Identification of peroxisomal proteins by using M13 phage protein VI phage display: molecular evidence that mammalian peroxisomes contain a 2,4-dienoyl-CoA reductase

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To elucidate unknown mammalian peroxisomal enzymes and functions, we subjected M13 phage expressing fusions between the gene encoding protein VI and a rat liver cDNA library to an immunoaffinity selection process *in vitro* (biopanning) with the use of antibodies raised against peroxisomal subfractions. In an initial series of biopanning experiments, four different cDNA clones were obtained. These cDNA species encoded two previously identified peroxisomal enzymes, catalase and urate oxidase, and two novel proteins that contained a C-terminal peroxisomal targeting signal (PTS1). A primary structure analysis of these novel proteins revealed that one, ending in the tripeptide AKL, is homologous to the yeast peroxisomal 2,4-dienoyl-CoA reductase (EC 1.3.1.34; DCR), an enzyme required for the degradation of unsaturated fatty acids, and that the other, ending in the tripeptide SRL, is a putative member of the short-chain dehydrogenase/reductase (SDR) family, with three isoforms. Green fluorescent protein (GFP) fusions encoding GFP–DCR-AKL, GFP–DCR, GFP–SDR-SRL and GFP–SDR were expressed in mammalian cells. The analysis of the subcellular location of the recombinant fusion proteins confirmed the peroxisomal localization of GFP–DCR-AKL and GFP–SDR-SRL, as well as the functionality of the PTS1. That the AKL protein is indeed an NADPH-dependent DCR was demonstrated by showing DCR activity of the bacterially expressed protein. These results demonstrate at the molecular level that mammalian peroxisomes do indeed contain a DCR. In addition, the results presented here indicate that the protein VI display system is suitable for the isolation of rare cDNA clones from cDNA libraries and that this technology facilitates the identification of novel peroxisomal proteins.

Key words: fatty acid oxidation, M13 phage, peroxisomal metabolism.

INTRODUCTION

Peroxisomes are found in virtually all eukaryotic cells ranging from eukaryotic micro-organisms to plants and animals. The identification of a group of inherited diseases associated with peroxisomal dysfunction indicates that peroxisomes have an essential part in cellular metabolism [1]. Some of the most important metabolic functions of peroxisomes include the synthesis of plasmalogens, bile acids, dolichols and cholesterol, and the oxidation of fatty acids [2,3]. At present, more than 50 metabolic enzymes have been identified in mammalian peroxisomes. In addition, 13 of the 21 peroxins are also conserved in mammals [4–9]. The characterization of genes encoding peroxisomal proteins at the molecular level has frequently guided the way towards identification of the defects in patients suffering from peroxisomal disorders [10,11].

To reveal as yet unknown mammalian peroxisomal enzymes and functions, we used phage display, a technique in which a foreign protein or peptide is displayed on the surface of a (filamentous) bacteriophage [12]. Although the phage display technology is already being used successfully in protein engineering, in the high-level expression of recombinant proteins such as phage bodies and in studies of protein–ligand interactions, the screening of actual cDNA libraries on the surface of filamentous phage has faced the problem that peptides and proteins had to be displayed on a filamentous phage by fusion to the N-terminus of protein III (pIII) or protein VIII. Unfortunately, these 5′-end fusions are unsuitable for the surface expression of full-length cDNA clones bearing stop codons. These problems were overcome by the development of other phagemid vectors that allow the functional display of cDNA clones on the surface of the M13 phage coat either (1) as a direct C-terminal fusion of the cDNA library to the minor coat protein VI (pVI) [13] or (2) through an indirect means involving anchoring cDNA-encoded proteins to filamentous phage protein pIII by co-expressing Fos–cDNA and Jun–pIII gene fusions [14,15]. Here we report experiments in which we subjected a phage library of pVI–rat-liver cDNA fusions to an immunoaffinity selection process *in vitro*, called biopanning, with antibodies that specifically recognize peroxisomal protein subfractions. We describe the cloning of genes encoding novel peroxisomal enzymic activities with the use of this approach.

EXPERIMENTAL

Animals, strains and vectors

Male Wistar rats (150–200 g) and White New Zealand rabbits were maintained on a diet of standard laboratory chow. Animal care approval was granted by the local institutional ethics committees. The phagemid vectors pG6A, pG6B and pG6C were kindly provided by Dr L. Jespers. These vectors differ from the

Abbreviations used: CHO, Chinese hamster ovary; DCR, 2,4-dienoyl-CoA reductase; DTT, dithiothreitol; EST, expressed sequence tag; GFP, green fluorescent protein; PTS1, C-terminal peroxisomal targeting signal; pVI, M13 phage protein VI; SDR, short-chain dehydrogenase/reductase; sorboyl-CoA, 2-*trans*,4-*trans*-hexadienoyl-CoA.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers AF044127 (human SDR-SRL) and AF044574 (rat DCR-AKL).

Table 1 List of oligonucleotides (restriction sites are underlined)

Name	Sequence
DCRf	5′-ATGACCCAGCAGCCGCCTG-3′
DCRrdel	5′-CGGCACAGCGGCCGCTAAGAGGAGGATTCAAACTCTAG-3′
GFPfw1	5′-ACGCGGATCCAAGATGGCGATGAGTAAAGGAGAAGAACTTTTC-3′
GFPrDCR	5′-AGGCGGCTGCTGGGTCATTTTGTATAGTTCATCCATGCC-3′
GFPrSDR	5′-GGGTCATCCCGGAGCTGGCCATTTTGTATAGTTCATCCATGCC-3′
M13f	5′-CGCCAGGGTTTTCCCAGTCACGA-3′
M13r	5′-AGCGGATAACAATTTCACACAGGA-3′
NotGT11r	5′-TTGACACGCGGCCGCCTGGTAATGGTAGCGACCG-3′
pAKL1	5′-GGGTAGAGGACGTAGTTCAGTTG-3′
pAKL2	5′-AGCCTTGGGCGGCCGCTTCAAACACTACAGCTTAGCAG-3′
pAKL3	5′-CGATCACTCCTCCATGGTCC-3′
pAKL4	5′-GTGGCAGCCATGCCTCATG-3′
pAKLf	5′-CACAGGATCCATGACCCAGCAGCCGCC-3′
pG6f	5′-CTCCCGTCTAATGCGCTTCCCTG-3′
pG6r	5′-GCTGCAAGGCGATTAAGTTGGGT-3′
SfiGT11f	5′-CCATATGGGCCCTGGTGGCCACGACTCCTGGAGC-3′
SDRf	5′-ATGGCCAGCTCCGGGATGACC-3′
SDRr	5′-CGGCACAGCGGCCGCTCAGAGGCGGGACGGGGTTCC-3′
SDRrdel	5′-CGGCACAGCGGCCGCTCACGGGGTTCCTCCACCCACCAC-3′
SRL1	5′-GGCCACCAGCCGCTCCCGG-3′
SRL2	5′-CCGGGAGCGGCTGGTGGCC-3′
SRL3	5′-GTCCCACACCTCCTCAGTGAC-3′
SRL4	5′-GTCACTGAGGAGGTGTGGGAC-3′

pDONG6 series of vectors [13] by the introduction of a unique *Sal*I site between the unique *Sfi*I and *Not*I sites.

Generation of the antisera ab-MF8 and ab-MF16

Highly purified rat liver peroxisomes [16] were resuspended in (hypotonic) lysis buffer A [10 mM Mops/1 mM EDTA/1 mM dithiothreitol (DTT)/0.1 % (v/v) ethanol (pH 8.0)] and sonicated in ice with a Branson Sonifier B15 P Cell Disrupter, equipped with a microtip (output 5, duty 50 %, six times 15 s). After centrifugation for 1 h at 100000 ***g***, the pellet was resuspended in buffer A and the entire procedure was repeated. The combined supernatant (S1) contained the soluble matrix proteins (71 % of total peroxisomal protein) and was used to raise the polyspecific, polyclonal antiserum ab-MF16 (see also [6]). The pellet (P1), containing the cores and membranes, was further subjected to extraction with carbonate [17] and the corresponding supernatant [containing peripheral membrane proteins and urate oxidase, an enzyme associated with the cores (18.6 % of total peroxisomal protein)] was used to generate the polyspecific, polyclonal antiserum ab-MF8. Rabbits were immunized with antigen as described previously [6].

Cloning procedures

Standard procedures were used for cloning [18]. PCR amplification was always started with 2 min of incubation at 94 °C, followed by 30 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 55 °C and 2 min of extension at 72 °C. The reaction was completed by 15 min of incubation at 72 °C. All oligonucleotide primers (Table 1) were purchased from Genset. PCR products were subcloned with the Topo TA cloning kit (InVitrogen). Direct cloning of DNA amplified by *Pfu* DNA polymerase (Stratagene) into the Topo TA cloning vector was performed by the addition of a 3′ A-overhang to the purified DNA fragments as described by the manufacturer. Plasmids encoding expressed sequence tags (ESTs) were obtained from the American Type Culture Collection and the corresponding cDNA sequences were confirmed by sequencing.

Construction of pVI–cDNA libraries and phage production

Proteins were displayed on the surface of filamentous phage M13 as fusions to the C-terminus of the minor coat protein pVI. For this, the cDNA inserts of a custom λGT11 cDNA library (rat-liver cDNA, 2.5×10^6 primary recombinants, bidirectionally cloned with an *Eco*RI/*Not*I adaptor) (InVitrogen) were transferred into the pG6A, pG6B and pG6C phagemid expression vectors, each encoding one of the three reading frames. The cDNA inserts were amplified by PCR with the oligonucleotide primers SfiGT11f and NotGT11r (Table 1). The amplified material was purified with the QIAquick PCR purification kit (Qiagen), digested with either *Eco*RI or *Not*I, gel-purified and ligated into a pool of the different pG6 vectors, digested either with *Eco*RI or *Not*I and dephosphorylated. The ligation mixture was used to transform *Escherichia coli* Top10F′ (InVitrogen) cells by electroporation yielding 1.5×10^6 and 2.0×10^6 independent clones for the '*Eco*RI' and '*Not*I' library respectively. Colonies were scraped off in Luria–Bertani medium supplemented with 50 μg/ml ampicillin and 20 μg/ml tetracycline, glycerol was added to a final concentration of 50 % (v/v) and 1 ml aliquots were stored at −80 °C. Inserts ranging from 500 to 1700 bp were found in 66 % of the clones by PCR screening with the primers pG6f and pG6r. Rat liver '*Eco*RI' and '*Not*I' phagemid particles displaying the cDNA-encoded proteins were generated by helper phage infection. For this, 20 μl of the library stock (approx. 5×10^9 colony-forming units) was grown for 3 h at 37 °C in Luria–Bertani medium supplemented with ampicillin and tetracycline, superinfected with the helper phage R408 (Stratagene) and grown further for 18–20 h. After centrifugation (5000 ***g***, 20 min), the supernatant was 'filter-sterilized' (0.45 μm filter) and the filtrate was used for biopanning. Before biopanning, the '*Eco*RI' and '*Not*I' fusion phage libraries were pooled.

Affinity selection of fusion phages

To screen phage-display libraries, we used a similar protocol to that described by Parmley and Smith [19], but to capture bound phages we used Protein A–Sepharose beads (Pharmacia) instead of polystyrene plates coated with streptavidin. In brief, 1 ml (10^{10}–10^{12} plaque-forming units) of phages expressing the rat-liver pVI–cDNA library was added to 2.5 μl of antiserum diluted in 10 ml of Tris-buffered saline/Tween 20 (TBST) supplemented with 1 % (w/v) BSA. This mixture was incubated with gentle rotation for 1 h at room temperature. Phages bound to the antibody were then captured by adding 50 μl of a 1:1 (v/v) slurry of Protein A–Sepharose beads [preblocked with 5 % BSA (w/v) in TBST] and rotating this mixture for an additional 1 h at room temperature. Non-bound phages were removed by collecting the Protein A–Sepharose beads by centrifugation and washing the particles ten times with TBST. After the last wash, the beads were transferred to a microcentrifuge tube and the bound phages were released under acidic conditions by the addition of 100 μl of elution buffer (1 mg/ml BSA/0.1 M HCl, adjusted to pH 2.2 with glycine) for 15 min. After a brief centrifugation, the eluate was immediately neutralized by pipetting 80 μl of the supernatant to another microcentrifuge tube containing 4.8 μl of 2 M Tris (pH unadjusted). The phage titres were determined by infecting 0.2 ml of mid-exponential-phase *E. coli* Top10F′ cells (plating bacteria) with 10 μl of the eluate; the bacteria were then left to stand for 30 min at 37 °C before being plated on Luria–Bertani agar plates containing ampicillin and tetracycline. Colonies were

Table 2 PCR fragments generated to create the SDR-SRL expression clones

Fragment	Template (EST)	Primers
1	R25850	M13r, SRL1
2	AA293652	M13f, SRL2
3	H63672	M13f, SRL4
4	AA293652	SRL2, SRL3

Table 3 PCR fragments generated to create the GFP–DCR-AKL, GFP–DCR, GFP–SDR-SRL and GFP–SDR clones

Fragment	Template (EST)	Primers
1	GFP(S65T)	GFPfw1, GFPrDCR
2	GFP(S65T)	GFPfw1, GFPrSDR
3	SDR-SRL2	SDRf, SDRr
4	SDR-SRL2	SDRf, SDRrdel
5	DCR-AKL	DCRf, pAKL2
6	DCR-AKL	DCRf, DCRrdel

left to grow overnight at 37 °C and counted. The rest of the eluted phages were amplified in a similar way by the addition of 0.3 ml of plating bacteria. After incubation overnight at 37 °C, the cells obtained were scraped into liquid Luria–Bertani medium supplemented with ampicillin and tetracycline, and the culture was shaken at 37 °C for 2 h. New 'sublibraries' were generated by helper phage superinfection as described above. The 0.45 μm filtrate was used for the next round of biopanning; after three to six rounds of selection, individual clones were selected and the insert size was determined by PCR-colony screening with the oligonucleotide primers pG6f and pG6r. Different cDNA inserts were sequenced.

Sequencing, computer analysis and alignments

DNA sequencing on both strands was performed by the method of Sanger [20], with vector- and gene-specific primers. Nucleic acid and protein database searches were performed at the National Center for Biotechnology Information server with the use of the BLAST algorithm [21]. Protein alignments and computing of similarity scores were performed with the ALIGN program at the GENESTREAM SSEARCH network server CRBM. Searches for protein motifs were done with the Expasy ScanProsit tool (Swiss Institute of Bioinformatics).

Cloning of full-length short-chain dehydrogenase/reductase (SDR)-SRL cDNA species and their expression in bacteria

To express the SDR-SRL1, SDR-SRL2 and SDR-SRL3 proteins (which end in the tripeptide SRL), the cDNA species containing the complete open reading frame of those three forms were subcloned into the Pinpoint expression system (Promega). Therefore the *Sma*I–*Not*I fragment of EST R25850, which encodes the full-length SDR-SRL2 clone, was transferred into the *Pvu*II–*Not*I-digested Xa1 vector. The full-length SDR-SRL1 and SDR-SRL3 clones were generated by fusion PCR. In a first PCR reaction, four PCR fragments (Table 2) were generated that were used as templates in a second PCR reaction to generate full-length SDR-SRL1 (fragments 1 and 2) and SDR-SRL3 (fragments 1, 3 and 4) clones respectively. After digestion with *Sma*I and *Not*I, these 'full-length' fragments were subcloned into the *Pvu*II–*Not*I-digested Xa1 vector. To check expression, bacterial lysates were fractionated by SDS/PAGE and transferred to nitrocellulose by semi-dry blotting; the biotinylated fusion proteins were detected with streptavidin–alkaline phosphatase or with the rabbit polyclonal antibody that was used for biopanning.

Cloning of full-length DCR-AKL cDNA and its expression in bacteria

The putative open reading frame of the cDNA-AKL clone (which ends in the tripeptide AKL) could not be composed by EST database probing. To amplify additional cDNA segments containing extensions of the 5′ end of the cDNA, rapid amplification of 5′ cDNA ends was performed with 1 μl of the custom λGT11 rat-liver cDNA library (3.8×10^7 phage-forming units) (InVitrogen) together with λGT11-specific and 5′ reverse gene-specific primers. The expression clone was generated by PCR with two sequential PCR reactions [first reaction, 1 μl of the rat liver cDNA-library as template, gene-specific primers pAKLf and pAKL1; second reaction, 1 μl of the first PCR reaction (diluted 1:1000) as template, gene-specific primers pAKLf and pAKL2]. The 913 bp PCR fragment obtained was gel-purified, digested with *Bam*HI and *Not*I and subcloned into the *Bam*HI/*Not*I-digested PinPoint Xa2 expression vector. Protein expression was performed as described for the SDR-SRL clones.

Cloning and expression of the green fluorescent protein (GFP) fusions encoding GFP–DCR-AKL, GFP–DCR, GFP–SDR-SRL and GFP–SDR

All the GFP fusions were generated by fusion PCR. In a first PCR reaction, six PCR fragments (Table 3) were generated that were used as template in a second PCR-reaction to generate the GFP–DCR-AKL (fragments 1 and 5), GFP–DCR (fragments 1 and 6), GFP–SDR-SRL (fragments 2 and 3) and GFP–SDR (fragments 2 and 4) cDNA fusions respectively (DCR stands for 2,4-dienoyl-CoA reductase). After digestion with *Bam*HI and *Not*I, these PCR fragments were subcloned into the *Bam*HI–*Not*I-digested pcDNA3 vector (InVitrogen). Chinese hamster ovary (CHO) cells were transiently transfected with the plasmids encoding the GFP fusions by using the Effectene Transfection Reagent (Qiagen). Immunofluorescence studies were done essentially as described previously [22]. Double immunofluorescence studies were performed with the rabbit anti-PMP70 antiserum [23] in conjunction with CyTM3-conjugated AffiniPure goat anti-rabbit IgGs (Jackson ImmunoResearch Laboratories). To avoid GFP fluorescence through the rhodamine channel after excitation with the FITC filter set, we detected the Cy™3 marker first [24,24a].

Synthesis of substrates

2-*trans*,4-*trans*-Hexadienoyl-CoA (sorboyl-CoA) was prepared enzymically as follows. Sorbic acid (50 μmol; Aldrich) was dissolved in 5 ml of 10 mM NaOH containing 1 % (v/v) Triton X-100, followed by the addition of 45 ml of reaction mixture whose final concentrations were 50 mM Tris/HCl buffer, pH 8.0, 1 mM EDTA, 6 mM $MgCl_2$, 3 mM ATP, 0.3 mM CoA and 0.3 mM DTT. The reaction was started by adding 2.5 units of acyl-CoA synthetase (yeast; Boehringer) and incubated at 30 °C. To monitor the reaction, the reaction medium was fortified with 0.8 mM NADH, 4 mM phosphoenolpyruvate, 30 units/ml myokinase (rabbit muscle; Boehringer), 15 units/ml pyruvate kinase (rabbit muscle; Boehringer) and 15 units/ml lactate dehydrogenase (rabbit muscle; Boehringer). After completion [after

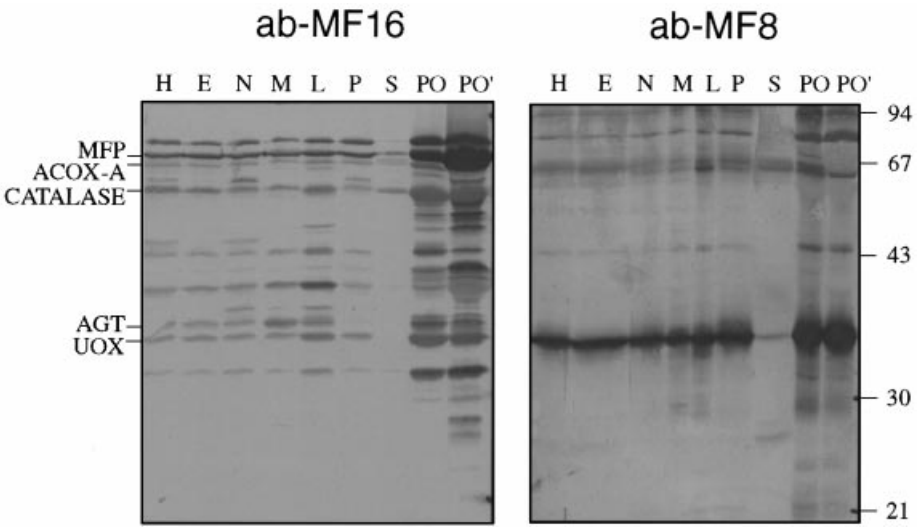

Figure 1 Ab-MF8 and ab-MF16 recognize many peroxisomal proteins

Protein (100 μg), present in rat liver homogenate (H), a post-nuclear fraction (E), a nuclear fraction (N), a heavy mitochondrial fraction (M), a light mitochondrial fraction (L), a microsomal fraction (P), cytosol (S) or purified peroxisomes of a control (PO) or a clofibrate-treated (PO′) rat, were subjected to SDS/PAGE, transferred to nitrocellulose and immunoblotted with ab-MF8 or ab-MF16. Reference proteins, identified by using antisera raised against individual peroxisomal proteins [29], are indicated at the left (ACOX-A, the 70 kDa subunit of palmitoyl-CoA oxidase; AGT, L-alanine:glyoxylate aminotransferase 1; MFP, the inducible multifunctional protein; UOX, urate oxidase). The migrations of molecular mass markers (their masses expressed in kDa) are indicated at the right.

approx. 2 h as followed at 365 nm (ϵ 3400 $M^{-1} \cdot cm^{-1}$)], the mixture was acidified with $HClO_4$ [2% (w/v) final] and cleared by centrifugation. The pellet was washed once with 5 ml of 0.5% $HClO_4$ and the combined supernatants were extracted twice with 25 ml of diethyl ether. The aqueous phase was applied to a reverse-phase solid-phase-extraction (SPE) column (C_{18} Bond Elut; 500 mg; Varian). The column was washed with 5 ml of water and the bound CoA ester was eluted with a step gradient of methanol in water. The eluates were monitored by TLC [Alugram; solvent butan-1-ol/acetic acid/water (5:3:2, by vol.)] with iodine vapour and phosphate spray for detection. Fractions containing pure CoA ester (R_F 0.5) were combined. Spectral analysis of these fractions, after removal of the methanol with a rotary evaporator, in 10 mM potassium phosphate buffer, pH 7.0, showed in addition to the 260 nm absorbance a second peak at 300 nm typical of 2,4-dienoyl-CoA species [25]. The ratio of A_{300} to A_{260} was close to 0.8, indicating a high degree of purity [26,27].

Measurement of DCR activity

Bacterial cultures, grown overnight, were centrifuged at 8000 ***g*** for 10 min. Pelleted cells were dissolved in 25 ml of Tris-buffered saline containing 1 mM DTT, 1 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml α-macroglobulin, 0.5 μg/ml leupeptin, and 10 μg/ml chymostatin, then sonicated. After sonication, Tween 20 was added [final concentration 0.005% (w/v)] and the lysate was centrifuged at 12500 ***g*** for 15 min. The pellet was dissolved in 3 ml of 50 mM potassium phosphate buffer, pH 7.4, containing 1 mM DTT, 1 mM EDTA and 1% (w/v) Thesit. The supernatant and pellet were analysed for protein levels and reductase activities as well as being subjected to SDS/PAGE and blotting. Reductase activity was determined kinetically by following the sorboyl-CoA-dependent NADPH decrease at 340 nm [28], in a final volume of 1 ml at 30 °C. Final concentrations were 50 mM potassium phosphate buffer, pH 7.4, 125 μM NADPH, 1 mg/ml BSA and 30 μM sorboyl-CoA.

RESULTS

ab-MF8 and ab-MF16 recognize peroxisomal proteins

We generated polyspecific polyclonal antisera against rat liver peroxisomal subfractions. Immunoblot analysis of rat liver fractions (Figure 1) confirmed that two of those antisera, ab-MF8 and ab-MF16, each recognized a number of proteins, most of which were highly enriched in peroxisomal fractions. In addition, by using antibodies raised against specific peroxisomal proteins [29], several of the cross-reactive proteins were indeed identified as genuine peroxisomal proteins (Figure 1). On the basis of these patterns of recognition, both ab-MF8 and ab-MF16 were used as bait in our biopanning experiments.

Biopanning of the pVI–cDNA library with ab-MF8 and ab-MF16

In a first experiment we performed six rounds of biopanning with ab-MF16 (Figure 2, conditions 1 and 2) as bait. Surprisingly, PCR analysis of ten independent phages showed that we had isolated only one single cDNA, which after sequencing was identified as a clone encoding the 124 C-terminal residues of rat liver catalase, a peroxisomal enzyme. Performing a similar experiment (only five rounds of biopanning) resulted again in the isolation of a single cDNA. Sequencing this partial DNA yielded a novel sequence with no significant homologies in the database, but judged by the facts that (1) the pVI–cDNA open reading frame had the C-terminal peroxisomal targeting signal (PTS1)-like sequence AKL and (2) the purified phagemid reacted specifically with ab-MF16 relative to the preimmune serum (Figure 2, condition 2), it seemed likely that the cloned cDNA encoded a peroxisomal matrix protein. In the next biopanning experiment, in which the ab-MF8 was used as bait, only three rounds of selective enrichment were performed. PCR analysis

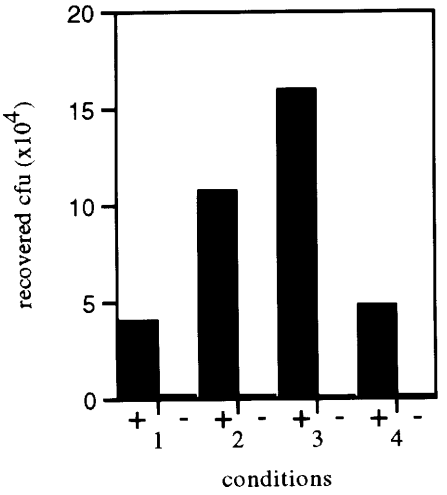

Figure 2 pVI fusion phage clones obtained by panning are specifically recognized by the antiserum that was used as bait

Individual phage particles [(1–2) × 10^9 cfu] were incubated with preimmune serum (−) or the antiserum used to biopan for the corresponding fusion phages (+) (ab-MF16 for conditions 1 and 2; ab-MF8 for conditions 3 and 4). The pure fusion phages were captured, washed, eluted and quantified as described in the Experimental section. The results of a representative experiment are shown. Condition 1, pVI–catalase (residues 404–528); condition 2, pVI–AKL (residues 264–292); condition 3, pVI–urate oxidase (residues 51–303); condition 4, pVI–SRL (residues 227–260).

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                10        20        30        40        50
RnDCR-AKL       M-TQQPPDVEEDDCLSEYHHLFCPDLLQDKVAFITGGGSGIGFRIAETFMRHGCHTVIVS
                : :..  .. .   ..:     . :::.. :::.:::.. :    .: ..  :....:.
ScSPS19         MDTMNTANTLDGKFVTEGS--WRPDLFKGKVAFVTGGAGTICRVQTEALVLLGCKAAIVG
                          10        20        30        40        50

                60        70        80        90        100       110
RnDCR-AKL       RSLPRVSEAAKKL--VAATGKRCLPLS-MDVRVPPAVMAAVDQALKEFGKIDILINCAAG
                :.  :. .::: .  .:       : .. .:::     :  :: ....:::::..:  :::
ScSPS19         RDQERTEQAAKGISQLAKDKDAVLAIANVDVRNFEQVENAVKKTVEKFGKIDFVIAGAAG
                 60        70        80        90        100       110

                  120       130       140       150       160       170
RnDCR-AKL       NFLCPASALSFNAFKTVVDIDTLGTFNVSRVLYEKFFRDHGGVIVNITATLSMRGQVLQL
                ::.:  . :: :::.:::::: ::.::.... ... ...:... ..:: . :  .:
ScSPS19         NFVCDFANLSPNAFKSVVDIDLLGSFNTAKACLKELKKSKGSILF-VSATFHYYGVPFQG
                120       130       140       150       160       170

                  180       190       200       210       220       230
RnDCR-AKL       HAGAAKAAVDAMTRHLAVEWGPQNIRVNSLAPGAISGTEGLRRLGGPKASSKFKYLSS-P
                :.::::::..:::...:::: :: .:: : .:::.:.::::.::: :  :  : :.. :
ScSPS19         HVGAAKAGIDALAKNLAVELGPLGIRSNCIAPGAIDNTEGLKRLAGKKY--KEKALAKIP
                180       190       200       210       220       230

                   240       250       260       270       280           290
RnDCR-AKL       IPRLGTKTEIAHSVLYLASPLASYVSGIVLVVDGGSWMTLPNDIGRLLEFE----SSSAKL
                . :::.  .::.:.:. :: ::::.:: ::::::: :  : ...:. : :  : .:::
ScSPS19         LQRLGSTRDIAESTVYIFSPAASYVTGTVLVVDGGMWH-LGTYFGHELYPEALIKSMTSKL
                 240       250       260       270       280       290
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Figure 3 Alignment of the rat peroxisomal DCR (RnDCR-AKL) and the *S. cerevisiae SPS19* gene product (ScSPS19)

Identical (:) and similar (.) amino acids between both proteins are indicated. The amino acid sequence present in the pVI–AKL clone that was obtained by biopanning is underlined once. The C-terminal targeting signals are underlined twice. The putative NADPH nucleotide-binding sequence is boxed.

demonstrated that we had obtained two different clones, which after sequencing were found to encode the peroxisomal marker enzyme urate oxidase (252 C-terminal residues) (four of the ten clones analysed), and another novel cDNA encoding a partial open reading frame with the PTS1-like sequence SRL (six of the ten clones analysed). Although both the pVI–urate oxidase and the pVI–SRL fusion phages were specifically recognized by ab-MF8 (Figure 2, conditions 3 and 4), subjecting the third round 'sublibrary' to a fourth round of biopanning yielded only the pVI–SRL clone (all 15 analysed clones). Possible explanations for this surprising result will be discussed below.

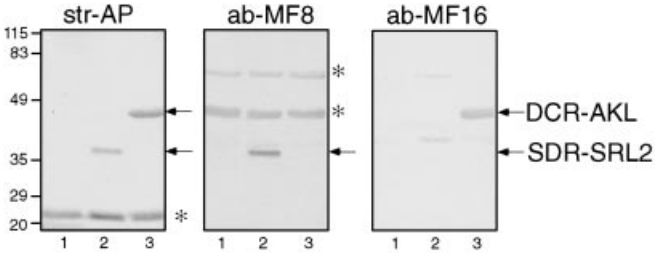

Figure 4 Recombinant human SDR-SRL2 and rat DCR-AKL are specifically recognized by ab-MF8 and ab-MF16 respectively

Equal amounts of bacterial lysates expressing no recombinant protein (1), biotinylated SDR-SRL2 (2) or biotinylated DCR-AKL (3) were subjected to SDS/PAGE and transferred to nitrocellulose. The membranes were then probed with streptavidin–alkaline phosphatase (str-AP), ab-MF8 or ab-MF16. The arrows indicate the recombinant expressed biotinylated proteins (the biotin tag added approx. 13 kDa to the molecular mass of the proteins). The stars indicate the positions of migration of non-specific bacterial proteins. The migrations of molecular mass markers (masses shown in kDa) are indicated.

Table 4 Recombinant expressed DCR-AKL has DCR activity

The supernatants (S) and pellets (P) of bacterial lysates expressing no recombinant protein (−) or biotinylated DCR-AKL (+) were analysed for DCR activity as described in the Experimental section. The results of a representative experiment are shown.

Fraction	Activity (m-units)	Specific activity (m-units/mg of protein)
S (−)	67	9
P (−)	36	3
S (+)	1063	193
P (+)	2044	178

The pVI–AKL clone encodes a putative peroxisomal DCR

The complete open reading frame encoding the rat cDNA of the pVI–AKL clone was obtained by rapid amplification of 5′ cDNA ends. The corresponding 31.2 kDa protein was 42 % identical with the yeast peroxisomal DCR, a protein encoded by the oleate-inducible gene *SPS19* [30] (Figure 3), and 29 % identical with the cloned rat liver mitochondrial DCR [31] (results not shown). Besides a putative PTS1, the 'AKL-protein' also contained a putative nucleotide-binding site that is present in nicotinamide-dependent enzymes and includes the motif HXH-XGXGXXGXXXHXHX (where H is a hydrophobic amino acid) [32] (Figure 3). The putative NADPH nucleotide-binding sequence deviates from the consensus sequence only at the third glycine residue and at the fourth hydrophobic amino acid. However, of the GXGXXG consensus only the first G is totally conserved; the second G can be replaced by S in some mercuric reductases and the final G is not conserved at all [33]. Expression of the recombinant version of this putative rat peroxisomal DCR (hereafter referred to as DCR-AKL) in *E. coli* resulted in the expression of an ab-MF16-immunoreactive protein (Figure 4).

Recombinant DCR-AKL has DCR activity

Expression of the putative reductase in *E. coli* as a biotinylated fusion protein resulted in a substantial increase in the sorboyl-CoA reductase activity (Table 4). Approx. one-third of the plasmid-expressed reductase activity was recovered in the soluble

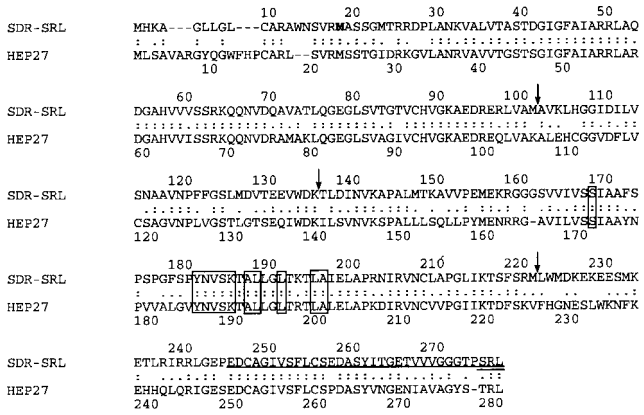

Figure 5 Alignment of the human SDR-SRL3 and HEP27 proteins

Identical (:) and similar (.) amino acids between both proteins are indicated. The methionine residue indicated in bold is the more likely candidate for the initiation of translation of SDR-SRL (see the Results section). The amino acid sequence present in the pVI–SRL clone that was obtained by biopanning is underlined once; the PTS1 is underlined twice. The SDR family signature is boxed. Residues 137–222 and 103–222 (flanked by vertical arrows) are not present in SDR-SRL1 and SDR-SRL2 respectively.

fraction after sonication of the bacteria. A similar distribution was observed when these fractions were analysed after SDS/PAGE and blotted for the presence of biotinylated proteins by means of streptavidin–alkaline phosphatase (results not shown). Whereas approx. 15% of the protein present in the pelleted material could be extracted by the detergent Thesit, only 3% of the reductase activity was solubilized, suggesting that the fusion protein, despite being active, was present within inclusion bodies. Control experiments with purified rat liver peroxisomes revealed that the reductase activity was not affected by Thesit.

cDNA of the pVI–SRL clone encodes a putative member of the SDR family

More than 50 overlapping human ESTs were found by probing the EST database with the rat cDNA encoding the rat partial 'SRL protein'. This allowed us to compose a human cDNA of 1282 bp. Given that the first initiation codon is preceded by a translational termination signal (TGA) and that the flanking nucleotides of the second ATG follow the Kozak rule [34], the second methionine residue is the more likely candidate for the initiation of translation (Figure 5). Database analyses showed that the encoded protein contained a SDR family signature (Prosite: PDOC00060) and is highly similar to the human HEP27 protein (Figure 5), a nuclear protein of unknown function that is synthesized in growth-arrested human hepatoblastoma cells [35]. Although the homology searches did not allow us to define a function for this SDR-SRL protein, a more detailed search of the EST database revealed the presence of three different forms (Figure 5), hereafter designated SDR-SRL1, SDR-SRL2 and SDR-SRL3. The shortest form, SDR-SRL2 (Figure 4), as well as SDR-SRL1 and SDR-SRL3 (results not shown), were expressed in bacteria as biotinylated fusion proteins and were recognized specifically by ab-MF8.

DCR-AKL and SDR-SRL contain a functional PTS1

To identify the subcellular locations of DCR-AKL and SDR-SRL, we expressed both proteins as C-terminal fusions to the

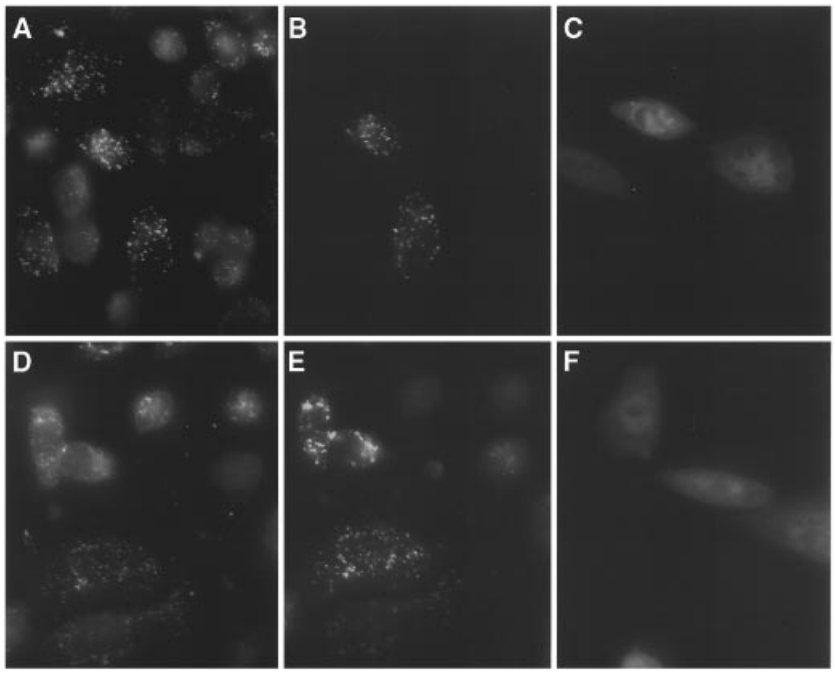

Figure 6 GFP–DCR-AKL and GFP–SDR-SRL are targeted to the peroxisomes by their C-terminal tripeptides

CHO cells were transfected with pcDNA3 derivatives encoding GFP–DCR-AKL, GFP–SDR-SRL, GFP–DCR and GFP–SDR. At 2 days after transfection, the cells were processed for immunofluorescence. Double localization studies of PMP70 (**A**) and GFP–DCR-AKL (**B**) or PMP70 (**D**) and GFP–SDR-SRL (**E**) demonstrated clearly that the cell organelles labelled with the GFP fusions were peroxisomes as judged by co-localization with the appropriate PMP70-positive particles. Cells expressing GFP–DCR (**C**) or GFP–SDR (**F**) gave rise to diffuse cytosolic staining.

heterologous reporter protein GFP. Punctate fluorescence was observed in transiently transfected CHO cells (Figures 6B and 6E). Double localization of GFP–DCR-AKL (Figure 6B) or GFP–DCR-SRL (Figure 6E) with the peroxisomal membrane protein PMP70 (Figures 6A and 6D) demonstrated clearly that the cell organelles marked with the GFP fusions were peroxisomes. Transfection of CHO cells with GFP–DCR (Figure 6C) and GFP–SDR (Figure 6F) resulted in diffuse fluorescence patterns. These results demonstrate that DCR-AKL and SDR-SRL are peroxisomal proteins and that the peroxisomal targeting of both proteins depends on the presence of the C-terminal three residues. The tripeptides AKL and SRL have previously been shown to be sufficient for the peroxisomal targeting of passenger proteins [36].

DISCUSSION

Because preliminary experiments demonstrated that the PTS1-binding domain of the human PTS1 receptor Pex5p [22], the *Pichia pastoris* PTS2-receptor Pex7p [37] and the *P. pastoris* peroxisomal membrane protein Pex3p [38] could be stably expressed on the phage coat as fusions with pVI (results not shown), we generated pVI–rat-liver cDNA libraries and a set of antisera that specifically recognized peroxisomal proteins, and used them in a series of biopanning experiments. These experiments yielded four cDNA clones encoding two known peroxisomal proteins, catalase and urate oxidase, and two novel proteins, a DCR and a protein with an SDR signature. Several characteristics of these novel proteins indicated they are localized to the peroxisomes: (1) the C-terminal three residues of the SDR and the DCR are SRL and AKL, respectively, tripeptides known to act as peroxisomal-matrix-targeting signals [36], (2) the antisera used as bait recognize peroxisomal proteins specifically and (3) the yeast counterpart of the putative DCR has been shown to be a peroxisomal matrix protein. Direct evidence that SDR-SRL and DCR-AKL are peroxisomal proteins and that their three C-terminal residues are required for targeting was obtained by immunofluorescence studies. In addition, there is experimental evidence at the enzymic and immunocytochemical levels that mammals possess, besides two mitochondrial DCRs [39,40], a third 33 kDa peroxisomal DCR [28,41,42]. However, until now the peroxisomal isoform has remained uncharacterized at the molecular level.

Because (1) the SDR superfamily is a very large family of enzymes and (2) SDRs tend to have a multifunctional nature, i.e. they catalyse dehydrogenations/reductions of seemingly disparate substrates, the functional significance of the SDR-SRL protein, as well as the relevance of the three isoforms, remains to be determined. However, from the EST entries we have evidence for the expression of the three SDR-SRL isoforms in various tissues and cells (results not shown). Proof that the pVI–AKL protein represents a DCR was obtained by expressing its cDNA in *E. coli* to yield a protein with this activity. DCR is necessary for the complete degradation by β-oxidation of unsaturated fatty acids with double bonds extending from either even-numbered [41] or odd-numbered carbon atoms [43,44]. The description by Roe et al. [45] that a disorder in which unsaturated fatty acid oxidation is affected is due to a deficiency of a (mitochondrial) DCR suggests that the novel peroxisomal DCR identified here could be important in peroxisomal diseases.

In contrast with the biopanning experiments performed by Crameri et al. [14], who obtained a number of different clones after six rounds of biopanning, we invariably ended up with one or two clones. Whether or not this was due to the use of the pVI phage display technology (direct fusions of cDNA clones) compared with the pIII phage display technology (indirect fusions of cDNA clones) is not clear at present. In general, successful display requires that the pVI fusion protein is expressed in *E. coli*, translocates to the periplasm, folds correctly and incorporates into the phage particles. Sequences that interfere with any of these steps will decrease or eliminate the growth of the corresponding phages. As a result, the enrichment factor gained by different fusion phages can probably be lost partly or completely in the amplification step. This phenomenon, in combination with the fact that ab-MF8 and ab-MF16 most probably have different affinities for the different cross-reactive peroxisomal proteins, might explain why only a limited number of clones were obtained in this study.

The results described here demonstrate that the pVI vectors are very attractive antibody-selectable expression vectors for the identification of novel (peroxisomal) proteins from actual cDNA libraries. Experiments are under way in which antibodies, raised against subfractions of peroxisomal membrane proteins that are capable of inhibiting the import of peroxisomal protein import *in vitro*, are used as bait to isolate specific molecules involved in peroxisomal protein import. A more complete inventory of peroxisomal proteins is critical for the identification and interpretation of the molecular defects that cause human peroxisomal diseases.

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