Localization of the FK506-binding protein, FKBP 13, to the lumen of the endoplasmic reticulum

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The function of the immunophilins, FKBP 12 and FKBP 13, which are binding proteins for the immunosuppressant drug FK506 and rapamycin, remains poorly defined, although it has been suggested that immunophilins and immunophilin-like proteins may play a role in protein sorting/folding and intracellular calcium ion regulation. As a first step towards understanding the function of FKBP 13, we studied its subcellular localization by immunoblotting of well-defined subcellular fractions from a canine pancreatic homogenate and immunocytochemical analysis of an overexpressed cloned cDNA for FKBP 13. Whereas FKBP 12 fractionated entirely into the cytosol, virtually all FKBP 13 was found in the rough microsomal fraction which consisted of highly purified rough endoplasmic

reticulum (ER), along with several well-characterized ER markers [the immunoglobulin heavy-chain binding protein (BiP), grp 94 and ribophorin I]. Moreover, FKBP 13 co-banded with the ER markers on isopycnic sucrose gradients. By immunofluorescence, the overexpressed cDNA for FKBP 13 in HeLa cells gave an ER-staining pattern highly similar to that of known ER proteins. Addition of the ligand FK506 did not appear to alter the distribution of FKBP 13. Separation of the ER luminal contents and membrane revealed FKBP 13 to be a luminal ER protein. Since the lumen of the ER is where the folding of membrane and secreted proteins occurs, as well as a major site of intracellular calcium storage, it seems possible that FKBP 13 may be involved in one of these functions.

INTRODUCTION

The immunophilins, a class of proteins which binds immunosuppressive drugs (including cyclosporin A (CsA), FK506, and rapamycin) has become the subject of considerable interest for investigators from a number of fields (see [1] for review). Apart from their clinical usefulness as immunosuppressants, the drugs appear to be useful probes for the study of signalling events in activated T lymphocytes [2] and probably, given the ubiquitous presence of their binding proteins, in other cells as well. In particular, FK506 and CsA appear to be potent inhibitors of calcineurin, a serine/threonine phosphatase [3,4]. Indirect evidence also suggests a role for immunophilins in intracellular calcium regulation, as some of the immunophilins may associate with proteins regulating different intracellular calcium pools. In this latter regard, it has been shown that s-cyclophilin co-localizes with the putative calcium-storage protein calreticulin [5], and that FK506-binding protein 12 (FKBP 12) associates with the ryanodine receptor, a calcium-release channel in skeletal muscle sarcoplasmic reticulum [6]. Accordingly, it has been suggested that the subcellular localization of immunophilins in non-muscle cells may provide important insights into the localization of the elusive InsP_3 -insensitive intracellular calcium pool(s) within the cell [7]. Finally, since it has been shown that the immunophilin-like product of the gene *ninaA* in the *Drosophila* photoreceptor cell plays an important role in the apparent sorting of rhodopsin through the secretory pathway [8], there has been considerable speculation on the possible involvement of immunophilins in analogous processes in mammalian cells.

FKBP 12 and FKBP 13 are highly similar proteins, of

molecular masses 12 kDa and 13 kDa respectively, with approx. 43% amino acid identity. FKBP 13 contains a 21-amino-acid signal peptide and appears to be membrane-associated [9], though its precise localization remains undefined. In addition to binding FK506, it also binds the immunosuppressant drug, rapamycin [1]. However, apart from the finding that FKBP 12, like some of the cyclophilins, possesses drug-inhibitable rotamase activity (the ability to catalyse, *in vitro*, *cis-trans* interconversion of peptidyl-prolyl bonds), little is known about their function within cells in the absence of the drugs.

As a first step towards understanding the constitutive function of FKBP 13, we have studied the subcellular localization of this protein. We now report that FKBP 13 is localized to the lumen of the endoplasmic reticulum (ER).

EXPERIMENTAL

Materials

Important commercial sources are listed below. Monospecific antisera were generous gifts from the following: David Bole, University of Michigan [against the Ig heavy-chain-binding protein (BiP)], David Meyer, U.C.L.A. (against ribophorin I) and Michael Green, St. Louis University [against the 94 kDa glucose regulated protein (grp-94)]. The characterization of monospecific antiserum against FKBP 12 and 13, as well as the isolation of a cDNA clone for FKBP 13, have been described previously [9,10].

Subcellular fractionation

The subcellular fractionation protocol employed has been de-

Abbreviations used: FKBP, FK506-binding protein; ER, endoplasmic reticulum; RER, rough ER; SM, smooth microsome; RM, rough microsome; BiP, immunoglobulin heavy-chain binding protein; grp 94, glucose regulated protein of 94 kDa; CsA, cyclosporin A; PMSF, phenylmethanesulphonic acid; TEA, triethanolamine.

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Figure 1 Subcellular localization of FKBP-13 to rough microsomes by fractionation of a canine pancreatic post-mitochondrial supernatant

A post-mitochondrial supernatant (devoid of nuclei, mitochondria, secretory granules, plasma membrane sheets, etc.) was obtained from a canine pancreatic homogenate by two differential-centrifugation steps as described in the Experimental section. This supernatant was further fractionated over a 1.3 M sucrose cushion to yield what have previously been shown to be three biochemically and morphologically distinct fractions: highly purified cytosol (C, approx. 120 μ g of protein), smooth microsomes (SM, containing 'smooth' vesicles and Golgi, approx. 20 μ g of protein) and rough microsomes (RM, consisting of highly purified RER, approx. 35 μ g of protein). 'Equivalent' fractions were loaded (see the Experimental section and [11–14] for definition) for analysis. Immunoblots using monospecific antisera and depicting the distribution of FKBP-13 (molecular mass 13 kDa) (a), FKBP-12 (12 kDa) (b), grp 94 (94 kDa) (c), BiP (78 kDa) (d) and ribophorin I (Ribo I) (66 kDa) (e) are shown.

scribed in detail previously [11–14]. Briefly, the post-mitochondrial supernatant (10000 *g* for 10 min) of a canine pancreatic homogenate [prepared in 0.25 M sucrose, 50 mM triethanolamine (TEA)/HCl, pH 7.5, 50 mM potassium acetate, 6 mM magnesium acetate, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethanesulphonyl fluoride (PMSF) plus aprotinin] was subfractionated over a 1.3 M sucrose cushion and centrifuged at 202000 *g* for 2.5 h to yield three distinct fractions: a high-speed supernatant consisting of highly purified cytosol, a somewhat heterogeneous smooth microsome (SM) fraction (containing Golgi and other 'smooth' membranes) and a rough microsome (RM) fraction consisting of highly purified rough ER (RER), as previously demonstrated by transmission electron microscopy [12]. Furthermore, these fractions have previously been shown to possess distinct biochemical profiles [11–14]. Equivalent fractions (defined in detail in [11]) were loaded for SDS/PAGE and immunoblotting. Thus, the amounts of post-mitochondrial fractions (cytosol, SM, RM) analysed in Figure 1 directly reflects the result, in the fractionation scheme, if a given amount of post-mitochondrial supernatant were further sub-fractionated to obtain cytosol, SM and RM. This method provides a semi-quantitative indication of the distribution of various proteins within the cell.

Isopycnic floatation through a sucrose gradient

Freshly prepared RM were layered at the bottom of a 35–65% (w/v) sucrose gradient in 150 mM potassium acetate/50 mM TEA/HCl, pH 7.5/5 mM MgCl_2 /0.5 mM PMSF as previously

described [11] and centrifuged to equilibrium (approx. 18 h) at 40 °C in a SW40 swinging-bucket rotor. Individual fractions were collected with a Haake–Buchler Autodensiflow 2C gradient former connected to a fraction collector. Fractions were analysed by SDS/PAGE and immunoblotting.

Separation of ER luminal contents

RMs were briefly (2 s) tip sonicated at the lowest energies (1–3 in a Fischer sonicator) on ice and then separated into supernatant (luminal) and pellet (membrane) fractions by centrifugation in a Beckman airfuge at 207 kPa (30 lbf/in²) for 10 min. The fractions were separated by SDS/PAGE and immunoblotted.

Immunoblots

Western blots and probing of nitrocellulose membranes with primary antisera were performed as previously described [11,14], except that FKBP 12 and FKBP 13 immunoblots were developed using the alkaline phosphatase method, whereas immunoblots for other (marker) proteins were developed using the Amersham enhanced chemiluminescent (ECL) system with horseradish-peroxidase-conjugated secondary antisera.

Transfection of the *c-myc*-tagged FKBP 13 cDNA construct

A human full-length cDNA clone encoding FKBP 13 was tagged with a sequence encoding ten amino acids of human *c-myc*. Briefly, the construct was made by PCR using the 5' primer GGCCAAGCTTGGCGAGGAGAGACATGA and the 3' primer GGCCGAATTCTTATAGGTCTTCTTCGCTAATGAGCTTCTGCTCCAGCTCAGTTCGTCGCTCTAT with an FKBP 13 template. The PCR products were digested with *Hind*II and *Eco*RI and ligated into the eukaryotic expression vector PECE [15]. HeLa cells were grown on coverslips and fed 3 h before transfection with the expression vector containing the *c-myc*-tagged FKBP 13 construct, which was performed by the method of calcium phosphate precipitation (0.1 M CaCl_2 , 140 mM NaCl, 25 mM Hepes, 750 μ M Na_2HPO_4 , pH 7.1) [16]. After a 4 h incubation cells were washed three times and placed in fresh medium. For some samples 1 μ M FK506 was added. Then, 14–16 h later, cells were fixed for indirect immunofluorescence.

Immunofluorescence

Cells were fixed with 3% (v/v) paraformaldehyde in PBS for 10 min at room temperature and then permeabilized with PBS/0.1% Triton X-100. Non-specific binding was blocked with 5% (v/v) fetal-calf serum in PBS/0.1% Triton X-100. Cells were then double stained using a mouse monoclonal antibody against the *myc* tag 9E10 [17] and a rabbit polyclonal antiserum against ER proteins (gift of Brian Burke, Harvard, MA, U.S.A.). (This latter antiserum possesses identical reactivities by immunoblotting and immunofluorescence to an extremely well-characterized ER antiserum [19] and reacts exclusively with four ER proteins of molecular masses 91, 66, 58 and 29 kDa [19]. One of these proteins (91 kDa) has recently been identified as the ER chaperone, calnexin [20].) The cells were then probed with the secondary antibodies, fluorescein-labelled goat anti-(rabbit IgG) and rhodamine-labelled goat anti-(mouse IgG).

RESULTS

To unambiguously determine the subcellular localization of FKBP 13, two approaches were used: a well-defined subcellular fractionation scheme was combined with immunocytochemical

Figure 2 FKBP-13 co-distributes with known ER markers on isopycnic sucrose gradients

RMs were layered at the bottom (last two fractions = load zone) of a continuous 35–65% sucrose gradient and centrifuged (floatation protocol) to equilibrium as described in the Experimental section. Individual fractions were immunoblotted with antisera against FKBP-13 (a), ribophorin I (Ribo I) (b) and grp 94 (c).

analysis of the overexpressed protein. The subcellular fractionation scheme has been described in detail elsewhere, and data from analysis of markers for a number of organelles, including RER, plasma membrane, nuclear envelope, secretory granules and cytoskeleton, have been presented [11–14]. Briefly, a post-mitochondrial supernatant of a canine pancreatic homogenate was subfractionated over a 1.3 M sucrose cushion. Based on previously published results, the post-mitochondrial fraction is expected to be devoid of nuclei and nuclear envelope, mitochondria, secretory granules and most of the plasma mem-

brane [12,14,18]. Three 'post-microsomal' fractions were obtained: cytosol, SM and RM. That the rough microsomal fraction consists of very highly purified RER is indicated by the fact that it is markedly enriched in several RER-specific membrane and luminal proteins and uncontaminated by a number of biochemical markers for other cellular components, including the plasma membrane, secretory granules, and the nuclear membrane [11–14]. Furthermore, electron microscopy of this fraction has revealed no evidence of contamination with non-ER membranes [12]. The smooth microsomal fraction, although heterogeneous, is largely free of cytosolic and RER contamination, and the cytosolic fraction does not appear to be contaminated by any of the forementioned membrane markers [11–14]. Thus, the fractions are highly enriched for particular cellular components (virtually pure in the case of the cytosolic and RM fractions) and possess distinct biochemical and/or morphological profiles.

FKBP 12 and FKBP 13 distributed unambiguously into different fractions: FKBP 12 was almost entirely in the cytosol (Figure 1b), whereas FKBP 13 (Figure 1a) was almost entirely in the RM (RER) fraction, in a pattern very similar to the well-characterized ER luminal proteins, BiP and grp 94, as well as the ER membrane protein, ribophorin I (Figure 1c–e). However, although the fractionation pattern of FKBP 13 was very similar to that of grp 94 and BiP, it did not appear to be identical (see the Discussion).

To localize FKBP 13 more clearly to the RER of pancreas, the RM fraction was further subfractionated on a continuous isopycnic 35–65% (w/v) sucrose gradient (Figure 2). Consistent

Figure 3 Immunolocalization of the over-expressed FKBP-13 reveals an ER staining pattern

A *c-myc*-tagged cDNA coding for FKBP-13 was overexpressed in HeLa cells and immunofluorescence (left-hand panels) performed with an anti-*c-myc* antibody (9E10) in the absence (top panels) and presence (bottom panels) of FK506 (1 μ M) (see the Experimental section). Double staining was performed with an antiserum against known ER proteins (right-hand panels). Neither non-immune serum nor the anti-*c-myc* serum gave ER staining in untransfected cells.

Figure 4 FKBP-13 fractionates with the luminal contents of the ER

The luminal contents (S) were separated from the RM membrane by sonication and centrifugation as described in the Experimental section. Immunoblot analysis was used to compare the distribution of FKBP-13 (a) with known luminal markers of the ER, BiP (b) and grp 94 (c), as well as an ER membrane marker, ribophorin I (d).

with an RER localization, FKBP 13 equilibrated at high sucrose densities and co-banded with the known RER proteins, grp 94 and ribophorin I.

Because the antisera raised against FKBP 13 gave no immunofluorescent staining, confirmation of an ER localization was obtained by immunocytochemical analysis of an overexpressed *c-myc*-tagged FKBP-13 cDNA construct in HeLa cells (Figure 3, top panels). Indirect immunofluorescence revealed a perinuclear reticular staining pattern extending well into the cytoplasm. Double labelling with antiserum against known ER proteins gave a very similar staining pattern. As can be seen in Figure 3 (bottom panels), the addition of FK506 did not seem to alter the distribution of FKBP 13. Neither non-immune antisera nor the anti-*c-myc* sera reacted with the ER by immunofluorescence in untransfected HeLa cells.

Since the cloned cDNA sequence for FKBP 13 does not possess a membrane-spanning hydrophobic domain, but contains a C-terminal Arg-Thr-Glu-Leu (RTEL) sequence reminiscent of the classical ER luminal retention sequence, Lys-Asp-Glu-Leu (KDEL), we sought to determine whether FKBP 13 was localized to the ER lumen. The ER luminal contents (supernatant in Figure 4) was separated from the membrane fraction (pellet in Figure 4) and the distribution of FKBP 13 was analysed along with marker proteins for the luminal contents [BiP and grp 94, Figures 4(b) and 4(c) respectively], and the ER membrane protein, ribophorin I (Figure 4d). As expected for an ER luminal protein, FKBP 13 was markedly enriched in the supernatant (luminal contents) fraction, along with the ER luminal (KDEL-containing) proteins, BiP and grp 94, whereas the ER membrane protein, ribophorin I, was found primarily in the pellet (membrane) fraction.

In an attempt to establish whether the C-terminal RTEL sequence might function as an ER luminal retention sequence in a fashion similar to KDEL [21], we deleted the RTEL motif and transfected the deletion mutant into HeLa cells. However, we were unable to detect any expression in either the transfected cells or the medium (the expected result, if RTEL serves as an ER luminal retention signal). One possible explanation for this is that, since FKBP 13 is such a small protein (13 kDa), the RTEL motif may not only be involved in retention but also in proper folding or interaction with another protein. If the folding/interaction is disrupted, the protein might be degraded within the ER and thus would not be detectable.

DISCUSSION

Using a combination of transient overexpression of a cloned cDNA and unambiguous subcellular fractionation, our data clearly demonstrate that FKBP 13 is almost exclusively localized to the lumen of the ER. FKBP 12 fractionated into a high-speed supernatant fraction and FKBP 13 fractionated, along with other ER proteins (BiP, grp 94 and ribophorin I), into an RM fraction previously demonstrated, both biochemically and morphologically, to consist of highly purified RER. However, the fractionation pattern of FKBP 13, though very similar to the other ER proteins, was not identical (Figure 1). For example, small amounts of BiP and grp 94 were present in non-ER fractions (probably due to leakage of luminal contents during homogenization), whereas FKBP 13 fractionated more clearly into the ER fraction. This may indicate an association of FKBP 13 with some other protein from the ER (possibly a membrane protein) which, unlike BiP and grp 94, was not released from the ER during homogenization. Transient overexpression of the cDNA encoding FKBP 13, followed by immunofluorescence with specific antisera, revealed that FKBP 13 was distributed in a pattern almost identical to that of known ER proteins (Figure 3). Separation of the ER luminal contents from the membrane fraction indicated that FKBP 13 is a luminal protein of the ER (Figure 4). Based on these findings, it seems possible that the four C-terminal amino acids of FKBP 13, RTEL, function as a variant of the classical ER luminal retention tetrapeptide sequence, KDEL [21].

The strong homology (43 % sequence identity) between FKBP 12 and FKBP 13 suggests that they may share similar biological functions, although, apart from rotamase activity, details relating to the function of either protein are scant. In the context of: (a) its rotamase (peptidyl-prolyl isomerase) activity [1]; (b) the known effect of immunosuppressants on calcium-dependent signalling events in activated T cells [2]; (c) the reported association of FKBP 12 with the skeletal muscle sarcoplasmic reticulum ryanodine receptor [6]; (d) the reported co-localization of at least one immunophilin, cyclophilin B, with proteins believed to define an intracellular calcium pool [5]; and (e) evidence that mutation in the immunophilin-like gene product of *ninaA* in *Drosophila*, an ER protein, results in altered processing of rhodopsin through the secretory pathway [8], the localization of FKBP 13 to the lumen of the ER suggests at least two possibilities regarding function. Luminal proteins of the ER such as BiP have been shown to be involved in protein folding/chaperone-like activities and as yet incompletely defined 'quality control' mechanisms within the ER lumen [22]. Some of these functions may be calcium-dependent [23]. These proteins, along with calreticulin, have also been implicated in calcium storage within the ER lumen, the primary site of the InsP_3 -sensitive intracellular calcium pool [7]. Thus in future studies it will be very important to determine whether FKBP 13 is involved

in protein folding (particularly given its likely peptidyl-prolyl isomerase activity) or in calcium regulation within the ER lumen, or in both.

Although we did not detect an unambiguous change in the distribution of FKBP 13 in the presence of FK506, it is possible that the drug exerts part of its immunosuppressive effect by binding to FKBP 13 in the ER lumen, where processing and presentation of antigen in the immune system are thought to take place [24]. If this is so, and since there is no obvious mechanism by which an ER luminal protein such as FKBP 13 could interact with calcineurin, which is believed to be a cytosolic enzyme, proteins other than calcineurin may also play a role in the mechanism by which FK506 exerts its immunosuppressive effect.

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