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UK114, a YjgF/Yer057p/UK114 family protein highly conserved from bacteria to mammals, is localized in rat liver peroxisomes

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Abstract

Mammalian UK114 belongs to a highly conserved family of proteins with unknown functions. Although it is believed that UK114 is a cytosolic or mitochondrial protein there is no detailed study of its intracellular localization. Using analytical subcellular fractionation, electron microscopic colloidal gold technique, and two-dimensional gel electrophoresis of peroxisomal matrix proteins combined with mass spectrometric analysis we show here that a large portion of UK114 is present in rat liver peroxisomes. The peroxisomal UK114 is a soluble matrix protein and it is not inducible by the peroxisomal proliferator clofibrate. The data predict involvement of UK114 in peroxisomal metabolism.

Keywords: Peroxisomes; UK114; YjgF/Yer057p/UK114 protein family; Clofibrate; Rat liver

Mammalian UK114 is a small protein (136 amino acids with a predicted molecular mass of 14.5 kDa, rat protein) forming a homotrimer and expressed predominantly in liver and kidney [1–4]. UK114 belongs to a highly conserved YjgF/Yer057p/UK114 family of proteins with a characteristic C-terminal stretch of amino acid residues. In addition to higher eukaryotes, members of this family have been identified in lower eukaryotes and in bacteria [5–8]. The precise function of UK114 and all proteins in the YjgF/Yer057p/UK114 family remains unknown, although several high-resolution crystal and NMR structures of the family members have been described showing that one subunit folds into pseudo α/β barrel with a narrow, deep grooves located at the intermonomer surfaces [4,9–12]. Search for functions of the proteins reveals their involvement in such diverse processes as isoleucine biosynthesis, translation inhibition, purine metabolism, chaperone and lipid-binding activities [1,2,5–7,13,14]. The abundance and versatility of the apparent functions led to suggestion that the YjgF/Yer057p/UK114 family members are implicated in binding and/or degrading of metabolites, possibly generated in side reactions by central metabolic enzymes [15].

So far the intracellular localization of UK114 has not been investigated in details. Initial studies on mammalian members of the YjgF/Yer057p/UK114 family described them as cytoplasmic components [1–3], though later results indicated a presence of UK114 in mitochondria [7]. Our research on a protein composition of highly purified peroxisomal fractions isolated from rat liver provided initial evidence that UK114 might be localized in peroxisomes. Here we present results of a more detailed investigation on the subcellular localization of UK114 indicating that it is a bona fide peroxisomal protein.

Abbreviations: BCA, bicinchoninic acid; 2-DE, two-dimensional gel electrophoresis; L-FABP, fatty acid binding protein (liver isoform); PEG, polyethylene glycol; SCP2, sterol carrier protein 2; thiolase, peroxisomal 3-oxoacyl-CoA thiolase.

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Materials and methods

Materials. The chemicals were from Sigma except those for which sources are indicated.

Subcellular fractionation. Male starved overnight 175–200 g Sprague–Dawley rats were used in accordance with the policies of the committee on animal experimentation at the University of Oulu. In some experiments rats were maintained on a standard diet containing 3% (v/w) clofibrate (Aldrich). The liver tissue was homogenized in isolation medium containing 0.16 M sucrose, 12% (w/v) poly(ethylene glycol) 1500 (PEG 1500), 10 mM Mops, pH 7.4, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, 0.1% ethanol and 0.1 mM PMSF. The isolation of highly purified peroxisomes was carried out exactly as described [16] using a two-step procedure which includes Percoll (Amersham) followed by Optiprep gradient centrifugations. For the one-step gradient centrifugation (see 'Results'), the homogenates were centrifuged at 1500*g* for 12 min and the resulting postnuclear supernatant was loaded on a multistep Optiprep gradient [32 ml of 20–50% (w/v) Optiprep] prepared with isolation medium containing no sucrose and PEG 1500. The samples were centrifuged in a vertical VTi 50 rotor (Beckman) at 100 000*g* for 90 min in slow acceleration and deceleration mode. To detect the subperoxisomal localization of UK114, the purified peroxisomes were diluted with 20 mM MOPS, pH 7.4 to 0.4–0.6 mg/ml, sonicated (6 cycles, 15 s each, amplitude 15 μm), and the main peroxisomal constituents (membrane, nucleoid and matrix proteins) were separated by sucrose-density-gradient centrifugation [16]. Proteinase treatment of purified peroxisomes was carried out as described [17].

Measurement of enzyme activities. Activities of marker enzymes for subcellular organelles: mitochondria (glutamate dehydrogenase), lysosomes (acid phosphatase), endoplasmic reticulum (esterase) and peroxisomes (L-α-hydroxyacid oxidase) and for peroxisomal constituents: nucleoid (urate oxidase), matrix (catalase), and membrane (NADH:cytochrome *c* reductase) were measured according to standard procedures (see [18] for details).

Antibody production. A specific antibody against UK114 was raised by immunizing rabbits with a chemically derived peptide NH_2-SSIIRK VISTSKAPAA corresponding to the N-terminus (amino acids 1–17) of the rat UK114 (Swiss-Prot Accession No. P52759) and coupled with KLH. IgG fractions were prepared from rabbit sera using Protein-A Sepharose (Bio-Rad) as described by the manufacturer. The sources of other antibodies used have been described elsewhere [17].

Electrophoresis and immunodetection of UK114 and other proteins. Proteins were separated by SDS–PAGE using 15% (w/v) acrylamide gels (Criterion Precast Gel; Bio-Rad) and transferred to nitrocellulose (0.45 μm Protran; Schlechter & Schuell) by means of a semi-dry electroblotting device. The blot was incubated with the primary antibodies, followed by detection of IgG with alkaline phosphatase-labeled anti-rabbit IgG antibodies [19]. A rabbit polyclonal antibody against the peroxisomal enzymes 3-oxoacyl-CoA thiolase and catalase, and the cytoplasmic enzyme glutathione-*S*-transferase A were used to detect the corresponding proteins as markers for peroxisomes and cytoplasm, respectively. Coomassie blue staining and silver staining of gels were made as described [17,19]. The protein content was measured using the BCA reagent (Pierce).

Two-dimensional gel electrophoresis (2-DE) and protein identification. The peroxisomal matrix proteins were precipitated with 80% (v/v) acetone and resuspended in urea buffer (8 M urea, 2 M thiourea, 1% [w/v] CHAPS, 20 mM DTT, 0.8% [v/v] carrier ampholytes 3–10, and CompleteMini protease inhibitor cocktail). The two-dimensional gel electrophoresis was performed as described [17]. The gels were stained with SYPRO Ruby (Bio-Rad) and the protein pattern analyzed with Ettan Progenesis Workstation 2003.1 (Nonlinear Dynamics). For protein identification the spots were excised, in-gel-digested and analyzed by mass spectrometry (VOYAGER DE-STR, Applied Biosystems; parameters: mass tolerance 20 ppm, sequence coverage >20%).

Immunoelectron microscopy. The immunogold technique was applied for detection of UK114 and peroxisomal 3-oxoacyl-CoA thiolase on liver sections as previously described [17]. The control samples were prepared using pre-immune serum or by carrying out the labeling procedure without primary antibodies.

Results

Cell fractionation

Fig. 1A and B shows the separation of a rat liver postnuclear homogenate on an Optiprep gradient. As expected, the cytosolic proteins remained on top of the gradient (fractions 13–16) as was revealed using an antibody against marker enzyme glutathione-*S*-transferase A (Fig. 1B). According to the distribution of the peroxisomal markers, L-α-hydroxyacid oxidase (Fig. 1Aa) and 3-oxoacyl-CoA thiolase (thiolase, Fig. 1B), these particles were sedimented near the bottom of the gradient (fractions 3–8) with traces noted in fractions 9–12. In addition, some activity of L-α-hydroxyacid oxidase (Fig. 1Aa) and traces of a thiolase protein (Fig. 1B) were found in fractions containing cytosol that indicates leakage of soluble matrix proteins from broken peroxisomes [16,18]. The mitochondria (marker—glutamate dehydrogenase, Fig. 1Aa), fragments of the endoplasmic reticulum (esterase, Fig. 1Aa), and the lysosomes (acid phosphatase, Fig. 1Ab) were concentrated predominantly in fractions 10–12. The distribution of UK114 over the Optiprep gradient showed that the protein is abundantly present in fractions containing components of the cytoplasm (fractions 13–16). A substantial portion of UK114 was also detected in fractions enriched with peroxisomes (fractions 6–7). In contrast, fractions enriched with mitochondria and endoplasmic reticulum (fractions 11–12) contained only a limited amount of UK114 especially if consider a high protein content in these fractions (see Fig. 1Ab).

Highly purified peroxisomal fraction was isolated using a procedure which includes Percoll followed by Optiprep gradient centrifugation [16]. The purity of the isolated peroxisomes was determined by measuring the activity of organelle-specific enzymes, immunodetection of marker proteins, and transmission electron microscopy. The composition of the purified fraction was deduced from the specific activities of marker enzymes [20] indicating that peroxisomes constituted more than 95% of the total protein content of the fraction. The electron microscopic examination revealed a nearly homogeneous population of peroxisomes (results not shown). The protein composition of the isolated peroxisomal fraction differed from that of the cytosol (Fig. 1C, left panel). However, both these fractions contained a prominent protein band of around 14 kDa (marked by asterisk on Fig. 1C). Immunodetection of UK114 revealed the presence of this protein in both, cytoplasmic and highly purified peroxisomal fractions (Fig. 1C, right panel). The mobility of the protein corresponded to the mobility of the 14 kDa band (Fig. 1C, compare left and right panels). Importantly, the relative content of UK114 in the peroxisomal fraction was higher than in the cytosol (Fig. 1C, right panel) implying that the protein is a truly peroxisomal constituent.

In order to reveal whether or not UK114 is localized inside peroxisomes, the purified peroxisomal fraction was treated with proteinase K (Fig. 1D). In the absence of

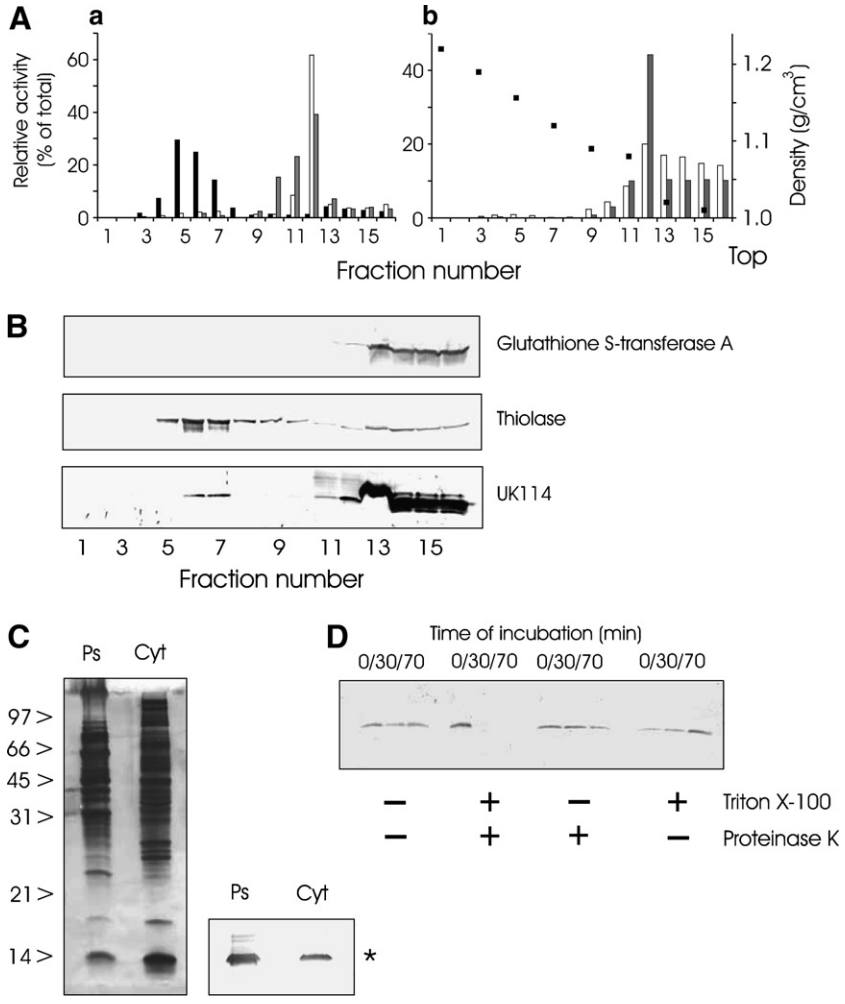

Fig. 1. Localization of a portion of UK114 in peroxisomes. (A,B) Optiprep gradient centrifugation of a post-nuclear supernatant from normal rat liver. (A) Fractions were analyzed for marker enzymes activities: L-α-hydroxyacid oxidase (a, filled bars), glutamate dehydrogenase (a, open bars), esterase (a, gray bars), acid phosphatase (b, gray bars) and for protein content (b, open bars). The ordinate axis (left) represents the relative enzyme activities in each fraction (percentage of the total activity loaded on the gradient). The recoveries of the enzyme activities and protein content were between 76 and 112%. (B) Proteins from equal volume of each fraction (see A) were separated by SDS–PAGE and transferred onto nitrocellulose for immunodetection using specific antibodies. (C) Silver staining of peroxisomal (Ps) and cytoplasmic (Cyt) proteins (left panel) and immunodetection of UK114 in the same fractions (right panel). Highly purified peroxisomes were isolated using Percoll and Optiprep gradient centrifugations as described in section 2. Samples with equal amounts of protein were loaded on the gel. The relative molecular mass (M_r) of marker proteins (in kDa) is indicated on the left side. The asterisk indicates the position of a 12–14 kDa protein band. (D) Protease treatment of peroxisomes. Purified peroxisomes were incubated at 37 °C in the presence of proteinase K (0.6 U/ml) and/or Triton X-100 (0.05%, w/v) for different time intervals and the presence of UK114 in the samples was detected using immunoblot technique.

detergent only limited degradation of UK114 by the protease was detected. In contrast, incubation of the detergent-treated peroxisomes with proteinase K led to total disappearance of the immunosignals corresponding to UK114. Damage of purified peroxisomes by sonication followed by separation of the peroxisomal fragments by sucrose-density-gradient centrifugation was accompanied by extensive leakage of UK114 from the particles indicating that the protein is located in the matrix of the organelles in a soluble form (data not shown).

2-DE of peroxisomal matrix proteins

To verify further the presence of UK114 in the peroxisomal fraction we applied 2-DE to separate the peroxisomal matrix proteins and identified UK114 in the gel by mass spectrometric analysis (Fig. 2A and B). These experiments allow us to confirm localization of UK114 to peroxisomes without using antibodies against this protein. The protein pattern obtained for normal rat liver peroxisomes includes several spots in the gel region of 12–14 kDa (Fig. 2A, marked by frame). Mass spectrometric analysis revealed that the spots in the gel region marked by a frame corresponded to UK114 (marked by asterisk on Fig. 2A and shown under number 1 in Fig. 2B), transthyretin-like protein (number 2); the liver isoform of fatty acid binding protein (L-FABP, number 3), and sterol carrier protein 2 (SCP2, number 4). Comparison of the quantities of these proteins in the gel (Fig. 2B) showed that the content of UK114 in normal rat liver peroxisomes is com-

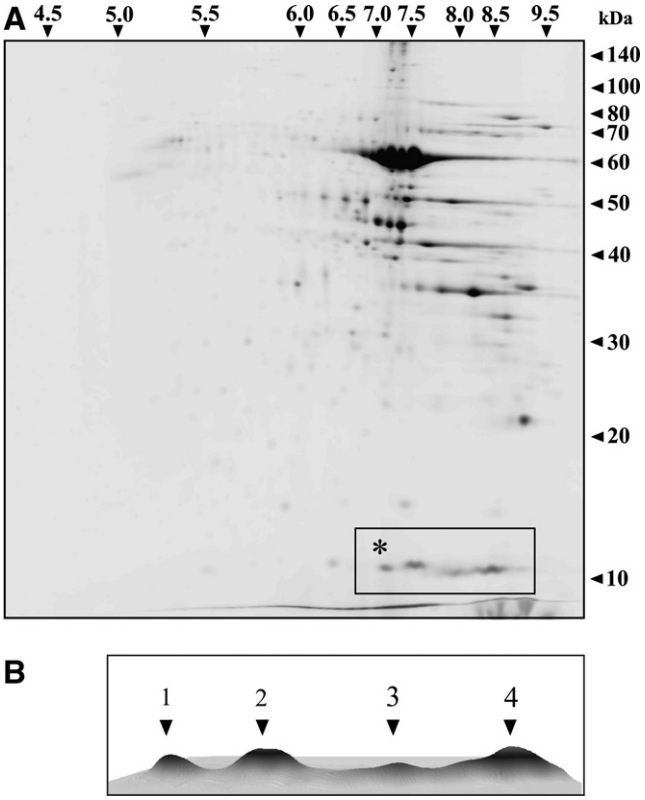

Fig. 2. 2-DE of matrix proteins from normal rat liver peroxisomes and detection of UK114. (A) Two-dimensional gel of matrix proteins (200 μg). Apparent pI and molecular mass values are indicated. The position of UK114 in the gel is shown by asterisk. (B) Comparison of the spot intensities of low molecular mass proteins detected in an area surrounded by a frame on the gel (see A). The proteins are: 1, UK114; 2, transthyretin-like protein; 3, L-FABP; 4, SCP2.

parable with the amount of well-established peroxisomal proteins such as transthyretin-like protein [21] and SCP2 [22].

Effect of clofibrate on the content of UK114 in rat liver

Treatment of rodents with hypolipidamic drug clofibrate is accompanied by profound proliferation of liver peroxisomes and by induction of peroxisomal proteins involved in the metabolic conversion of lipids, especially in the oxidative degradation of long-chain fatty acids [23]. Indications on the peroxisomal localization of UK114 prompted us to assess the effect of clofibrate on the content of this protein in rat liver (Fig. 3). The immunodetection of UK114 was carried out using the postnuclear homogenate, the preparations of isolated peroxisomes, and the cytosol. As a control we analyzed the amount of peroxisomal marker proteins catalase and thiolase. In accordance with previous reports [19,23], the content of thiolase in all preparations tested had increased severalfold after treatment by clofibrate (Fig. 3). At the same time clofibrate only slightly affects the amount of catalase. Unexpectedly, the level of UK114 in the preparations isolated from clofibrate-treated

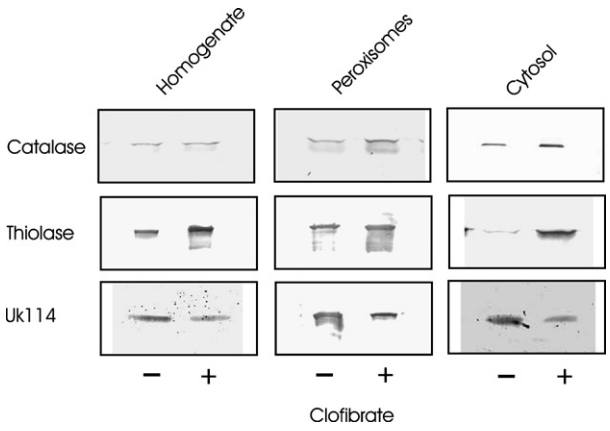

Fig. 3. Effect of clofibrate treatment on the content of UK114 in rat liver. Equal amount of proteins (control *versus* clofibrate) from corresponding fractions were separated by SDS–PAGE and immunoblotted. The aliquots of fractions 6 and 15 from the Optiprep gradients (see Fig. 1A) were used as peroxisomal and cytosolic samples, respectively. Post-nuclear supernatant was used as a homogenate sample.

rat liver was significantly lower relative to control (normal rat liver). The clofibrate-dependent decrease in the amount of UK114 was registered also after 2-DE of peroxisomal matrix proteins (data not shown).

Immunoelectron microscopy

Upon applying the protein A-gold immunocytochemical technique using tissue sections from normal rat liver and the anti-UK114 antibody gold particles were primarily detected in peroxisomes (Fig. 4A, C, and D). The matrix of the organelles was distinctly stained (Fig. 4C). Gold particles were found also in the cytosol throughout the cell (Fig. 4A, C, and D). However, mitochondria were devoid of labeling (Fig. 4B–D). As a control, we performed immunogold labeling on the liver sections with antibodies generated against the peroxisomal marker protein thiolase (Fig. 4E and F). As expected, the label was located in the matrix of peroxisomes, whereas only very few gold particles were detected in the cytosol (Fig. 4E). The labeling of thiolase in peroxisomes was greatly enhanced after treatment of rats with clofibrate (Fig. 4F). In contrast, the drug did not affect the labeling of peroxisomal UK114 (Fig. 4B).

Discussion

Our results obtained by means of largely independent experimental approaches: analytical subcellular fractionation, immunoelectron microscopy, and mass spectrometry of peroxisomal matrix proteins separated by 2-DE, clearly demonstrate that a significant portion of UK114 is indeed present in the matrix of rat liver peroxisomes. Moreover, the data suggest that the concentration of the protein in the particles is higher than in the cytosol. In contrast, our study failed to produce evidence on the mitochondrial localization of mammalian UK114. Initial reports have associated the UK114 protein with the cytoplasmic com-

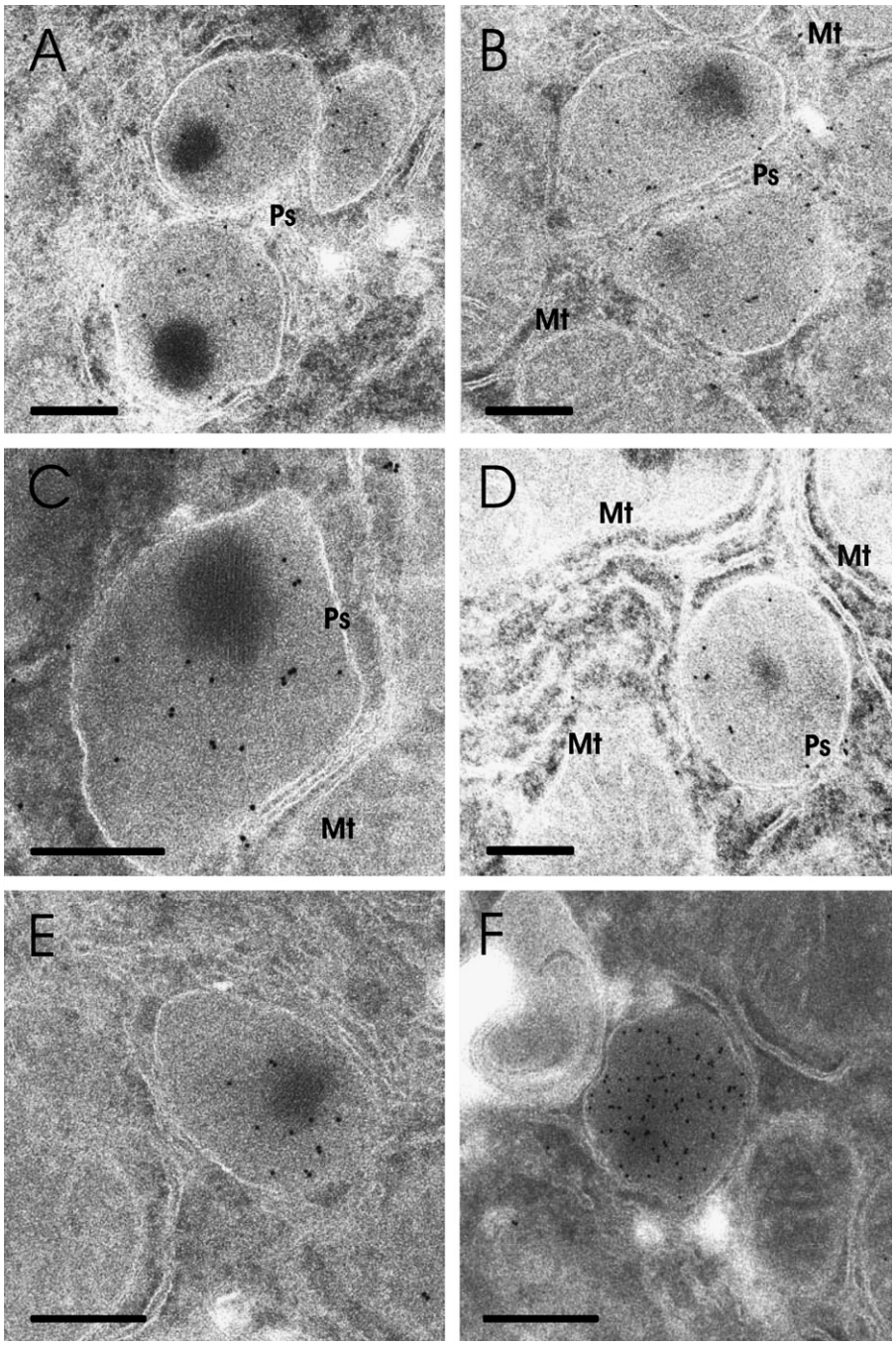

Fig. 4. Localization of UK114 in rat hepatocytes using the immunogold technique. Protein A-gold immunolabeling (10 nm gold particles) of UK114 (A–D) or peroxisomal thiolase (E,F). Sections of normal (A, and C–E) or clofibrate-treated (B,F) rat livers are shown. The gold particles revealing UK114 antigenic sites are visible over the peroxisomes (Ps) and in the cell cytoplasm but were not observed over the mitochondria (Mt). Original magnification: 15,000× (A, B, and D) or 20,000× (C, E, and F); scale bar, 300 nm. Control preparations (incubation without primary antibodies) did not contain immunogold labels (results not shown).

partment of the cell [1–3]. Later on it has been found that lower eukaryotes, e.g. yeast *Saccharomyces cerevisiae,* may contain two proteins homologous to mammalian UK114. One of these proteins was located to mitochondria [7]. Attempts have been made to detect UK114 in the mitochondrial fraction isolated from rat liver [7]. However, the authors did not present an assessment of the purity of this fraction. As known, the mitochondrial fractions are usually contaminated by peroxisomes, lysosomes, and fragments of endoplasmic reticulum [16,20,24].

An estimation of the content of peroxisomal matrix proteins separated by 2-DE revealed that UK114 is one of the abundant proteins in normal rat liver peroxisomes. This fact indicates the importance of UK114 for peroxisomal metabolism that includes such pathways as purine degradation, oxidation of fatty acids, and formation of α-ketoacids

[25,26]. The metabolites involved in these pathways have been associated with apparent functions of some proteins in the YjgF/Yer05/UK114 family [5,6,8,12,14,15].

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