s-Cyclophilin Is Retained Intracellularly Via a Unique COOH-Terminal Sequence and Colocalizes with the Calcium Storage Protein Calreticulin

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Abstract. Cyclophilins (cyclosporin A–binding proteins) are conserved, ubiquitous, and abundant proteins that accelerate the isomerization of XaaPro peptide bonds and the refolding of proteins in vitro. s-Cyclophilin is a member of the cyclophilin family with unique NH_2- and COOH-terminal extensions, and with a signal sequence. We now report that s-cyclophilin is retained in the cell, and that the conserved s-cyclophilin-specific COOH-terminal extension VEKPFAIAKE is sufficient to direct a secretory protein to s-cyclophilin containing structures.

Antibodies to s-cyclophilin-specific peptides were produced and the location of the protein was determined by an immunocytochemical study at the light microscopic level. s-Cyclophilin colocalized with the Ca^{2+}-binding protein calreticulin and, to a lesser extent, with the microsomal Ca^{2+}-ATPase in the myogenic cell line L6, and with the Ca^{2+}-binding protein calsequestrin in skeletal muscle. In activated platelets, s-cyclophilin immunoreactivity was detected in a ring-like structure that might correspond to the Ca^{2+}-storing and -releasing dense tubular network. In spreading cells, s-cyclophilin containing vesicular structures accumulated at actin-rich protrusion sites. While s-cyclophilin consistently codistributed with Ca^{2+} storage site markers, the distribution of s-cyclophilin immunoreactivity was not identical to that of ER markers.
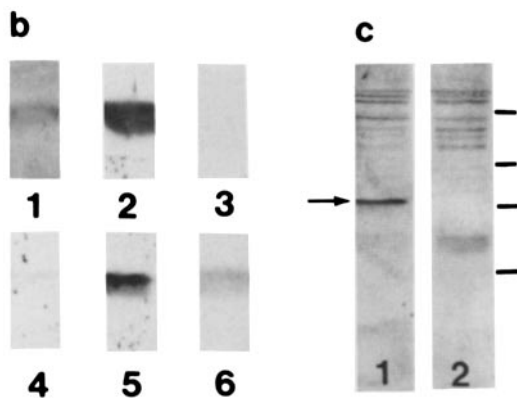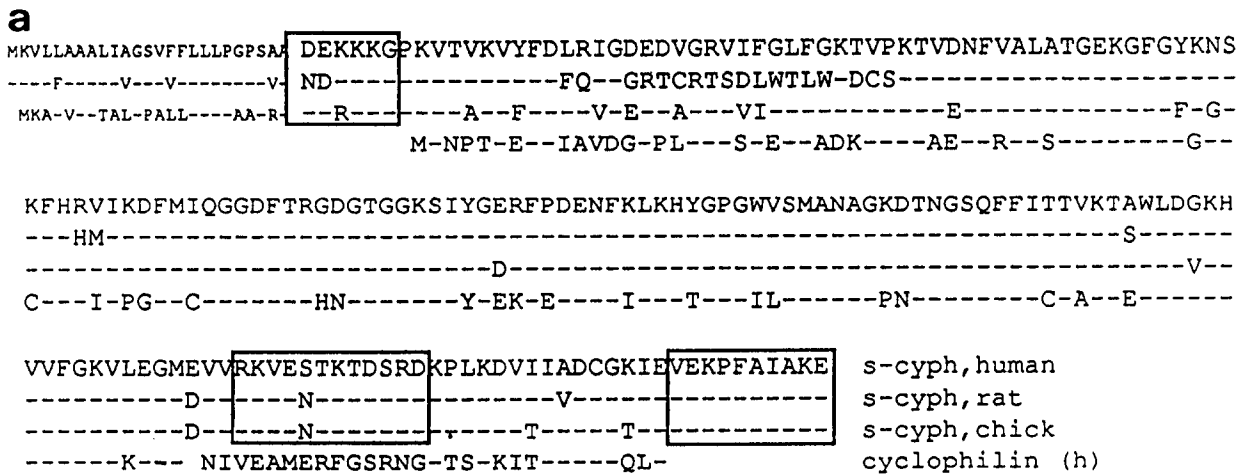
To determine whether the COOH-terminal extension of s-cyclophilin was involved in its intracellular transport we added this sequence to the COOH-terminus of the secretory protein glia-derived nexin. Appropriate constructs were expressed transiently in cultured cells and proteins were detected with specific antibodies. We found that glia-derived nexin with the COOH-terminal sequence VEKPFAIAKE (but not with the control sequence GLVVMNIT) colocalized with endogenous s-cyclophilin, indicating that the sequence contained retention information. These results indicate that s-cyclophilin is a retained component of an intracellular organelle and that it may accumulate in specialized portions of the ER, and possibly in calciosomes. Because of its conserved structure, widespread distribution, and abundance s-cyclophilin may be a useful marker to study the biogenesis and distribution of ER subcompartments.

CYCLOPHILIN is an ubiquitous, highly conserved, and abundant 18-kD cytosolic protein (Hohman and Hultsch, 1990; Stamnes and Zuker, 1990). In vitro, it greatly accelerates the isomerization of Xaa-Pro peptide bonds (Fischer et al., 1989; Takahashi et al., 1989). This observation led to the proposal that the peptidyl-prolyl *cis-trans* isomerase activity of cyclophilin may enable it to promote protein folding (Fischer and Schmid, 1990).

The function of cyclophilin in vivo is presently not clear, but indirect evidence suggests that it might be involved in Ca^{2+}-mediated signal transduction processes. Thus cyclophilin is a major cellular target of the immunosuppressant cyclosporin A (CsA)[1], a potent inhibitor of several Ca^{2+}-mediated responses (Hohman and Hultsch, 1990). CsA

blocks the enzymatic activity of cyclophilin. The best-studied action of CsA is its suppression of T-helper lymphocyte activation, leading to immunosuppression. In lymphocytes, CsA blocks the transcriptional activation of a subset of genes essential for the activation process. The affected genes are apparently all activated by signaling pathways involving Ca^{2+} (Trenn et al., 1989; Mattila et al., 1990). CsA is also a potent inhibitor of several regulated secretion processes, where it has been shown to interfere with Ca^{2+} signaling, rather than with the exocytosis process itself (Cirillo et al., 1990; Hultsch et al., 1990). Finally, CsA blocks the activation of a Ca^{2+}-conducting pore in mitochondria, probably because of its binding to a mitochondrial matrix-associated cyclophilin (Halestrap and Davidson, 1990; McGuiness et al., 1990). Interestingly, the cellular target of the structurally unrelated immunosuppressant FK-506 is a cytosolic protein that does not resemble cyclophilin in structure,

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1. *Abbreviations used in this paper*: CsA, cyclosporin A; GDN, glia-derived nexin; KLH, keyhole lympet hemocyanin.

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MKVLLAAALIAGSVFFLLLPGPSA DEKKKGPKVTVKVYFDLRIGDEDVGRVIFGLFGKTVPKTVDNFVALATGEKGFGYKNS
----F-----V---V--------V ND---------------FQ--GRTCRTSDLWTLW-DCS--------------------
MKA-V--TAL-PALL----AA-R- --R-------A--F----V-E--A---VI----------E------------F-G-
                             M-NPT-E--IAVDG-PL---S-E--ADK----AE--R--S--------G--

KFHRVIKDFMIQGGDFTRGDGTGGKSIYGERFPDENFKLKHYGPGWVSMANAGKDTNGSQFFITTVKTAWLDGKH
---HM----------------------------------------------------------------S------
-------------------------------D-----------------------------------------V--
C---I-PG--C-------HN-------Y-EK-E----I---T---IL------PN--------C-A--E------

VVFGKVLEGMEVV RKVESTKTDSRD KPLKDVIIADCGKIE VEKPFAIAKE    s-cyph,human
----------D-- ----N-------- --------V----- ----------    s-cyph,rat
----------D-- ----N-------- -+----T-----T-- ----------    s-cyph,chick
------K--- NIVEAMERFGSRNG-TS-KIT-----QL-                 cyclophilin (h)
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Figure 1. Specificity of antisera to s-cyclophilin specific peptides. (*a*) Comparison of human (Spik et al., 1991; Price et al., 1991), rat (Iwai and Inagami, 1990), and chick (Caroni et al., 1991) s-cyclophilin (*s-cyph*), and human cytosolic cyclophilin sequences. s-Cyclophilin specific sequences used for the production of antibodies are boxed. Signal peptide sequences are printed in small characters. The published rat s-cyclophilin sequence extends for 23 amino acids from the indicated COOH-terminal end. However, since it is almost identical to human and chick s-cyclophilin, and since the corresponding nucleotide sequence would possess a stop codon, but for a one-base insertion at nucleotide 684, we assumed that rat s-cyclophilin does stop at Glu_{208}. (*b*) Detection of s-cyclophilin with antisera to s-cyclophilin specific peptides. Purified recombinant signal peptide-free human s-cyclophilin (100 ng) was fractionated and blotted. Blots were then either stained with amido black to detect protein (lane *1*, in this case 1 μg) or reacted with antibodies to: (lane *2*) a mixture of the three uncoupled s-cyclophilin specific peptides; (lane *3*) non-immune mouse serum; (lane *4*) NH₂-terminal peptide; (lane *5*) internal peptide; (lane *6*) COOH-terminal peptide. Unless indicated, the peptides were coupled to KLH for immunization. Antisera were diluted 1:200, except in (lane *2*), where the antiserum to the mixture of uncoupled peptides was diluted 1:2,000. (*c*) Specific detection of s-cyclophilin with anti-peptide mixture antiserum. 100 μg of L6 cell homogenate protein were separated by SDS-PAGE, blotted, and reacted with 1:2,000 dilutions of either s-cyclophilin antiserum (lane *1*) or nonimmune mouse serum (lane *2*). A few major Coomassie-staining bands were detected with all sera, but only s-cyclophilin (*arrow*) was detected with the immune serum. Marks indicate migration position of 68, 31, 21, and 14-kD standards.

but that possesses similar peptidyl–prolyl isomerase activity (Standaert et al., 1990; Tropschug et al., 1990). FK-506 blocks the enzymatic activity of the FK-506-binding protein. Cytosolic cyclophilins with highly homologous structures have been found in eukaryotes from man to yeast, and in bacteria (Hohman and Hultsch, 1990; Stamnes and Zuker, 1990). In addition, a mitochondrial cyclophilin has been described in Neurospora crassa, where alternative splicing of a mitochondrial-localization sequence yields two cellular cyclophilins from one single gene (Tropschug et al., 1988). Additional genes coding for cyclophilins with a signal sequence have been described in yeast (Koser et al., 1990) and in Drosophila (Schneuwly et al., 1989; Shieh et al., 1989). While the yeast variant has not been further characterized, the Drosophila variant is encoded by the mutated gene in photoreceptor-deficient ninaA flies. NinaA is a tissue-specific, integral membrane cyclophilin-like protein required for the proper synthesis of a subset of Drosophila rhodopsins (Stamnes et al., 1991).

We have recently reported the identification of chick s-cy-

clophilin, a cyclophilin-like protein with an NH₂-terminal signal sequence (Caroni et al., 1991). Cytosolic cyclophilin and s-cyclophilin are transcribed from separate genes in the chick genome, and s-cyclophilin mRNA was ubiquitous and abundant throughout chick development. When overexpressed with the baculovirus system, a significant proportion of the recombinant s-cyclophilin was secreted by infected insect cells (Caroni et al., 1991). Almost identical proteins were also identified in man (Spik et al., 1991; Price et al., 1991; Hasel et al., 1991), mouse (Hasel et al., 1991), and rat (Iwai and Inagami, 1990). Like cytosolic cyclophilin, human s-cyclophilin possesses peptidyl–prolyl *cis–trans* isomerase activity, and binds CsA with high affinity, indicating that the two proteins are also functionally related (Spik et al., 1991; Price et al., 1991; Hasel et al., 1991). The sequences of human, mouse, rat, and chick s-cyclophilin are almost identical in regions where these secretory forms differ in sequence from the cytosolic forms. There are three conserved s-cyclophilin specific sequences: (*a*) a seven amino acid large highly charged NH₂-terminal extension; (*b*) a 10-residue
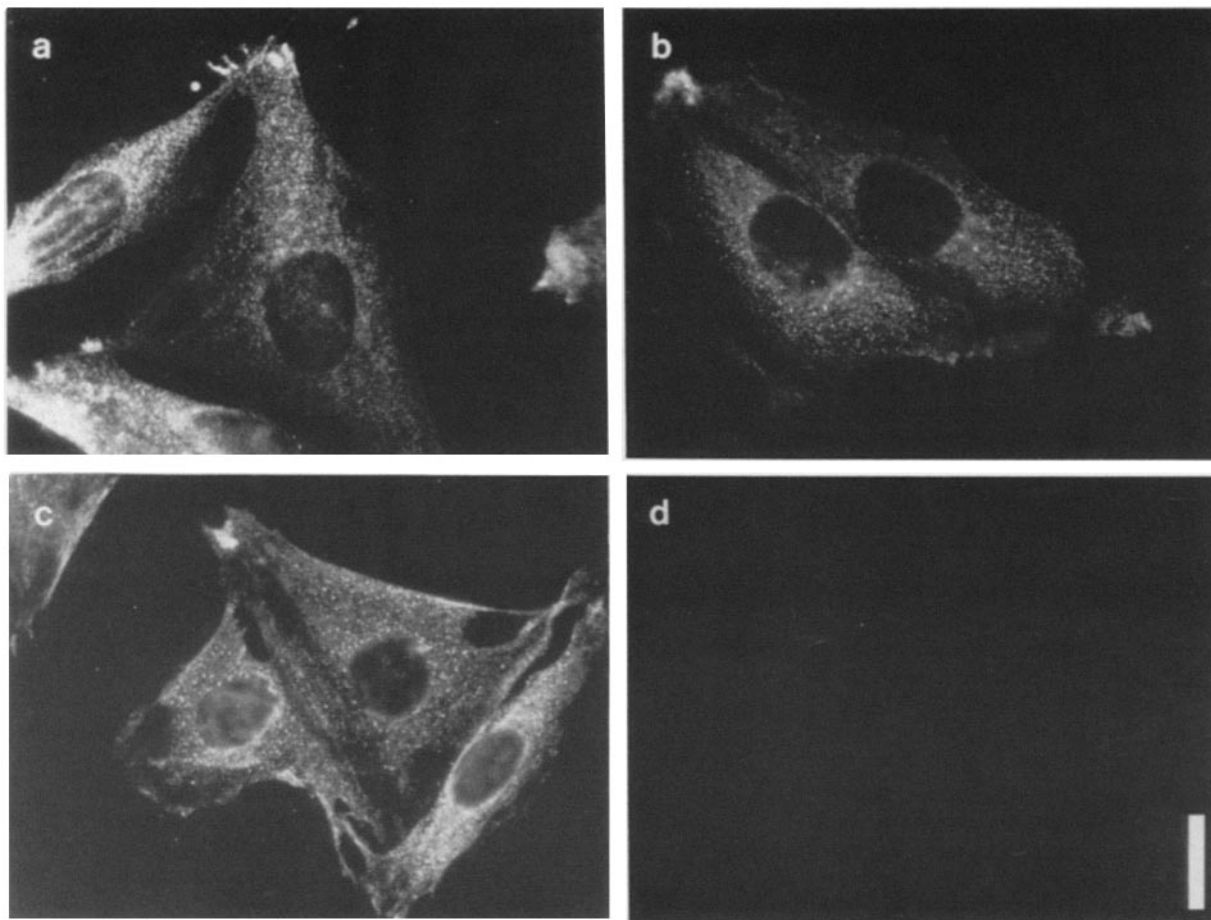
Figure 2. Antibodies to non-overlapping s-cyclophilin specific peptides detect similar intracellular vesicular structures. Permeabilized L6 cells were stained with antisera to the following antigens: (*a*) KLH-coupled NH2-terminal peptide; (*b*) KLH-coupled COOH-terminal peptide; (*c*) uncoupled peptide mixture (all three peptides); (*d*) non-immune mouse serum. Bar, 15 μm.

stretch of high surface probability in the COOH-terminal half of the shared cyclophilin sequence; and (*c*) a 10-residue COOH-terminal extension. The COOH-terminal extensions are identical in chick, mouse, rat, and human s-cyclophilin but do not resemble the long COOH-terminal extension that carries the hydrophobic sequence responsible for membrane anchoring of the ninaA gene product.

In the present study we have localized s-cyclophilin in the cell and identified sequences important for localization. s-Cyclophilin is an intracellularly retained protein, and it colocalizes with calreticulin (Krause et al., 1989), and to a lesser extent with microsomal Ca^{2+}-ATPase and SR calsequestrin, i.e., with calcium-binding ER and calciosomal proteins (Hashimoto et al., 1988; Volpe et al., 1988; Meldolesi et al., 1990). On the other hand, s-cyclophilin and ER antibodies (Louvard et al., 1982; Bole et al., 1986) yielded different labeling patterns. The unique COOH-terminal extension of s-cyclophilin is necessary to direct it to its intracellular location and is sufficient to efficiently sort a secretory protein to the same intracellular compartment.

Materials and Methods

Reagents and Cells

Cycloheximide, tunicamycin, and lucifer yellow-CH were from Fluka (Buchs, Switzerland). Calcium-ionophore A23187 was from Sigma Chem-

ical Co. (St. Louis, MO). TRITC–phalloidin was a gift of Dr. K. Ballmer (Friedrich Miescher Institute, Basel, Switzerland). CsA (stock solution of 20 mg/ml in ethanol) was a kind gift of Dr. S. Alkan (Ciba-Geigy, Basel, Switzerland). Oligonucleotides were synthesized using a 308B DNA synthesizer (Applied Biosystems Inc., Foster City, CA). The glia-derived nexin (GDN) full-length clone was a kind gift of Dr. D. Monard (Friedrich Miescher Institute, Basel, Switzerland). Signal peptide-free recombinant human s-cyclophilin (Ala25 to Glu208) was expressed and purified from *Escherichia coli* according to standard techniques (Hasan and Szybalski, 1987).

Rabbit antiserum to ER membrane proteins (Louvard et al., 1982) was a gift of Dr. D. Louvard (Pasteur Institute, Paris, France). mAb to Gl/93 antigen (Schweizer et al., 1988) was a gift of Dr. H.-P. Hauri (Biocenter, University of Basel, Switzerland). Rabbit antiserum to chick calsequestrin (Choi and Clegg, 1990) was a gift of Dr. D. Clegg, (University of California, Santa Barbara, CA). Chicken antiserum to rat GDN (Halfter et al., 1989) was a gift of Dr. D. Monard (Friedrich Miescher Institute, Basel, Switzerland). Rabbit antiserum to peptide GLVVMNIT (internal sequence of the extracellular matrix protein tenascin) detected this peptide only when it was located at the COOH-terminal end of proteins, and was a gift of Dr. R. Chiquet-Ehrismann (Friedrich Miescher Institute). Antibodies against human calreticulin and human Ca^{2+}-ATPase were produced by immunization of rabbits with synthetic peptides coupled to keyhole limpet hemocyanin (KLH) (Sigma Chemical Co.). The 20 NH2-terminal amino acids were deduced from the cDNA sequence (Ca^{2+}-ATPase) or from protein sequence and cDNA cloning (calreticulin). The antibodies used in this study specifically detected their respective antigens on Western blots. FITC- and TRITC-coupled goat anti–mouse, and goat anti–rabbit were from Milan Analytica (LaRoche, Switzerland). TRITC-coupled goat anti–chicken IgG was from Jackson Immuno Research Laboratories Inc. (Avendale, PA).

Cell lines were cultivated in DME with 10% FCS. Monkey kidney epithelial cells (COS-7), and rat myogenic L6 cells were from American Type
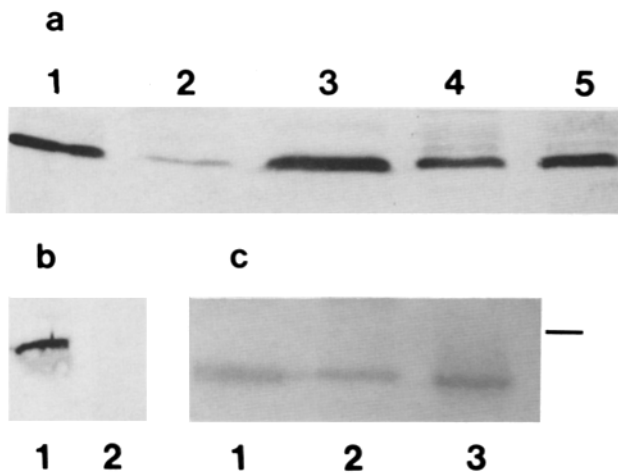
Figure 3. Distribution of s-cyclophilin in crude subcellular fractions. s-Cyclophilin was detected on Western blots with antiserum to the uncoupled peptide mixture. (*a*) Rat liver fractions. 100 μg of the following crude protein fractions were applied to the gel: (lane *1*) homogenate; (lane *2*) high-speed supernatant; (lane *3*) microsomes; (lane *4*) mitochondria; (lane *5*) nuclei. s-Cyclophilin was enriched in the microsomal fraction. (*b*) Bovine anterior pituitary membrane fractions. 100 μg of crude low-density membranes (lane *1*), and of purified secretory granules (lane *2*) were applied to the gel. s-Cyclophilin was not detected in secretory granules. (*c*) Microsome associated s-cyclophilin comigrates with mature recombinant human s-cyclophilin. Samples contained 50 μg of rat liver microsome protein plus 100 ng of pure recombinant s-cyclophilin (lane *1*), 50 μg of liver microsomes (lane *2*), and 100 ng of pure recombinant s-cyclophilin (lane *3*). The estimated migration position of recombinant s-cyclophilin with signal peptide (plus 2.7 kD is indicated by the bar.

Cell Culture (Rockville, MD). Human platelets were cultivated in 137 mM NaCl, 2.6 mM MgCl$_2$, 5.5 mM glucose, 15 mM Hepes, pH 7.4 (10^8 platelets/ml), activated by substrate contact, and examined 30 min after activation.

Production of Antibodies to s-Cyclophilin

The following s-cyclophilin specific peptides were synthesized and purified: DERKKGPKVTAK-Y (NH$_2$-terminal extension after signal peptide cleavage), RKVENTKTDSRD-Y (internal sequence of high surface probability), Y-TIEVEKPFAIAKE (COOH-terminal extension). The peptides were separately coupled to KLH with bis-diazo-benzidine (2 h, 4°C reactions; 2 mg/ml of KLH, 0.1 mg/ml of peptide) (Doolittle, 1986). 6-wk-old BALB/c mice were immunized either with 100 μg of each KLH-coupled peptide separately, or with 200 μg of an equimolar mixture of the three uncoupled peptides. The first i.p. injection in complete Freund's adjuvant was followed by boosts after 3 and after 5 wk (100 μg of antigen in incomplete Freund adjuvant). Sera were collected and tested 5 d after the second boost. Initially, antisera were tested by ELISA and on Western blots with BSA (Sigma)-coupled peptide as the antigen. Positive antibodies were further tested for detection of non-denatured purified recombinant human s-cyclophilin on ELISA, and for the specific detection of s-cyclophilin on Western blots of recombinant baculovirus infected Sf9 cell homogenate, and of rat myogenic L6 cell homogenate. All four immunization protocols produced antibodies that satisfied these criteria. The best antibodies for Western blots were obtained with the uncoupled peptide mixture as antigen, whereas the best antibodies for immunocytochemistry experiments were against the internal peptide coupled to KLH.

Immunocytochemistry

Chick tissues were fixed overnight at 4°C in 4% paraformaldehyde in PBS, cryoprotected by a 4–6-h incubation in PBS with 20% sucrose at 4°C, cut in 12-μm sections on a cryostat (Carl Zeiss, Zurich, Switzerland), and sections were collected on subbed cover slides. All further incubations were

carried out at room temperature. Non-specific-binding sites were blocked by a 30-min incubation in PBS with 5% BSA and 0.1% NP-40 (Sigma Chemical Co.), and further incubations were as described for tissue culture cells. Cultured cells were fixed for 30 min at room temperature in freshly prepared 4% paraformaldehyde in PBS. Fixative was then removed, cells were rinsed in PBS, and permeabilized by incubating for 10 min in PBS with 0.1% NP-40. Non-specific-binding sites were blocked by a 30-min incubation in PBS with 5% BSA. Subsequent procedures were carried out in a basic medium consisting of PBS with 5% BSA. Cells were treated with 1:200–1:500 dilutions of anti-s-cyclophilin antibodies, with or without additional antibodies as indicated. Unbound antibodies were then removed, and cells were exposed to 1:100 dilutions of the appropriate fluorescent second antibodies (alone or in combination, as indicated). After extensive washing, cultures were mounted in Gelvatol (Monsanto, St. Louis, MO), and fluorescence was detected with an Axiovert-10 microscope (Carl Zeiss) equipped with the appropriate filters for independent detection of FITC and of TRITC. Variant protocols included fixation and permeabilization in methanol/acetone (1:1) for 3 min at room temperature, or the inclusion of 0.1% saponin in all incubation buffers. Control experiments with appropriate non-immune antisera were routinely performed to verify the specificity of the signals. For double-labeling experiments, appropriate control experiments were performed to verify that specific independent labeling was detected.

To detect endocytotic compartments, cells were incubated for periods of 5–60 min in 10 mg of lucifer yellow per ml of culture medium (Swanson et al., 1985; Brown et al., 1986), washed on ice, fixed, and processed for immunocytochemistry as described above. F-actin was detected with FITC-phalloidin (Sigma Chemical Co.). N-linked glycosylation was blocked by the addition of 0.5 μg/ml of tunicamycin to the culture medium for a period of 18 h. For protein synthesis blocking experiments, 0.1 μg/ml of cycloheximide were added to the culture medium; cells were incubated in inhibitor containing medium for periods of up to 6 h, and then processed for immunocytochemistry as described above. In temperature block experiments, cells were incubated at 20°C for 3 h, and then fixed and labeled as described above. Where indicated 7 μM of A23187 was included in the culture medium for a period of 3 h before fixation.

Transfection Experiments

Mutant s-cyclophilin and GDN sequences were produced by the polymerase chain reaction, with appropriate primers. Briefly, homologous five prime end sense primers were used in combination with partially overlapping mutant three prime antisense primers in three consecutive reactions that progressively produced the complete, mutated three prime sequence. Products were cloned into appropriate vectors for double-stranded sequencing with SP6 and T7 primers, using the chain termination method and the Sequenase 2 kit (United States Biochemicals Corp., Cleveland, OH). Inserts with the mutated sequence were cloned directionally into the eukaryotic expression vector pECE (Ellis et al., 1986), plasmid DNA was isolated, and transfected into L6 or COS-7 cells with the calcium–phosphate precipitation method (Chen and Okayama, 1987). Cells were exposed to a glycerol shock 16 h after transfection, fixed 48 h after transfection, and finally processed for immunocytochemistry as described above. Transfection efficiencies ranged from 1–5%.

Other Methods

Rat liver subcellular fractions were isolated by a procedure that had been specifically devised to yield Ca^{2+} transport competent microsomes (Dawson, 1982). Bovine adrenal medulla derived secretory granules were prepared as described (Schweizer et al., 1989).

SDS-PAGE fractionation and Western blot analysis of protein containing fractions were performed according to standard methods. Bound antibody was detected with alkaline phosphatase-coupled goat anti-mouse (Milan Analytica, LaRoche, Switzerland).

Protein amounts were determined by the filter binding assay (Schaffner and Weissman, 1973), with BSA (Sigma Chemical Co.) as a standard.

Results

s-Cyclophilin Is an Ubiquitous Intracellularly Retained Protein: Colocalization with the ER and Calciosomal Protein Calreticulin

s-Cyclophilin possesses a classical signal sequence and is

secreted from cells (Caroni et al., 1991; Spik et al., 1991). The NH₂-terminal end of s-cyclophilin isolated from human milk begins at Ala₂₅, indicating that the signal sequence of s-cyclophilin is functional (Spik et al., 1991). We noticed however, that even in insect cells that had been infected with recombinant baculovirus and that were therefore massively overexpressing s-cyclophilin, only ~60% of the newly synthesized protein was secreted, and that most of that secretion had taken place within the first 30 min after synthesis (Caroni et al., 1991). This suggested to us that a significant proportion of the protein was removed from the export pathway, indicating that the prevalent location of s-cyclophilin might be intracellular.

To determine the cellular location of s-cyclophilin we produced antibodies to conserved, nonoverlapping s-cyclophilin-specific peptides (Fig. 1 a). As shown in Fig. 1 a, the peptides did not overlap with cytosolic cyclophilin sequences, thus excluding crossreactivity of the antibodies with this related protein. Antisera to three different peptides all bound s-cyclophilin on Western blots (Fig. 1 b). The NH₂-terminal peptide produced weak antibodies for immunoblot experiments (Fig. 1 b, lane 4) and for ELISA, but importantly, all antisera detected vesicle-like structures of similar distribution in permeabilized L6 myogenic cells (Fig. 2, a and b). An antiserum produced against a mixture of the three uncoupled peptides yielded similar results (Fig. 2 c), demonstrating that the antibodies did not detect a shared epitope that had been produced in the process of coupling the peptides to KLH. The peptide mixture antiserum gave the strongest signals on immunoblots and was therefore used for most such experiments. The specific detection of s-cyclophilin in L6 cell homogenates (immunoblot data, Fig. 1 c) and the essential identity of the immunocytochemical results obtained with all antibodies to s-cyclophilin peptides (Fig. 2, see also Fig. 4) led us to conclude that the antibodies specifically bind s-cyclophilin in the immunocytochemical experiments.

The experiments shown in Fig. 2 did not reveal a Golgi-like pattern of immunoreactivity, and therefore suggested that a significant proportion of s-cyclophilin might be retained in intracellular vesicular structures. To obtain an estimate of s-cyclophilin contents in subcellular fractions, crude fractions of nuclei, mitochondria, microsomes, and of soluble proteins were prepared from rat liver homogenate. As shown in Fig. 3 a, while s-cyclophilin was recovered in all crude membrane fractions, this protein was enriched in the microsomal fraction. On the other hand, low levels of s-cyclophilin were detected in a non-membrane-associated soluble protein fraction. Also shown in Fig. 3 are Western blot data of adrenal medulla derived membrane fractions: high levels of s-cyclophilin were detected in a low density membrane fraction but no s-cyclophilin was found associated with secretory granules (Fig. 3 b). In a recent study, Sutcliffe and colleagues (Hasel et al., 1991) have determined the association of s-cyclophilin (called cyclophilin-2 in that study) with purified subcellular fractions by immunoblot analysis. These authors could demonstrate that s-cyclophilin is nearly exclusively associated with ER-enriched fractions. Our results indicate that tissue s-cyclophilin tends to codistribute with low-density membrane-bound structures. That s-cyclophilin was actually located inside these structures in situ is indicated by the Western blot data shown in Fig. 3 c: micro-

some-associated rat s-cyclophilin and recombinant human s-cyclophilin that had been expressed in E. coli as a signal peptide-free protein had very similar apparent molecular weight (difference of less than 0.4 kD, i.e., much less than the 2.7 kD difference predicted for the signal peptide of s-cyclophilin). E. coli–derived recombinant s-cyclophilin was not glycosylated, and the apparent molecular weight of L6 cell s-cyclophilin was not affected by the inhibitor of N-linked glycosylation tunicamycin (data not shown). Therefore, microsome-associated s-cyclophilin has lost its signal sequence, and hence reached the compartment after translocation across the ER membrane.

To localize s-cyclophilin-containing membrane-bound structures in the cell, we performed double-labeling experiments at the light microscopic level. The endocytotic pathway was labeled by incubating cells in a lucifer yellow derivative, which is a marker for bulk phase endocytosis (Swanson et al. 1985; Brown et al., 1986). Cells were then fixed and reacted with s-cyclophilin specific antibody. No overlap between the early endocytotic pathway (5-min uptake) and s-cyclophilin positive structures could be detected (Fig. 4 a). Negative results were also obtained when later endocytotic structures were visualized by the lucifer yellow method (data not shown). We conclude, therefore, that s-cyclophilin is not sorted to components of the endocytotic pathway, and that it does not accumulate in cells by reentering these after secretion. When cells were treated with the inhibitor of protein synthesis cycloheximide, s-cyclophilin immunoreactivity only declined very slowly (see Fig. 4 c; little change was observed after 6 h, data not shown), indicating that this protein has a long half-life in the cell, and suggesting that most of it does not reach the constitutive secretory pathway. This interpretation was also supported by temperature block experiments, where exit of proteins from the trans-Golgi was inhibited by incubating the cells at 20°C for 3 h: as shown in Fig. 4 d, the distribution of s-cyclophilin was apparently not affected by the temperature block, indicating that it did not pass the trans-Golgi compartment to reach its intracellular location.

We then compared the distribution of s-cyclophilin with that of the ER: for this purpose, we used an antiserum directed against ER membrane antigens (Louvard et al., 1982). As shown in Fig. 4, e–h, a pattern resembling that of s-cyclophilin was obtained. However, the typical reticular structure obtained with the ER antiserum was never observed with s-cyclophilin antibodies. In addition, while the overall distribution of ER and s-cyclophilin immunoreactivity were comparable, s-cyclophilin-containing vesicular structures frequently did not overlap with ER signal, and occasional peripheral reticular structures did not overlap with s-cyclophilin "spots". Similar results were obtained when the ER was visualized with a mAb against the soluble lumenal ER protein, BiP (Bole et al., 1986) (data not shown). In contrast, we found that s-cyclophilin and the calcium storage protein calreticulin (Smith and Koch, 1989; Treves et al., 1990; Milner et al., 1991) had very similar distributions (Fig. 5 a–f). Both calreticulin- and s-cyclophilin-containing structures accumulated in the vicinity of the nucleus in the presence of the microtubule depolimerizing drug nocodazole (3 h of treatment, Fig. 5, c and d), and were little affected by the calcium ionophore A23187 (3 h of treatment, Fig. 5, e and f). The localization of s-cyclophilin was also compared
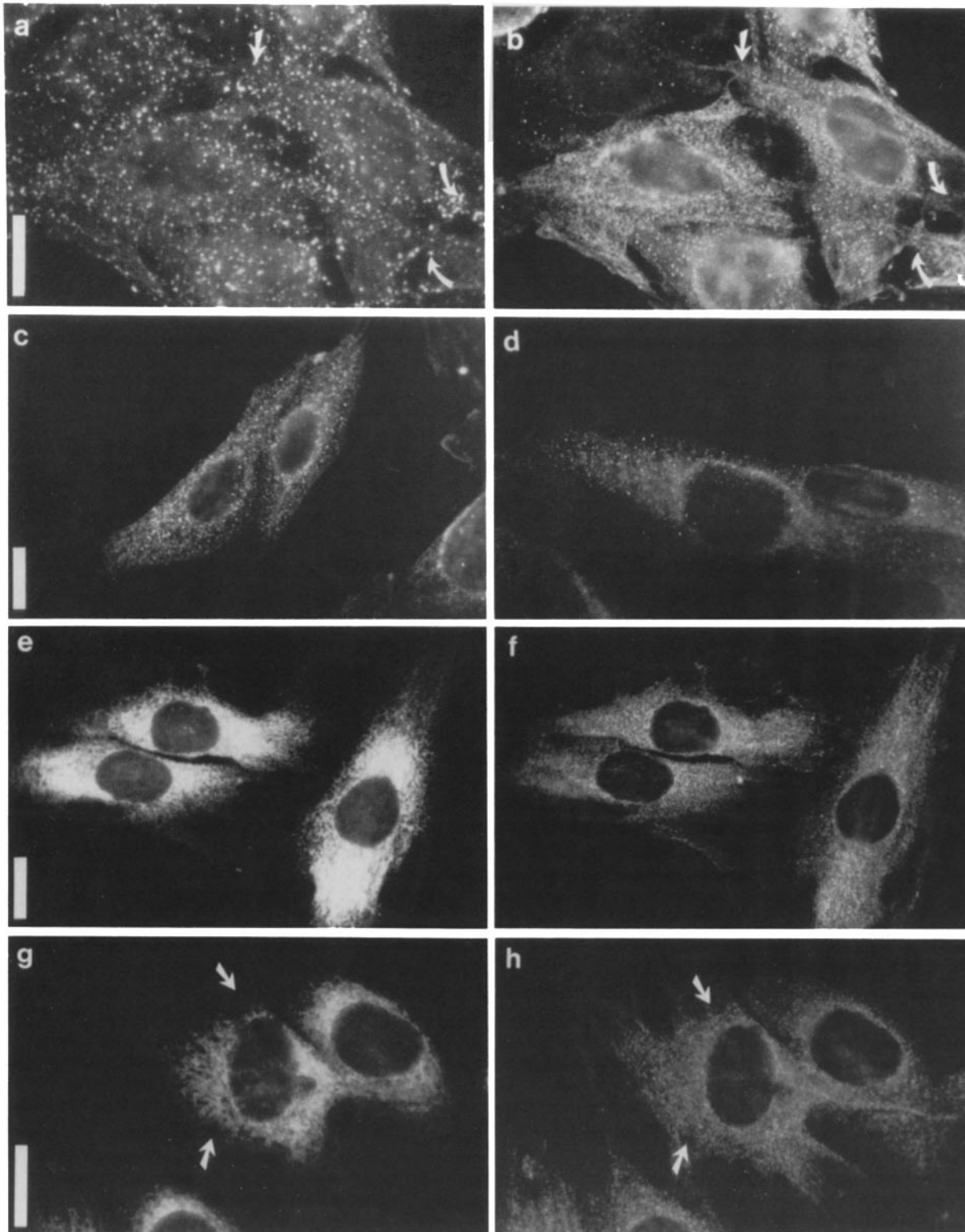
Figure 4. s-Cyclophilin is not detected in early endocytotic compartments, nor does it codistribute with ER antigens. (*a* and *b*) s-Cyclophilin is not detected in early endocytotic compartments. Cells were exposed to lucifer yellow for 5 min, washed, fixed, and reacted with s-cyclophilin antibody (internal peptide). Double-labeling experiment: (*a*) lucifer yellow; (*b*) s-cyclophilin detected with TRITC-anti–mouse. Three endosomes and the corresponding position on the s-cyclophilin micrograph are indicated by the arrows. (*c* and *d*) The distribution of s-cyclophilin immunoreactivity is not affected by a 2-h incubation in the presence of the inhibitor of protein synthesis cycloheximide (*c*), nor by a 3-h incubation at 20°C (*d*). s-Cyclophilin was detected with the internal peptide antiserum in (*c*), and with the uncoupled peptide mixture antiserum in *d*. (*e–h*) Double-labeling experiments to compare the distribution of the ER (*e* and *g*) to that of s-cyclophilin containing vesicle-like structures (*f* and *h*). ER was visualized with an established antiserum, and s-cyclophilin was detected with the internal peptide antiserum. To facilitate comparison of the two distributions, arrows indicate corresponding positions in (*g* and *h*). All experiments were performed in L6 cells. Bars: 12 μm.

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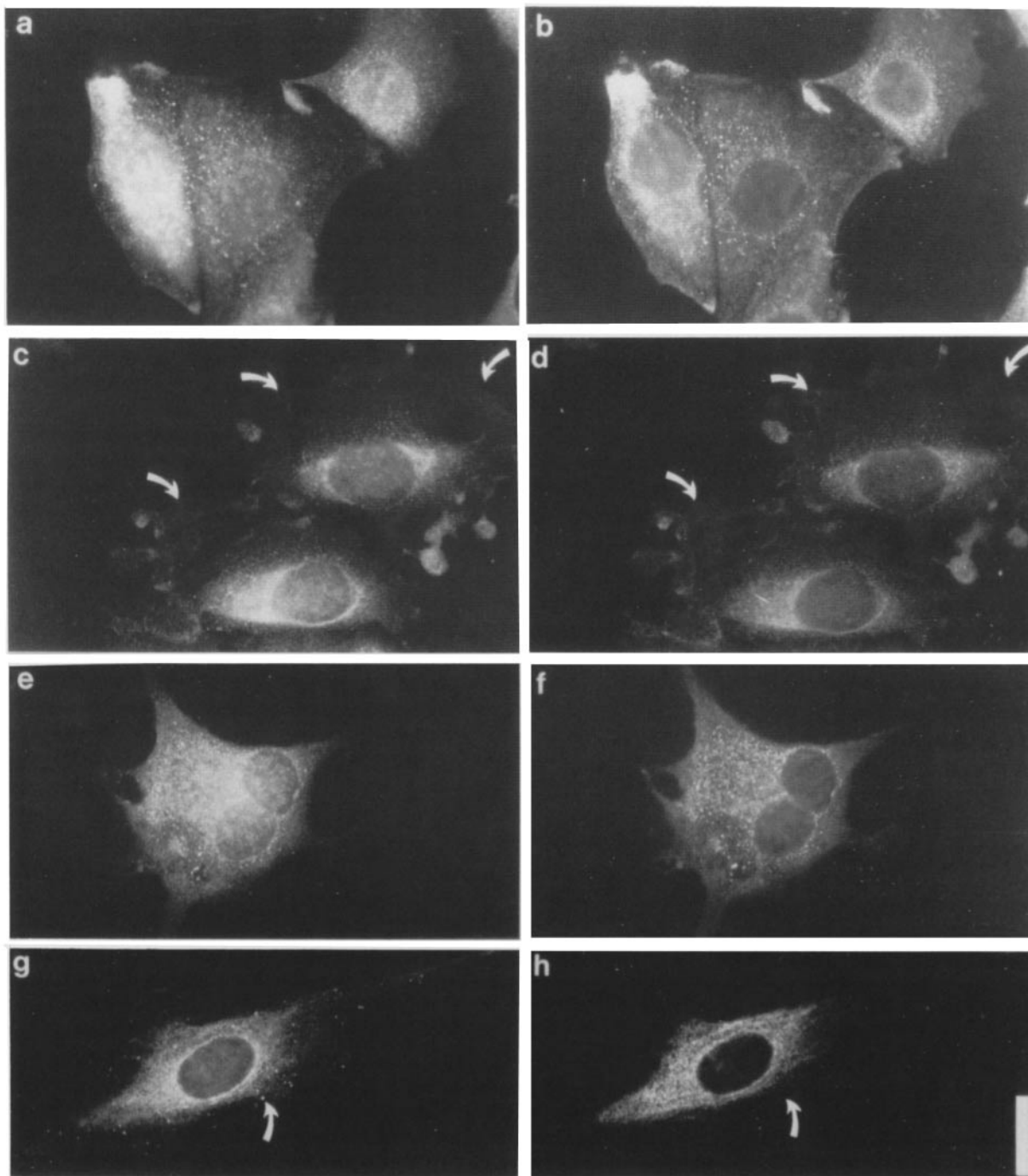
Figure 5. s-Cyclophilin codistributes with the ER calcium-binding proteins calreticulin and Ca²⁺-ATPase. (*a–f*) Codistribution of calreticulin (*a, c,* and *e*) and of s-cyclophilin (*b, d,* and *f*) in L6 cells. Double-labeling experiments were performed on untreated control cells (*a* and *b*), on cells that had been exposed to the microtubule depolimerizing drug nocodazole for 3 h (*c* and *d*), and on cells that had been exposed to the calcium ionophore A23187 for 3 h (*e* and *f*). Arrows in (*c* and *d*) indicate the edge of the cells: in the presence of nocodazole, calreticulin, and s-cyclophilin immunoreactivity was concentrated in the vicinity of the nucleus. (*g* and *h*): Ca²⁺-ATPase (*g*) and s-cyclophilin (*h*) in L6 cells. Arrows point to a vesicle-like structure that was not detected with the s-cyclophilin antibody. Bars: 15 μm.

to that of microsomal Ca²⁺-ATPase (Campbell, 1986; Burgoyne et al., 1989; Kaprialian et al., 1989). As shown in Fig. 5, *g* and *h*, the labeling pattern with the Ca²⁺-ATPase antibody was spotted and the two proteins had similar distributions. However, calcium-ATPase and s-cyclophilin spots frequently did not overlap, suggesting that the two proteins may colocalize only partially. A similar conclusion was

reached when the distributions of s-cyclophilin and of the sarcoplasmic reticulum cisternae-associated Ca²⁺-binding protein calsequestrin (Jorgensen et al., 1983; Campbell, 1986; Fliegel et al., 1987; Choi and Clegg, 1991) were compared (see Fig. 7, *f* and *g*): similar labeling patterns were obtained, but prominent spots of calsequestrin immunoreactivity were not emphasized by the s-cyclophilin antibody. We
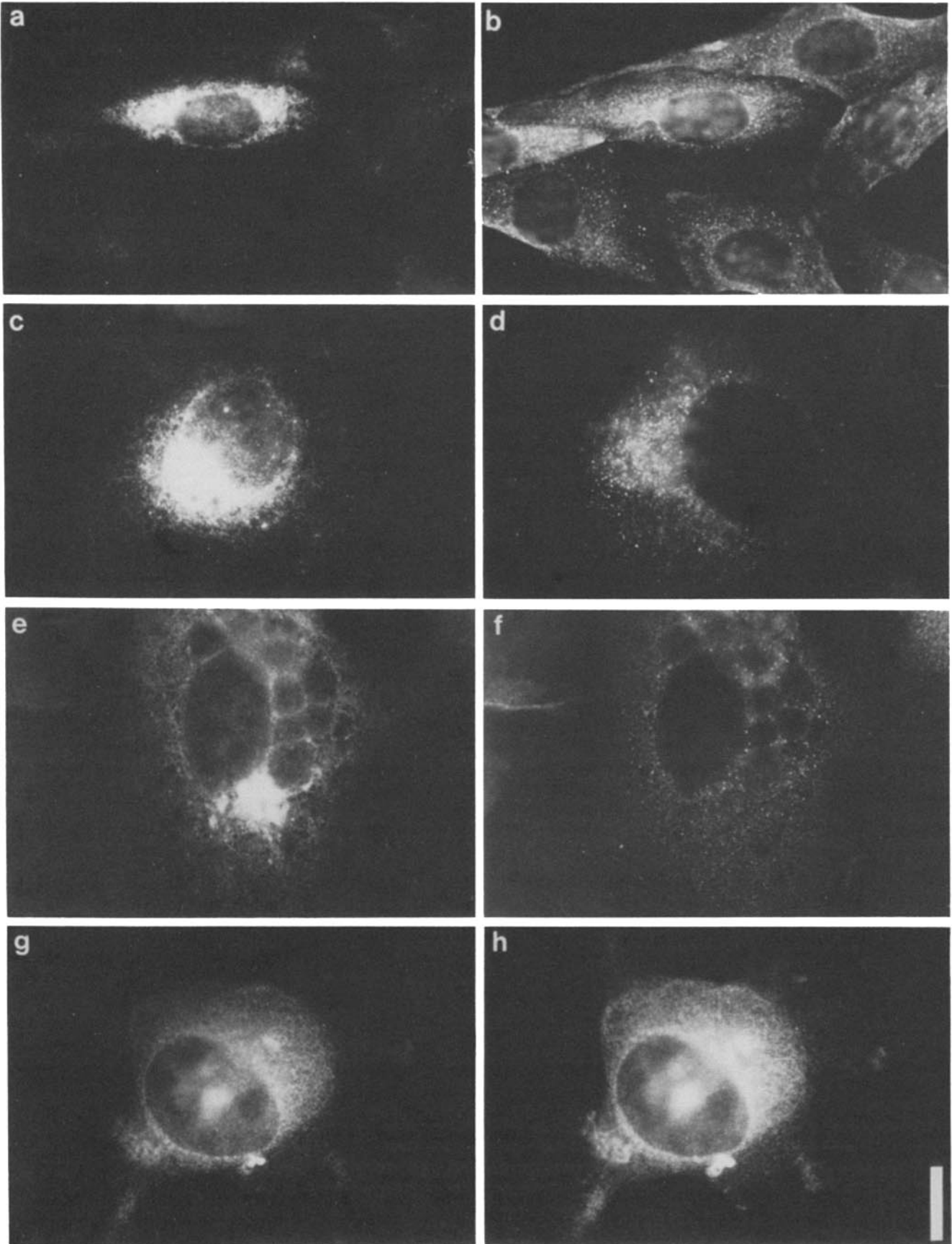
therefore concluded that s-cyclophilin is an ER protein, where its distribution resembles most closely that of the calcium-binding protein calreticulin.

The Conserved s-Cyclophilin-specific COOH-Terminal Amino Acid Extension Contains Intracellular Localization Information

The soluble ER protein calreticulin possesses the COOH-terminal ER retention sequence KDEL (Munro and Pelham, 1987; Pelham, 1989); this sequence is not found in the soluble protein s-cyclophilin which shows a similar distribution. On the other hand, s-cyclophilins in man, mouse, rat, and chick possess identical COOH-terminal extensions VEKPFAIAKE, extending from the highly conserved stop signal of the cytosolic cyclophilins (Fig. 1 a). In addition, the membrane bound cyclophilin-like protein encoded by the ninaA gene in Drosophila possesses a (different) COOH-terminal extension that includes a hydrophobic membrane anchoring sequence (Stamnes et al., 1991). We therefore performed experiments to determine whether the COOH-terminal extension of s-cyclophilin was required for its intracellular location and, if so, whether it could affect the intracellular transport of a secretory protein. In a first series of experiments we replaced the wild-type COOH-terminal sequence of s-cyclophilin with the sequence GLVVMNIT, to which a specific antibody was available. This allowed us to detect mutant (anti-GLVVMNIT antibody) and wild-type s-cyclophilin (antibody to s-cyclophilin COOH-terminal peptide) independently from each other. When the mutant s-cyclophilin (s-cyclophilin-GLVVMNIT) was expressed in L6 cells, it was retained in the reticular structures characteristic of the ER, possibly because it failed to fold properly (Pelham, 1989; Rothman, 1989) (Fig. 6, a and b). However, when the fate of newly synthesized s-cyclophilin-GLVVMNIT was visualized by the addition of cycloheximide to the culture medium to block further protein synthesis, a Golgi-like pattern of immunoreactivity tended to appear, suggesting that s-cyclophilin-GLVVMNIT did eventually reach the secretory pathway (data not shown). At no time, and irrespective of whether the cells were treated or not with cycloheximide, did s-cyclophilin-GLVVMNIT colocalize with wild-type s-cyclophilin.

We then determined whether the sequence VEKPFAIAKE could by itself affect the sorting of a soluble secretory protein by transferring it to the COOH terminus of GDN, a potent inhibitor of serine proteases with neurite promoting activity (Guenther et al., 1985). GDN was selected because it was known from cristal structure data that its COOH terminus was probably exposed to the surface of the protein, where it did not appear to interact with other residues, implying

that it might not be essential for proper folding of GDN in the ER. For these experiments, monkey kidney epithelial (COS-7) cells had to be transfected because L6 cells contained relatively high levels of endogenous GDN, thus complicating the specific detection of recombinant protein. When GDN-GLVVMNIT was transfected into GDN-negative COS-7 cells, the intracellular recombinant protein was mainly detected in the Golgi area (Fig. 6 c), indicating that, like wild type GDN, it displayed the typical behavior of a secretory protein (Halfter et al., 1989). A substantial amount of GDN-GLVVMNIT was detected in reticular structures (Fig. 6 c), possibly reflecting slow exit of mutant protease inhibitor from the ER. Importantly, GDN-GLVVMNIT did not codistribute with endogenous s-cyclophilin (Fig. 6, e and f). Therefore, GDN-GLVVMNIT appeared to behave like a less efficiently folding, but otherwise unaltered GDN. A very different result was obtained when GDN-VEKPFAIAKE was transfected into COS-7 cells. In this case, no reticular ER and no Golgi pattern were observed: instead, the mutant protein was localized to vesicular structures that tended to accumulate in the vicinity of the nucleus, but could also be detected in the periphery of the cell (Fig. 6 d). When these cells were counterstained for s-cyclophilin with the internal peptide detecting antibody, thereby excluding any crossreactivity with GDN-VEKPFAIAKE, a good correspondence of the signals was observed (Fig. 6, g and h). Therefore, the COOH terminus sequence VEKPFAIAKE was sufficient to drastically alter the intracellular transport of GDN, directing the mutant protein to s-cyclophilin containing structures.

s-Cyclophilin in ER-free Structures: Platelets and Pseudopodia of Spreading Cells

We have evaluated the distribution range of s-cyclophilin by performing Western blot analysis of chicken, rat, and bovine tissues, and by immunocytochemistry of frozen sections of chick tissue, and of various human, monkey, rat, and chicken-derived cells and cell lines. These experiments led us to conclude that s-cyclophilin is a fairly abundant, conserved and ubiquitous protein (data in part not shown). Importantly, antibodies to non-overlapping s-cyclophilin-specific peptides detected a protein of same apparent molecular weight and/or localization. Therefore, anti-s-cyclophilin antibodies are valuable reagents to study the distribution of ER components in many, and possibly in all types of mammalian and bird cells (lower vertebrates were not tested).

Because CsA was consistently found to interfere with processes involving calcium, and because s-cyclophilin colocalized with ER Ca^{2+}-binding proteins, we analyzed s-cyclophilin immunoreactivity in cellular structures known to be sites of elevated calcium accumulation and release, i.e.,

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Figure 6. The s-cyclophilin specific COOH-terminal sequence VEKPFAIAKE can direct a secretory protein to s-cyclophilin containing vesicular structures. Proteins with mutated COOH-terminal sequences were expressed transiently in L6 (a and b) and in COS-7 (c–h) cells. (a and b) s-Cyclophilin-GLVVMNIT transfection: double labeling for recombinant mutated protein with anti-GLVVMNIT antiserum (a), and for endogenous wild-type protein with internal peptide antiserum (b). s-Cyclophilin-GLVVMNIT accumulated in the ER. (c and d) Comparison of the distributions of GDN-GLVVMNIT (c) and of GDN-VEKPFAIAKE (d). In both experiments cells were labeled with the anti-GDN antibody. (e and f) Double labeling of GDN-GLVVMNIT transfected COS-7 cell. (e) Anti-GLVVMNIT antibody to detect recombinant GDN; (f) anti-s-cyclophilin antibody to detect endogenous s-cyclophilin; (g and h) Double labeling of GDN-VEKPFAIAKE transfected COS-7 cell; (g) anti-GDN antibody to detect recombinant GDN; (h) anti-s-cyclophilin antibody (internal peptide) to detect endogenous s-cyclophilin. GDN-GLVVMNIT was localized to the Golgi and the ER, whereas GDN-VEKPFAIAKE was detected in vesicular structures that codistributed with endogenous s-cyclophilin (see double-labeling in g and h). Bar: 15 μm.
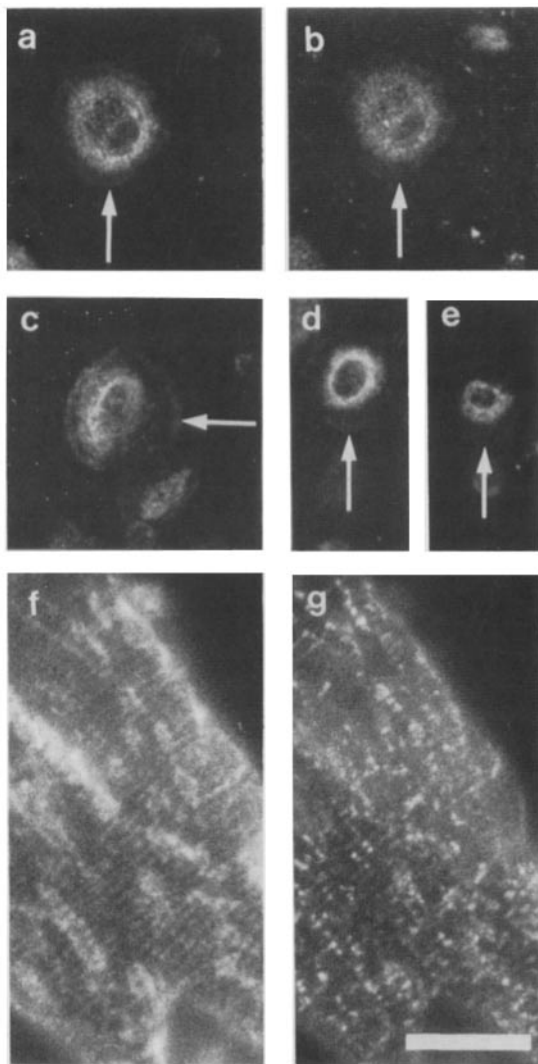
Figure 7. Codistribution of s-cyclophilin and calreticulin immunoreactivity in spreading platelets (*a* and *e*), and similar distributions of s-cyclophilin and calsequestrin immunoreactivity in skeletal muscle (*f* and *g*). Platelets were either double-labeled with s-cyclophilin (*a*) and with calreticulin antibody (*b*), or labeled for s-cyclophilin alone (*c–e*). Both proteins were detected within a ring-like structure and, although less intensively, towards the periphery of activated platelets. These localizations correspond to the putative location of the dense tubular network in activated platelets. Arrows point to the edge of the platelets. (*f* and *g*) s-cyclophilin (*f*) and calsequestrin (*g*) immunoreactivity in frozen sections of embryonic day 20 chick hind limb skeletal muscle. Bar, 10 μm.

the dense tubular network in activated platelets (Crawford, 1985), the sarcoplasmic reticulum of fast skeletal muscle (Campbell, 1986), and the pseudopodia of spreading cells. Platelets were selected because they are devoid of RER, and because the unique localization of calcium-releasing organelles (the dense tubular network) during the activation process has been described (Crawford, 1985). As shown in Fig. 7 (*a–e*), most s-cyclophilin in spreading platelets was restricted to a ring of immunoreactive material that was also detected with antibodies against calreticulin. Additional less intense signal was detectable towards the platelet periphery. The distribution of s-cyclophilin and calreticulin was similar

to the expected distribution of the dense tubular network, but was markedly different from the distribution of most other membrane-bound structures of platelets. Most membrane-bound structures, including secretory vesicles, are concentrated in the central part of the activated platelet (Crawford, 1985). Therefore, the distribution of s-cyclophilin in platelets was consistent with it being present in stimulus-sensitive calcium storage sites.

As mentioned above, we also compared the distributions of s-cyclophilin and of calsequestrin in skeletal muscle. Calsequestrin is a calcium-binding protein of the sarcoplasmic reticulum that is particularly enriched in terminal cisternae, i.e., the ryanodine receptor containing main sites of Ca^{2+} release in skeletal muscle (Campbell, 1986). As shown in Fig. 7 (*f* and *g*), s-cyclophilin and calsequestrin had similar distributions. However, the more uniform distribution of the s-cyclophilin signal may indicate that, unlike calsequestrin, s-cyclophilin is not concentrated in SR-terminal cisternae.

Finally, we determined whether s-cyclophilin-containing structures were present in pseudopodia of spreading cells, a specialized cellular region that might be an important site of calcium-mediated signaling. As shown in Fig. 8, s-cyclophilin was clearly present in pseudopodia of spreading L6 cells. s-Cyclophilin-containing organelles therefore appeared to be widely distributed inside the cell, including several, and perhaps all peripheral cellular regions and structures. These peripheral regions frequently appear to contain relatively high amounts of s-cyclophilin- (and calreticulin-) positive vesicular structures, possibly reflecting elevated demands for local calcium-mediated signaling.

Discussion

s-Cyclophilin is an ubiquitous, abundant, and conserved member of the cyclophilin family. Like cytosolic cyclophilin, s-cyclophilin is a soluble protein that possesses peptidyl-prolyl *cis-trans* isomerase activity and that binds to CsA with high affinity. Unlike its cytosolic counterpart, however, s-cyclophilin possesses a classical and functional signal sequence. We show that most of s-cyclophilin is not secreted, but is retained in an ER compartment. Retention is probably promoted by an s-cyclophilin-specific COOH-terminal extension, as this amino acid sequence is sufficient to direct an otherwise secretory protein to at least a part of the s-cyclophilin-containing vesicular structures.

That s-cyclophilin is associated with an ER compartment is demonstrated by its consistent colocalization with calreticulin, a soluble Ca^{2+}-binding protein that possesses the ER retention sequence KDEL and has been shown to copurify with ER markers (Smith and Koch, 1989; Krause et al., 1989; Treves et al., 1990; Milner et al., 1991). That s-cyclophilin is localized inside ER membranes is supported by the following observations: (*a*) microsome-associated s-cyclophilin comigrated with signal peptide-free recombinant s-cyclophilin, indicating that it had been previously translocated across the ER membrane (Fig. 3 *c*); (*b*) s-cyclophilin-GLVV-MNIT was retained in the ER (Fig. 6 *a*), implying that the signal sequence of s-cyclophilin did direct its translocation across the ER membrane; and (*c*) a minor proportion of s-cyclophilin was secreted from cells and was recovered in the extracellular space as a signal peptide-free species. The latter observation can probably be explained by leakage of

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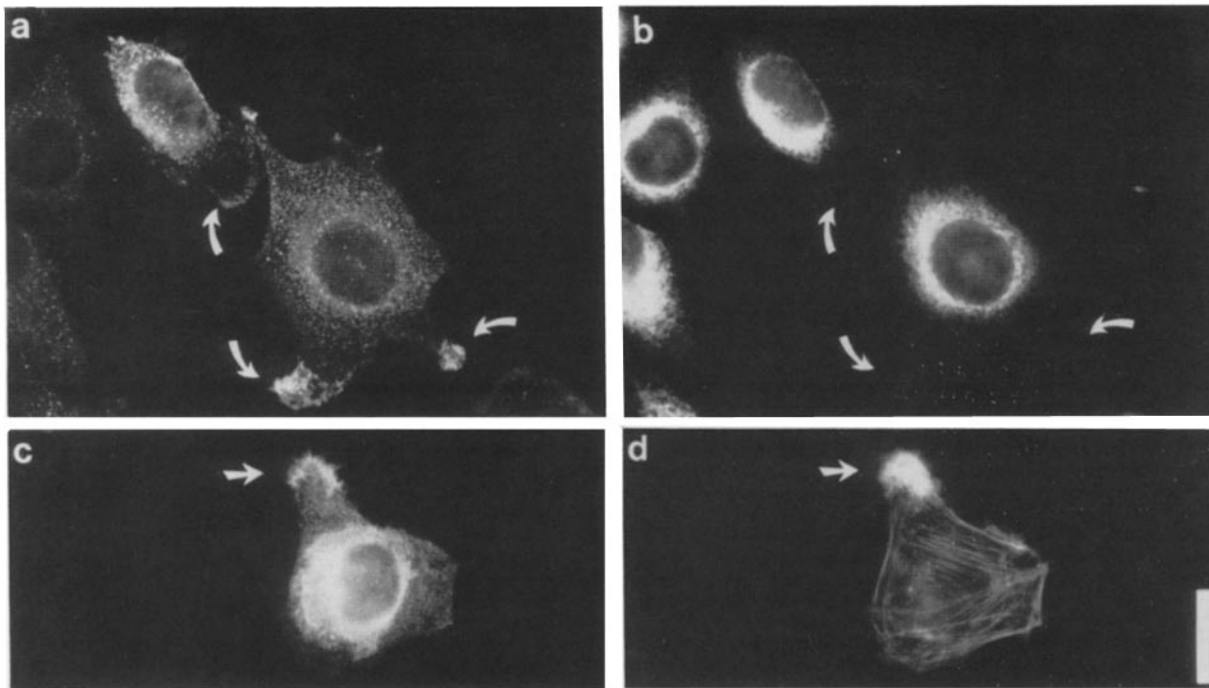
Figure 8. s-Cyclophilin containing vesicular structures in pseudopodia of spreading L6 cells. Cells were seeded onto tissue culture plastic, and allowed to spread for 50 min before fixation. Cells were then double labeled for either s-cyclophilin (*a*) and the ER (*b*), or for s-cyclophilin (*c*) and f-actin (*d*). Note that s-cyclophilin containing structures were particularly enriched in the vicinity of f-actin rich regions (*arrows* in *c* and *d*). Bar, 15 μm.

s-cyclophilin to the constitutive secretory pathway, a phenomenon that was also reported for the KDEL-bearing ER protein BiP (Pelham, 1989).

s-Cyclophilin and calreticulin antibodies yielded very similar labeling patterns, indicating that these proteins may colocalize throughout the cell. On the other hand, less precise codistribution was observed between s-cyclophilin and Ca^{2+}-ATPase or calsequestrin, suggesting the existence of differences in the distributions of these proteins within the ER system. Finally, striking differences were observed when the distribution of s-cyclophilin immunoreactivity was compared to that of a mixture of ER membrane proteins. These variations in the labeling patterns with antibodies to different ER proteins imply the presence of significant heterogeneity in the ER system. That some heterogeneity does exist was demonstrated for cerebellar Purkinje cells in a recent immunocytochemical study at the electron microscopic level (Villa et al., 1991). Our spreading cell data (Fig. 8) clearly show that pseudopods contain significant amounts of s-cyclophilin but little or no ER antigens. On the other hand, it was not possible to determine whether s-cyclophilin spots in the perinuclear region may correspond to "hot spots" of this protein and calreticulin within the ER, or whether they may belong to separate, possibly communicating structures. Clearly, our study at the light microscopic level does not provide the kind of resolution that would be required to answer these important questions. Unfortunately, our attempts to study the distribution of s-cyclophilin at the EM level have failed: several methods, including cryosection immunogold labeling, failed to yield appreciable signals, possibly due to the particular lability of s-cyclophilin containing structures. Such lability was reflected in the different sensitivity of the

vesicular pattern to various permeabilization protocols. As expected from the prevalently intracellular localization of s-cyclophilin, no labeling was observed when the antibody was applied to living cells at 4°C (data not shown). On the other hand, while all permeabilization protocols yielded a punctate labeling pattern to some degree, only a brief exposure to detergent after paraformaldehyde fixation led to the reproducible, homogeneous distribution of vesicular structures shown in this work. This sensitivity to permeabilization conditions was characteristic of the s-cyclophilin-type signal: while it was also observed for calreticulin, and to a lesser extent for Ca^{2+}-ATPase immunoreactivity, it was not observed for ER antigens with reticular labeling pattern. Therefore, while our data point to further heterogeneity in the ER system, only a corresponding study at the electron microscopic level will show whether and to what extent these ER proteins are localized to different ER subcompartments.

Our COOH-terminal substitution experiments demonstrate that the s-cyclophilin specific tail VEKPFAIAKE can direct a normally secreted protein to s-cyclophilin containing, vesicular structures. This finding suggests that the sequence VEKPFAIAKE might mediate binding to either an ER component or to a component of transport vesicles to the ER. This interaction could be either specific for s-cyclophilin, or be shared by additional soluble ER proteins. No COOH-terminal sequences related to VEKPFAIAKE could be identified in a database search (Gen/EMBL, April 1991), and it is therefore presently not possible to determine whether additional proteins might use this putative binding mechanism. It is, on the other hand, unlikely that the sequence VEKPFAIAKE may exclusively function as a KDEL substitute, because of the documented specific requirements of this

retention mechanism (Pelham, 1989), and because, in that case, one might have predicted an ER-type distribution of GDN-VEKPFAIAKE immunoreactivity. Finally, GDN-VEK-PFAIAKE was frequently not detected in peripheral vesicular structures that were detected with the s-cyclophilin, Ca^{2+}-ATPase, and calreticulin antibodies. While this observation may be due to comparatively low expression levels of the recombinant protein, the more central localization of GDN-VEKPFAIAKE did not reflect localization of the mutant protein in the transport vesicles that shuttle between ER and *cis*-Golgi: in agreement with the data of Hauri and colleagues (Schweizer et al., 1988), the G1/93 antigen that specifically identifies that compartment was detected in densely packed vesicular structures that were restricted to a narrow and highly unsymmetrical juxtanuclear region of COS-7 cells (data not shown), whereas GDN-VEKPFAIAKE was detected in comparatively widely spaced vesicles that were distributed over a much larger proportion of the cell.

The localization of s-cyclophilin in an ER compartment that may not coincide with the RER is unexpected in view of the proposed function of this protein in protein folding (Fischer and Schmid, 1990). Our findings may indicate that s-cyclophilin is not involved in biosynthetic protein folding, i.e., in the auxiliary reactions that promote proper protein folding after synthesis, and after membrane translocation of a polypeptide chain (Rothman, 1989). While a general protein folding promoting role of s-cyclophilin in the ER is possible, our findings are consistent with a specialized role of cyclophilins in processes involving Ca^{2+}. Such a role is suggested by the actions of CsA on Ca^{2+}-mediated processes, and by the association of s-cyclophilin with Ca^{2+}-binding proteins concentrated within a specialized ER subcompartment. Whether this subcompartment coincides, at least in part, with calciosomes (Hashimoto et al., 1988; Volpe et al., 1988; Krause et al., 1989; Meldolesi et al., 1990; Koch, 1990) remains to be investigated.

In summary while the function of cyclophilins, including s-cyclophilin, is presently not clear, our findings point to an additional potential target for CsA action, and provide a potentially valuable marker to study protein transport to the ER and to analyze behavior and heterogeneity of this important membrane system.

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