domain for ScPex5p and ScPex14p are formed by similar sets of building blocks, a small antiparallel β sheet segment (Pex5p, β1-β2; Pex14p, β3-β4) flanked by two or three loops. Because the Pex14p PXXP binding site is flanked by two large loops (RT, N-src) oriented toward this site, it appears as a deeper binding site cleft compared to the Pex5p binding site, which is flanked by the short distal loop and the C terminus of the RT loop (Fig. 30, 50).

These structural conformations of the two binding pockets for ScPex5p and ScPex14p suggest that the binding of ScPex13p to ScP14p is relative stronger than to ScPex5p, which was consistent with the binding constants determined by NMR. The NMR experiments yielded 640μM of the dissociation constants for the binding of the ScPex13p SH3 domain with the ScPex5p peptide (2mM if with the short ScPex5p peptide), while the dissociation constants for the binding of the ScPex13p SH3 domain with a typical PxxP ligand ScPex14p peptide was 50 ± 10 μM.

These results of the dissociation constants of the binding of the ScPex13p SH3 with ScPex5p or ScPex14p are in agreement with the fact that ScPex14p and ScPex13p form a stable docking base for the peroxisomal protein import, while the interaction between ScPex13p and ScPex5p should be unstable and transitory.

However, very interestingly, our NMR competition experiments showed that the ScPex14p peptide could be dissociated from the binding complex, if an excessive mount of ScPex5p peptide was introduced (Fig. 44); in contrast, the ScPex5p peptide could not be dissociated from the binding complex, if an excessive mount of ScPex14p peptide was introduced (Fig. 45). Similar results were observed also by in vitro binding experiments carried out on the affinity chromatography, in which the full length protein ScPex5p, ScPex14p – in stead of the peptides – and the ScPex13p SH3 domain with a longer flank on both sides were used (Fig. 43).

The concentrations used in these studies were far higher than the average concentration of ScPex5p in yeast cell (see Fig. 49). However, this can not exclude

that a high local concentration of ScPex5p is maintained on the peroxisomal membrane. Any way, these experimental data should be interpreted carefully because in both cases the excessive amount of ScPex5p (peptide or the purified protein) was used, which could not represent the physiological condition in vivo in certain cases. Nevertheless these results are partially in agreement with some of the earlier studies (Otera et al., 2002), which showed that the PTS1 receptor Pex5p loaded with its cargo protein could dissociate the Pex13p/Pex14p complex. This was supposed to be step of the peroxisomal protein import.

## *4.4 Speculation on the Biological Function of the ScPex13p SH3 Domain*

The model of the peroxisomal protein import proposed and modified in earlier studies (Erdmann and Blobel, 1996; Otera et al., 2002; Urquhart et al., 2000) suppose that the peroxisomal matrix proteins are synthesized on free ribosomes in cytosol and post translationally imported into peroxisomes. After the matrix proteins were captured by the receptors in cytosol, docking of the cargo-receptor complex onto the peroxisomal membrane is one of the key steps involved in the peroxisomal matrix protein import.

The initialization of the docking of the import receptor Pex5p loaded with its cargo protein is thought to take place at Pex14p, subsequently Pex13p might bind Pex5p after the cargo-receptor complex has released its cargo to the peroxisomal lumen or to another component of the protein import machinery (Otera et al., 2002; Urquhart et al., 2000). BIAcore studies showed that the dissociation constant of the complex ScPex5-ScPex14 (33.5 pM) is far smaller than that (55 μM) of the complex ScPex5p-ScPex13p (SH3 domains), support these hypotheses in the earlier studies.

Recent evidences including our data suggest that Pex13p and Pex14p do not form a permanent complex but that the proteins associate or dissociate during the protein import process (Otera et al., 2002; Urquhart et al., 2000). In this scenario, binding of the full length ligands may modulate the interaction of Pex13p with Pex14p, which might trigger the dissociation of the docking complex Pex13p-Pex14p.

Our data are consistent with the idea that Pex13p does not simply reflect another docking site for Pex5p but is also directly involved in the protein import process.