Cab45, a Novel Ca^{2+}-binding Protein Localized to the Golgi Lumen

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Abstract. We have identified and characterized Cab45, a novel 45-kD protein from mouse 3T3-L1 adipocytes. Cab45 is ubiquitously expressed, contains an NH_2-terminal signal sequence but no membrane-anchor sequences, and binds Ca^{2+} due to the presence of six EF-hand motifs. Within the superfamily of calcium-binding proteins, it belongs to a recently identified group of proteins consisting of Reticulocalbin (Ozawa, M., and T. Muramatsu. 1993. *J. Biol. Chem.* 268:699–705) and ERC 55 (Weis, K., G. Griffiths, and A.I. Lamond. 1994. *J. Biol. Chem.* 269:19142–19150), both of which share significant sequence homology with Cab45 outside the EF-hand motifs. In contrast to reticulocalbin and ERC-55 which are soluble components of the endoplasmic reticulum, Cab45 is a soluble protein localized to the Golgi.

Cab45 is the first calcium-binding protein localized to the lumenal portion of a post-ER compartment; Cab45 is also the first known soluble protein resident in the Golgi lumen. Cab45 can serve as a model protein to determine the mechanism of retention of soluble proteins in the Golgi compartment.

CALCIUM ions are involved in a myriad of cellular processes including signaling, secretion, and contraction. Intracellular levels of calcium are tightly controlled. As phosphate esters are highly abundant in cells and calcium phosphates are rather insoluble, calcium is typically stored complexed to proteins. One superfamily of calcium-binding polypeptides is a group of proteins known as EF-hand proteins (see Heizmann and Hunziker, 1991 for a recent review). Originally identified as loops E and F in the crystal structure of parvalbumin, a 12-kD carp muscle protein, this motif is responsible for co-ordination of a Ca^{2+} ion in a large number of proteins (Kretsinger and Nockolds, 1973). The calcium-binding site is formed by a helix, a loop and another helix. The loop is particularly rich in oxygen-containing amino acids that complex the calcium ion. Most EF-hand proteins contain two to eight copies of the helix-loop-helix domain, arranged in such a way that two domains are in close spatial proximity; this seems to increase the affinity for calcium. Some EF-hand proteins such as calmodulin are ubiquitously expressed, whereas the great majority shows tissue-specific expression. The bulk of these EF-hand proteins localize to the cytosol, but some are found in the endoplasmic reticulum and the sarcoplasmic reticulum. Only in a few instances do we know the functional implications of binding of one or more Ca^{2+} ions to the EF-hands. In the case of calmodulin, a protein containing four EF-hands, calcium binding triggers conformational changes within the entire protein and thereby plays a major regulatory role in interactions of calmodulin with other proteins (Wallace et al., 1982; Weinstein and Mehler, 1994).

The secretory pathway, particularly the ER, is a major storage compartment for intracellular calcium. The functional integrity of the ER seems to be critically dependent on calcium, as depletion of ER calcium interferes with folding and maturation of certain secretory proteins (Suzuki et al., 1991; Wileman et al., 1991; Lodish et al., 1992). All of the calcium-binding proteins identified so far in the secretory pathway are soluble constituents of the ER lumen. Examples include immunoglobulin heavy chain binding protein (BiP)[1] (Bole et al., 1986; Munro and Pelham, 1986), protein disulfide-isomerase (PDI) (Edman et al., 1985) and endoplasmin (GRP94) (Mazzarella and Green, 1987; Sorger and Pelham, 1987). All these proteins are retained in the ER through the interaction of a carboxy-terminal tetrapeptide with a membrane-bound receptor.

Here, we report the cloning and characterization of Cab45, a novel member of the EF-hand family of proteins that is a resident protein of the Golgi lumen.

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1. *Abbreviations used in this paper*: BiP, binding protein; EndoH, endoglycosidase H; PDI, protein disulfide isomerase; BFA, brefeldin A; ERGIC, ER/Golgi intermediate compartment; LRSC, lissamine rhodamine; PDI, protein disulfide isomerase; PNGaseF, N-glycosidase F; UTR, untranslated regions.

Materials and Methods

Materials

DME tissue culture medium lacking methionine, cysteine and glutamate was purchased from ICN Radiochemicals (Irvine, CA). The express protein labeling reagent, a mixture of ^{35}S-labeled methionine and cysteine, and $^{45}Ca^{2+}$ were purchased from New England Nuclear (Boston, MA). Brefeldin A was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

3T3-L1 mouse fibroblasts (American Type Culture Collection, Rockville, MD) were propagated and differentiated according to the protocol described in (Frost and Lane, 1985). For brefeldin A treatments, cells were treated for 3 h at 37°C with medium containing 5 μg/ml brefeldin A.

Isolation of Cab45 cDNA Clones

The original clone isolated from the adipocyte-specific subtractive cDNA library was used to isolate a full-length clone from a 3T3-L1 day 8 adipocyte cDNA library (Baldini et al., 1992). To do this, the original clone was labeled with digoxigenin (Boehringer-Mannheim Biochemicals) according to the manufacturer's instructions. Two positive clones (Cab45a and Cab45b) were isolated and subjected to automated sequencing on a sequencer (model 373-A; Applied Biosystems, Foster City, OR). The inserts were sequenced at least two independent times on one strand and once on the complementary strand. Sequence analysis was performed with the DNAstar package and showed an open reading frame of 1,083 bp encoding a protein of 38 kD. The sequence has been submitted to Genbank/EMBL/DDBJ. Homology searches were performed at NCBI using the BLAST network service, and alignments were performed with the Megalign program from DNAstar using the Clustal algorithm.

mRNA Isolation and Northern Blot Analysis

Isolation of mRNA from tissues and from 3T3-L1 cells at various stages of differentiation was as described by (Baldini et al., 1992), as was agarose gel electrophoresis of mRNA and its transfer to nylon membranes. Hybridizations were performed overnight at 42°C in 50% formamide, 5× SSC, 25 mM Na-phosphate, pH 7.0, 10× Denhardt's solution, 5 mM EDTA, 1% SDS, and 0.1 mg/ml PolyA; the [^{32}P] labeled DNA probes were used at concentrations of 2×10^6 cpm/ml. The filters were subsequently washed in 2× SSC/0.1% SDS and 0.1× SSC/0.1% SDS at 50°C before autoradiography.

Expression of Recombinant Cab45 and ^{45}Ca Overlay Assays

Glutathione *S*-transferase fusion DNA constructs were made by in-frame fusions of Cab45 cDNA fragments generated by PCR to the vector pGEX-4T (Pharmacia Fine Chemicals, Piscataway, NJ). Constructs were expressed in the *Escherichia coli* strain BL21 and total extracts were prepared by pelleting the cells for 10 min at 15,000 *g* followed by lysis in 2× SDS-PAGE sample buffer (250 mM Tris, pH 6.8, 4 mM EDTA, 4% SDS, 20% sucrose). Samples were passed several times through a 26-gauge needle to shear chromosomal DNA and directly analyzed by 12% SDS-PAGE. One set of samples was subjected to Coomassie brilliant blue staining to visualize total protein. The other set of samples was transferred to nitrocellulose membranes and a ^{45}Ca-overlay was performed as described in (Maruyama et al., 1984).

Antibodies

Antibodies against two Cab45 peptides were generated in rabbits by Research Genetics (Huntsville, AL). Peptide 1 (amino acids 44–56): RERAANREENEIM was synthesized on a poly-lysine backbone (MAP technology) and directly injected. Peptide 2: (carboxy-terminal 15 amino acids): GSKLMDYARNVHEEF was coupled to keyhole limpet hemocyanin and injected. Peptide 2 elicited a much stronger immune response, and this antiserum was used in all subsequent experiments. Rabbit anti-total ER and anti-total Golgi antisera were generously provided by Dr. Daniel Louvard (Pasteur Institute, Paris, France) and were described in (Louvard et al., 1982). A monoclonal antibody (G1/93) against ERGIC-53 (Schweizer et al., 1988) was provided by Dr. H.-P. Hauri (Biozentrum, University of Basel, Basel, Switzerland). Rabbit anti-mouse α-mannosidase II antibodies (Moremen and Robbins, 1991) were supplied by Dr. K. Moremen (Complex Carbohydrate Research Center, University of Georgia, Athens, GA). Anti-protein disulfide isomerase monoclonal antibodies were purchased from StressGen (Victoria, B.C., Canada). For affinity purification of antibodies, immune peptides were coupled to CNBr-Sepharose (Pharmacia Fine Chemicals) and affinity purification was performed according to standard methods (Harlow and Lane, 1988). Affinity purified Cy3-conjugated, lissamine rhodamine (LRSC)-conjugated and FITC-conjugated goat anti-rabbit and anti-mouse IgG was from Jackson Labs (West Grove, PA). Unconjugated affinity purified goat anti-rabbit IgG was from Cappel, Organon Teknika N.V. (Turnhout, Belgium).

Cos-cell Transfections, In Vivo Labeling and Immunoprecipitations

Both Cab45 cDNA clones in the pcDNA I vector that were isolated from the adipocyte cDNA library could be directly used for transfections, as both of the cDNA inserts were in the correct orientation with respect to the CMV-promoter. Plasmids were transiently transfected into Cos7-cells (10 cm dishes) by the DEAE-dextran method (Seed and Aruffo, 1987). At the specified times after transfection, cells were labeled, for the time indicated in the specific experiments, in 3 ml DME lacking methionine and cysteine and supplemented with 0.5 mCi (1,000 Ci/mmol) of Express protein labeling reagent. Cells were thereafter washed three times with chase medium (DME containing unlabeled methionine and cysteine at 1 mM and cycloheximide at 300 μM). Cells were then incubated for the indicated periods of time with 2 ml of chase medium. At the end of the chase period, the cell culture media were collected and centrifuged at 15,000 *g*. The cells were washed twice with cold PBS and then scraped into lysis buffer (1% Triton X-100, 60 mM octylglucoside, 5 mM EDTA, 20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM PMSF). Insoluble debris was removed by centrifuging for 10 min at 15,000 *g*. The cleared tissue culture supernatant and the cell lysate were then incubated for 30 min at 4°C with protein A–Sepharose (Pharmacia Fine Chemicals). The protein A–Sepharose was removed by centrifugation, and fresh protein A–Sepharose was added along with the corresponding antibody. Immunoprecipitations were performed for 3 h at 4°C; immunoprecipitates were then washed five times with lysis buffer (lacking octyl-glucoside) and subjected to either deglycosylation reactions (see below) or directly analyzed by SDS-PAGE.

Deglycosylation of Cab45

For Endo H digestions immunoprecipitates were washed once in PBS and then resuspended in Endo H buffer (0.1 M Na-citrate, pH 6.0, 1% SDS) and boiled for 5 min. For PNGaseF reactions, samples were resuspended in 0.5% SDS, 1% β-mercaptoethanol, boiled for 5 min, and then supplemented with NP-40 (1% final concentration) and sodium phosphate buffer (50 mM final concentration, pH 7.0); samples were incubated for 2 h in presence of 500 U Endo H (New England Biolabs) or 1 U PNGaseF (Boehringer-Mannheim Biochemicals) at 37°C. Reactions were stopped by boiling in 2× sample buffer (250 mM Tris, pH 6.8, 4 mM EDTA, 4% SDS, 20% sucrose), and analyzed by SDS-PAGE.

Immunofluorescence

The procedures used were essentially as described previously (Amara et al., 1989) with the following modifications. Fixation and permeabilization of the cells grown on coverslips was done by sequential incubation with methanol and methanol/acetone 1:1, for 5 min each at −20°C. The anti-Cab45 primary antibody used was affinity-purified anti-peptide 2 described above. Cy3-conjugated and in some cases LRSC-conjugated goat anti-rabbit IgG was used instead of the TRITC conjugate to insure no overlap of fluorescence when observing double labeled samples with the FITC filter.

Photography on an Axiomat fluorescence microscope (Carl Zeiss Inc., Thornwood, NY) was done at identical exposure times between samples to be able to compare signal intensities.

Extraction of Cells with Sodium Carbonate

Extraction of 3T3-L1 adipocytes with sodium carbonate was done according to a described protocol (Fujiki et al., 1982). The adipocytes were washed three times in phosphate-buffered solution and once in a solution containing 150 mM NaCl. The cells were then scraped from the culture

dish into 1 ml of 100 mM sodium-bicarbonate buffer, pH 11.5, and homogenized by five strokes in a 2 ml Dounce homogenizer. After 30-min incubation at 4°C, the homogenates were centrifuged at 100,000 rpm at 4°C in the TLA 100.2 rotor of a Beckman table-top ultracentrifuge. The pellets were resuspended in 0.5 ml of 100 mM sodium-bicarbonate buffer, pH 11.5, and pellet and supernatant fractions were immediately mixed with an equal volume of SDS-PAGE sample buffer. Any DNA in the pellets was sheared by several passages through a 26-gauge needle to decrease the viscosity; these samples were then used directly for SDS-PAGE and Western blot analysis.

Other Methods

Separation of proteins by SDS-PAGE, fluorography, immunoblotting, protein determinations and densitometric scanning of the gels was performed as described previously (Scherer et al., 1994).

Results

Cloning and Sequencing of Cab45

To identify novel adipocyte-specific proteins, we randomly sequenced portions of 1,000 clones from a subtractive cDNA library enriched in mRNAs induced during adipocyte differentiation of 3T3-L1 fibroblasts. One of the short DNAs recovered showed an open reading frame that was predicted to encode an EF-hand domain in which all consensus positions were conserved (see Fig. 2 *b*) but which otherwise had no matching counterpart within all database entries searched. We isolated a full-length version of this clone from a 3T3-L1 adipocyte library. Two independent clones were isolated (Cab45a and Cab45b) and completely sequenced. Both clones predicted identical open reading frames encoding a 361–amino acid protein (Fig. 1) with a single exception (position 281 was Lys in clone a and Arg in clone b). They also had identical 3′ untranslated sequences. However, they differed completely in their 5′ untranslated regions (5′UTRs). Cab45a had a 337-bp 5′-UTR and clone Cab45b had a 205-bp 5-UTR. Both clones converged into the same sequence just upstream of the starting methionine. Neither encoded an open reading frame containing a methionine upstream of the initiator methionine. Whether the existence of two distinct clones reflects a library artifact, differential splicing intermediates, or functionally different entities cannot be determined at this stage. It is unlikely, however, that these two forms are encoded by two different genes, as the nucleotide sequences of the two clones 3′ to the initiator methionine are 99.9% identical.

There are two potential initiator methionines within a distance of six amino acids. Neither of these methionines is surrounded by a sequence that matches perfectly the consensus for initiator methionines as defined by Kozak (1991). The use of the second methionine as the starting residue would be more in line with the structure of a classical signal sequence with a positively charged amino acid (arginine in position +4) close to the NH_2-terminus. Nevertheless, we refer to the more 5′ methionine as position +1 in this paper, because we believe that the first methionine is used as the translational start site in vivo. This is based on the observation that transient transfection into Cos7 cells of Cab45 cDNA starting with methionine 1 is translated far more efficiently than a cDNA construct starting with methionine +6 (data not shown).

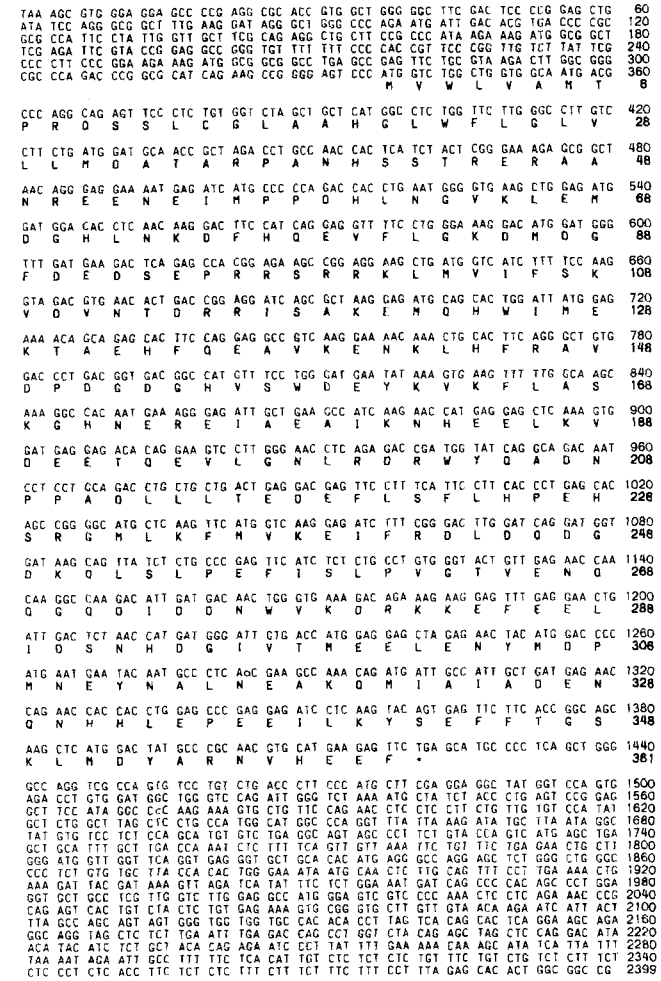

Figure 1. Nucleotide and predicted amino acid sequences of Cab45a. These sequence data are available from GenBank/EMBL/DDBJ under accession number U45977. Cab45b can be found under accession number U45978.

Cab45 Has 6 Potential EF Hands and Shows Greatest Similarity to Two Calcium-binding Proteins Localized to the Endoplasmic Reticulum

Cab45 has a characteristic NH_2-terminal signal sequence with an arginine preceding a stretch of hydrophobic amino acids (Fig. 2 *a*). As there is no stretch of hydrophobic amino acids long enough to anchor the protein within the membrane, we expected the protein to be completely translocated into the lumen of the ER. A further landmark is the predicted N-glycosylation site at position 39. A motif search suggests the presence of six EF-hands, a motif known to be involved in binding calcium. A database search reveals similarity to a large number of calcium-binding proteins with sequence homology concentrated within the EF-hand motifs. Two proteins, however, show significant sequence homology outside the calcium-binding domains: Reticulocalbin (Ozawa and Muramatsu, 1993) and Erc55 (Weis et al., 1994). These proteins are comparable in length and are also structurally very similar, as both also contain six EF-hands. Fig. 2 *b* shows a comparison between the six EF-loops of each of these proteins and the four EF-hands found in calmodulin. Fig. 2 *c* dem-

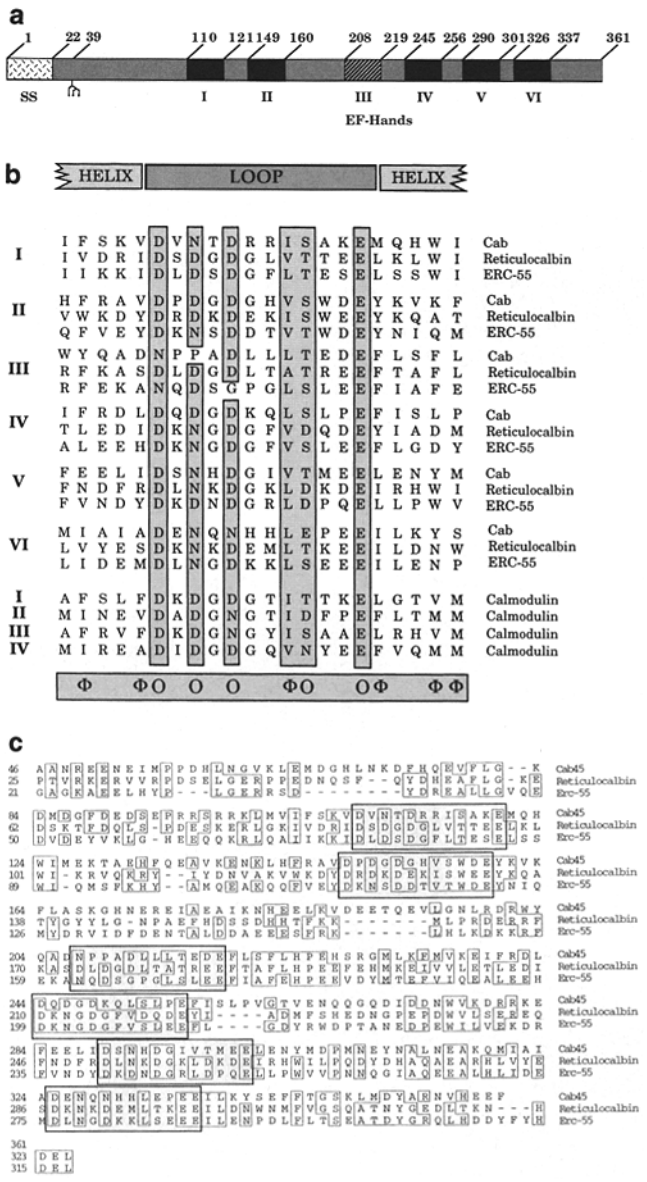

Figure 2. Cab45 has multiple EF-hands and is homologous to the resident ER-proteins Reticulocalbin and Erc-55. (*a*) Schematic representation of Cab45 structure. Cab45 has an NH_2-terminal signal sequence (*SS*), a predicted N-glycosylation site at position 39 and a total of six EF-hands. Arabic numbers indicate positions of amino acids at these landmarks. Roman numbers refer to EF-hands. EF-hand III has a slight aberration in one of the consensus positions (see *b*). (*b*) Alignment of the six EF-hands found in Cab45, Reticulocalbin, and ERC 55 and the four EF-hands found in calmodulin. The sequences of five amino acids from the flanking helices and the central loop constituting the calcium-binding domain from each of the indicated proteins were aligned. (ϕ) Consensus hydrophobic amino acid; and (*O*) Amino acid with an oxygen-containing side chain. Both Cab45 and Erc55 differ in one position from the corresponding consensus residue in EF-hand III. (*c*) Sequence alignment of Cab45 with Reticulocalbin and Erc 55. All three family members share significant sequence homology outside the EF-hand motifs. EF-hands are shaded in gray, and homologous residues conserved in at least two family members are boxed.

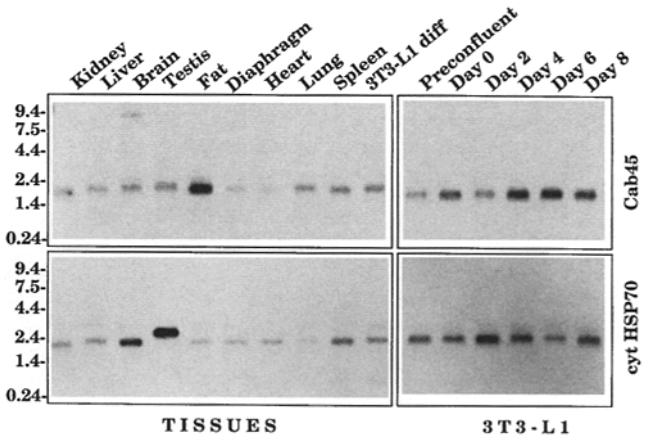

Figure 3. Cab45 is ubiquitously expressed in all tissues and slightly induced during differentiation of 3T3-L1 fibroblasts into adipocytes. (*Top*) 0.5 mg of polyA-RNA isolated from different tissues (*left*) or from 3T3-L1 cells at different days during differentiation of 3T3-L1 fibroblasts into adipocytes (*right*) were separated by agarose gel electrophoresis, blotted onto a nylon membrane, and probed with a DNA fragment comprising the open reading frame of Cab45 according to the protocols described in Materials and Methods. Numbers on the left indicate molecular weight markers (in kb). (*Bottom*) The same blots were stripped and reprobed with the constitutively expressed cytosolic hsp70 cDNA to control for equal loading of mRNA.

onstrates the overall sequence conservation between Erc55, Reticulocalbin, and Cab45. These proteins constitute a novel family of calcium-binding proteins found within the secretory pathway.

Cab45 Is Expressed in All Tissues

Since the original partial clone was isolated from a subtractive cDNA library presumably enriched in adipocyte–specific mRNAs, we wondered whether Cab45 expression would indeed be limited to adipocytes. Thus Northern blot analysis on mRNA isolated from a number of different tissues as well as from 3T3-L1 fibroblasts at different times during differentiation into adipocytes was performed (Fig. 3). While there is a slight increase in Cab45 expression during adipogenesis, Cab45 mRNA is expressed in all tissues. In line with the slight induction during adipogenesis, adipose tissue is the most predominant source of Cab45. As a control, the same blots were stripped and reprobed with a cDNA encoding one of the cytosolic hsp70s (Hunt and Calderwood, 1990), a protein expressed at comparable levels in all tissues and with constant expression during differentiation of fibroblasts into adipocytes (Scherer et al., 1994).

The cDNA for Cab45 Encodes a 45-kD Glycoprotein

To generate a tool for the biochemical analysis of Cab45, specific anti-peptide antibodies were generated as described in Materials and Methods. These peptides were chosen such that potential cross-reactivity with Reticulocalbin and Erc55 would be excluded, as the proteins share very little sequence homology in these regions. These antibodies were affinity-purified and tested on Cos cells transfected with the corresponding cDNAs. Since the original

library was made in the pcDNA1 vector and since the cDNA inserts in both Cab45a and Cab45b were in the correct orientation with respect to the CMV-promoter, these full-length clones were transiently transfected into Cos7 cells; the Cab45 protein was metabolically labeled and immunoprecipitated. Fig. 4 *a* shows that the endogenous Cab45 in Cos cells cannot be detected under these conditions (lane *1*), whereas both Cab45a and Cab45b cDNAs gave rise to immunoreactive proteins of 45 kD (lanes *2* and *3*). Reproducibly, Cab45b was expressed at much higher levels than Cab45a, suggesting that there is a functional difference between the two 5'UTRs. The affinity-purified antibodies were also tested by Western blot analysis on total cellular extracts from 3T3-L1 fibroblasts. The Western blots in Fig. 4 *b* show that the antibody recognizes a 45-kD protein in 3T3-L1 adipocytes; more than 95% of the signal is accounted for by the 45-kD band, indicating that this antibody preparation is suitable for use in immunofluorescence studies.

We found it difficult to metabolically label endogenous Cab45 in 3T3-L1 fibroblasts and adipocytes, suggesting that the protein has a rather long half-life. Further biochemical analysis of the protein was therefore performed in transiently transfected Cos7 cells.

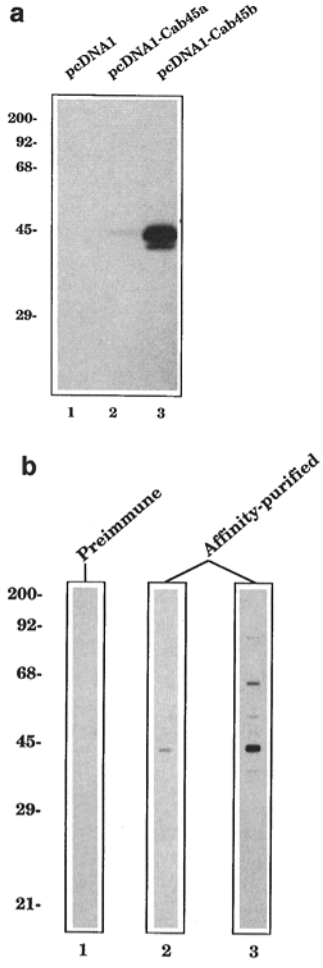

Figure 4. Cab45 cDNA encodes a 45-kD protein. (*a*) Transfection of Cab45 into Cos7 cells. The two clones (Cab45a and Cab45b) isolated from the day 8 adipocyte library and a control plasmid (pcDNA1) were transfected into Cos7 cells. 48 h after transfection the cells were labeled with ^{35}S-Express for 30 min. The cells were lysed, immunoprecipitated with affinity-purified anti-Cab45 antibodies, and analyzed by SDS-12%PAGE as detailed in Materials and Methods. (*b*) Western blot analysis of 3T3-L1 adipocyte extracts. 3T3-L1 adipocytes were solubilized in lysis buffer (see Materials and Methods), insoluble debris was removed by centrifugation, and the supernatant was diluted with an equal volume of SDS-PAGE sample buffer. In each lane, 50 μg of protein was analyzed by SDS-12.5%PAGE followed by transfer to nitrocellulose and Western blotting with affinity-purified anti-Cab45 antibody. (Lane *1*) Preimmune serum; (lanes *2* and *3*) two different exposures of strips incubated with anti-Cab45 antibody. The long exposure is shown to demonstrate that >95% of the total signal is due to the Cab45 signal. Numbers on the left indicate molecular weight standards (in kD).

Cab45 Is Not an Integral Membrane Protein

The amino acid sequence of Cab45 does not contain any hydrophobic stretches long enough to span a membrane as an α helix. To test whether Cab45 is indeed a soluble protein, we subjected 3T3-L1 adipocytes to an alkaline extraction procedure. Treatment of isolated membranes at pH 11.5 removes all but integral membrane proteins (Fujiki et al., 1982). Similar to its ER counterparts, Cab45 is not an integral membrane protein, as it is quantitatively recovered in the supernatant after alkaline extraction and centrifugation at 100,000 *g*. (Fig. 5).

Cab45 Is a Calcium-binding Protein

To determine whether Cab45 is indeed a calcium-binding protein, we tested its calcium-binding ability qualitatively in an overlay assay. EF-hands form structurally highly stable entities that retain the ability to bind calcium even under denaturing conditions. We expressed Cab45 in *E. coli* as a recombinant fusion protein with glutathione *S*-transferase. Extracts from cells either expressing glutathione *S*-transferase alone, as a fusion to Cab45, or as a fusion to an irrelevant protein were separated by SDS-PAGE, transferred to nitrocellulose, and overlaid with $^{45}Ca^{2+}$ (Fig. 6). In the extract originating from cells expressing the Cab45-glutathione *S*-transferase fusion, a protein of the expected molecular weight (68 kD) showed significant binding of $^{45}Ca^{2+}$, suggesting that Cab45 indeed contains functional calcium-binding domains. No $^{45}Ca^{2+}$ binding was detected in extracts of the control cells. Upon Western blot analysis (not shown) a co-migrating 68-kD polypeptide was recognized by our anti-Cab45 antibody. Further experiments are needed to determine the stoichiometric ratio between the six predicted EF-hands and the number of calcium ions bound per Cab45 molecule.

Cab45 Resides in a Post-medial Golgi Compartment

The cDNA sequence of Cab45 predicts an amino-terminal signal sequence that would direct Cab45 to the secretory pathway. To test whether this sequence can indeed function as a signal sequence, we transiently transfected Cab45 into Cos7 cells and monitored its biogenesis through the secretory pathway by its sensitivity to the glycosidases

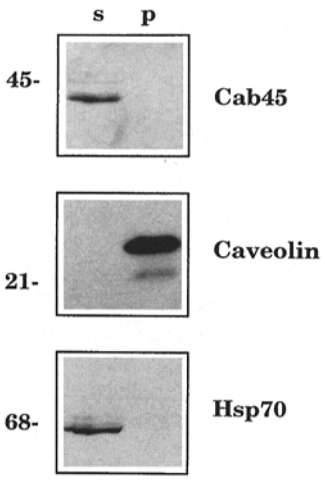

Figure 5. Cab 45 is not an integral membrane protein. Day 8 3T3-L1 adipocytes were subjected to an alkaline extraction procedure as described in Materials and Methods and analyzed by SDS-PAGE. The gel was subsequently subjected to Western blot analysis with antibodies against Cab45 (*top*), the integral membrane protein caveolin (*middle*) and cytoplasmic hsp70 as soluble cytoplasmic control protein (*lower*). *s*, soluble fraction; *p*, integral membrane proteins.

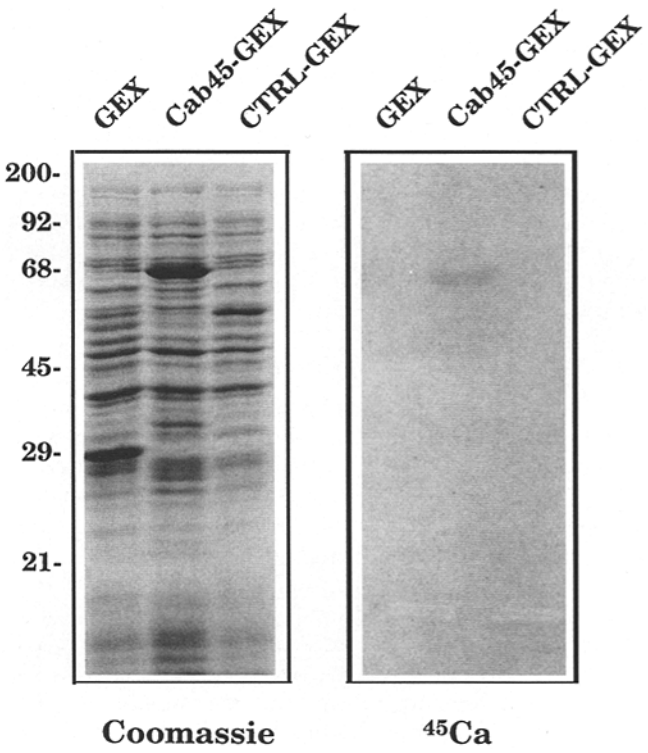

Figure 6. Cab45 binds calcium in vitro. Total cellular extracts were prepared from *E. coli* strain BL21 expressing either glutathione *S*-transferase alone (*GEX*), a glutathione *S*-transferase fusion with Cab45 (*Cab-GEX*) or another, irrelevant fusion protein (*CTRL-GEX*). The extracts were separated by SDS-12.5%PAGE and either stained with Coomassie brilliant blue (*left*) or transferred to Nitrocellulose and overlaid with $^{45}Ca^{2+}$ (*right*). Except for the Cab45-GST fusion, none of the proteins found in the extract showed significant binding to $^{45}Ca^{2+}$.

EndoH and PNGase F. Cab45 has one predicted N-glycosylation site at position 39 (see Fig. 2 *a*). EndoH cleaves high mannose oligosaccharides found on proteins localized in the endoplasmic reticulum but does not cleave complex oligosaccharides generated by enzymes localized in and after the medial Golgi. Therefore, glycoproteins that have exited the ER and the *cis*-Golgi are generally resistant to EndoH. PNGase F (or N-glycosidase F), in contrast, will cleave most N-linked sugars and is therefore used to determine the molecular weight of a protein completely devoid of N-linked oligosaccharides. Cos7 cells transfected with pcDNA1-Cab45 or pcDNA1 alone were pulse-labeled for 20 min with a mixture of ^{35}S-labeled methionine and cysteine and then chased for 2 h in the presence of cycloheximide to stop further protein synthesis. Tissue culture medium was then harvested, the cells were lysed and both medium and cell lysate were immunoprecipitated with anti-Cab45 antibodies (Fig. 7 *a*). The great majority of Cab45 is EndoH resistant (lane *2*), but sensitive to PNGaseF (lane *4*), suggesting the protein has left the ER and the *cis*-Golgi. Only trace amounts of Cab45 are seen in the tissue culture supernatant even after the three hour chase (lanes *5–8*).

To follow the time course of exit from the ER, cells were pulse-labeled for 5 min, chased for various amounts of time, lysed and subjected to EndoH treatment. As shown in Fig. 7 *b*, Cab45 has a rather short residence time in the ER, as within 20 min essentially all of the newly made Cab45 has become resistant to digestion with EndoH.

Taken together, these results suggest that Cab45 is a resident protein of a post-medial Golgi compartment. Although it rapidly undergoes carbohydrate processing events characteristic of proteins that do not reside within the ER, it is not secreted from the cells.

Cab45 Is Localized to the Golgi Complex in 3T3 Fibroblasts

Since biochemical evidence from transiently transfected Cos7 cells suggested a post-ER localization, we tested the localization of Cab45 endogenously expressed in 3T3 fibroblasts by immunofluorescence. As seen in Fig. 8 *a*, a clear asymmetric, juxtanuclear staining of Cab45 is observed that is reminiscent of Golgi staining. Indeed, double labeling with a Golgi-specific antibody preparation reveals complete co-localization (Fig. 8, *a* and *b*). To further corroborate the biochemical evidence that Cab45 is not secreted and accumulates in the Golgi, the cells analyzed in Fig. 8 (*c–e*) were treated for 8 h with cycloheximide, in order to stop further protein biosynthesis, before immunolocalization. If Cab45 were secreted or rapidly degraded, we would expect a drastically reduced immunofluorescence signal after this eight hour chase period. However, the Cab45 signal hardly decreased during the chase period (Fig. 8 *c*), and much like the Golgi marker proteins (*d*), the pattern of Cab45 staining became more compact during the chase period (see *c* and *e*). As an essential control, Cab45 pre-immune serum does not generate detectable immunofluorescence (*f*). Furthermore, the signal obtained with the affinity-purified anti-Cab45 antibody was abolished when the incubation was performed in the presence of the peptide used for immunization (*g*), while a nonrelevant peptide corresponding to a different region of Cab45 had no effect on the staining pattern (*h*). Further controls insured that there is no overlap in spectral emission between the two differentially labeled fluorescent probes (not shown).

Additionally, Fig. 9 shows the co-localization of Cab45 with the medial-Golgi protein α-mannosidase II in 3T3 cells. Panel *a* represents the staining pattern obtained with Cab45; the pattern completely overlaps that obtained with an antiserum specific for Golgi α-Mannosidase II (panel *b*). Thus, using antisera against a specific Golgi protein as well as an antiserum specific for a purified Golgi fraction, we showed that Cab45 is localized in the Golgi complex.

Cab45 Does Not Co-localize with ER-marker Proteins in 3T3 Fibroblasts and Cos7 Cells

Since the two closest homologs of Cab45 (Erc55 and Reticulocalbin) are resident ER proteins, we needed to establish that the staining pattern observed for Cab45 does not correspond to the endoplasmic reticulum. We therefore performed a double-labeling experiment with antibodies against Cab45 and ER marker proteins. Fig. 10 *a* demonstrates the labeling of 3T3 cells with the anti-Cab45 antibody; the compact staining pattern is completely different from the diffuse staining pattern in *b* obtained with an antiserum raised against an ER subcellular fraction

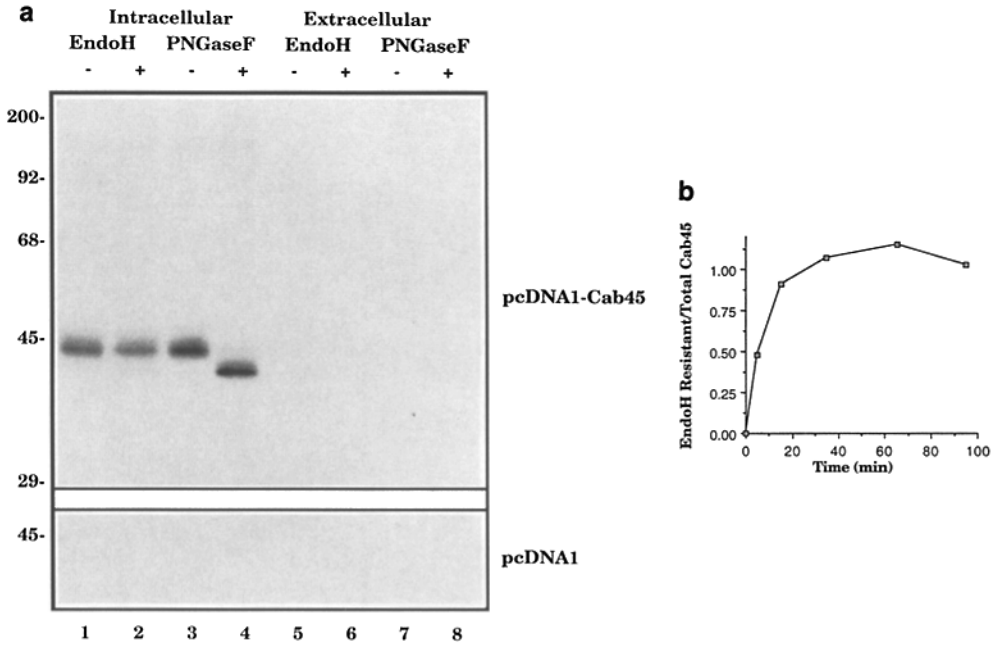

Figure 7. Cab45 undergoes carbohydrate modifications characteristic of a post-medial Golgi compartment and has a very short transit time in the ER. (*a*) Cab45 is EndoH-resistant, but PNGaseF sensitive. pcDNA1-Cab45b (*top*) or pcDNA1 (*lower*) was transfected into Cos7 cells. 40 h after transfection, cells were pulse-labeled with ^{35}S-Express for 20 min and chased for 2 h in the presence of cycloheximide. Intracellular material and the tissue culture supernatant were subjected to immunoprecipitation with anti-Cab45 antibodies and treated with EndoH or PNGaseF. Samples in lanes *1*, *3*, *5*, and *7* were incubated in the corresponding deglycosylation buffer in the absence of enzyme, samples in lanes *2*, *4*, *6*, and *8* were incubated in the presence of the listed enzyme. (*b*) Time-course of exit from ER. Cos7 cells transfected with pcDNA1-Cab45b were pulse-labeled for 5 min 40 h after transfection with ^{35}S-Express. Cells were then chased for various amounts of time in the presence of cycloheximide and lysed. The extracts were immunoprecipitated with anti-Cab45 antibodies, treated with EndoH, and analyzed by SDS-PAGE and autoradiography. The amount of EndoH-resistant Cab45 was determined with a Molecular Dynamics densitometer and plotted as a function of time. 0 min corresponds to the beginning of the pulse and 5 min corresponds to the beginning of the chase. Most of Cab45 exits the ER within 10–20 min.

which recognizes a complex mixture of ER proteins (Louvard et al., 1982). The marginal overlap observed in some cells is likely to be due to trace contaminations of Golgi proteins in the original preparation used to generate the ER antiserum. To substantiate the difference in staining profiles of Cab45 and ER proteins, we performed a double labeling experiment in Cab45-transfected Cos7 cells. PDI is a well-characterized resident protein of the ER lumen (Edman et al., 1985) and the staining pattern obtained with a monoclonal anti-PDI antibody exhibited the expected, diffuse, ER staining profile (Fig. 10 *d*). In contrast, panel *c* shows the expected asymmetric perinuclear staining for Cab45. The arrows in panels *c* and *d* highlight regions that stain positive for Cab45 but that are specifically absent from the ER-like staining pattern of PDI. Small amounts of Cab45 appear to localize to the ER; possibly this represents newly made protein en route to the Golgi. Cab45 therefore displays a staining pattern distinctively different from that obtained for either total ER proteins (*a* and *b*) or a resident protein of the ER lumen (*c* and *d*).

Brefeldin A Induces Redistribution of Cab45

BFA induces the rapid redistribution of Golgi into the ER, leaving no definable Golgi structure remaining in the cell (Lippincott-Schwarz et al., 1989). If the structures observed in immunofluorescence experiments with anti-Cab45 antibodies indeed represent the Golgi, we would predict that the pattern of Cab45 staining should, after BFA treatment, be converted to a much more diffuse staining pattern corresponding to that of the ER. Treatment of 3T3 cells for 3 h with brefeldin A redistributes both Cab45 and the Golgi protein α-mannosidase II to a much more diffuse, ER-like pattern (Fig. 9, *c* and *d*), indicating that Cab45 resides in a brefeldin A–sensitive compartment.

Cab45 Resides in a Compartment Distinct from the ER/Golgi Intermediate Compartment (ERGIC)

The ER-Golgi intermediate compartment (ERGIC) comprises a membrane system interposed between the rough ER and the *cis* side of the Golgi apparatus. It has been shown to mediate ER-to-Golgi exocytic protein transport (Schweizer et al., 1990; Lotti et al., 1992). The ERGIC was originally defined by a 53-kD type I transmembrane protein termed ERGIC-53 (Schweizer et al., 1988). Interestingly, ERGIC-53 displays a strikingly different sensitivity to brefeldin A treatment than do Golgi marker proteins. While in untreated cells ERGIC-53 is localized to numerous small vesicles concentrated around and partially overlapping with the Golgi, brefeldin A treatment causes ERGIC-53 to accumulate in larger sized vesicles. Typical Golgi marker proteins, such as galactosyltransferase, in contrast, redistribute from a single complex next to the nucleus into the ER, seen as a fine, punctate reticulum all over the cell (Lippincott-Schwartz et al., 1990).

We have subjected Cab45 and ERGIC-53 to a similar kind of analysis in Cab45-transfected Cos7 cells (Fig. 11). In agreement with previously published data, antibodies to ERGIC-53 stain in a halo-like pattern around the Golgi complex (*b*). In other instances, ERGIC-53 assumes a more compact distribution (*d*). The distribution observed for Cab45 (*a* and *c*) is clearly distinct from that seen for ERGIC-53, but there is significant overlap between the two proteins. However, upon brefeldin A treatment,

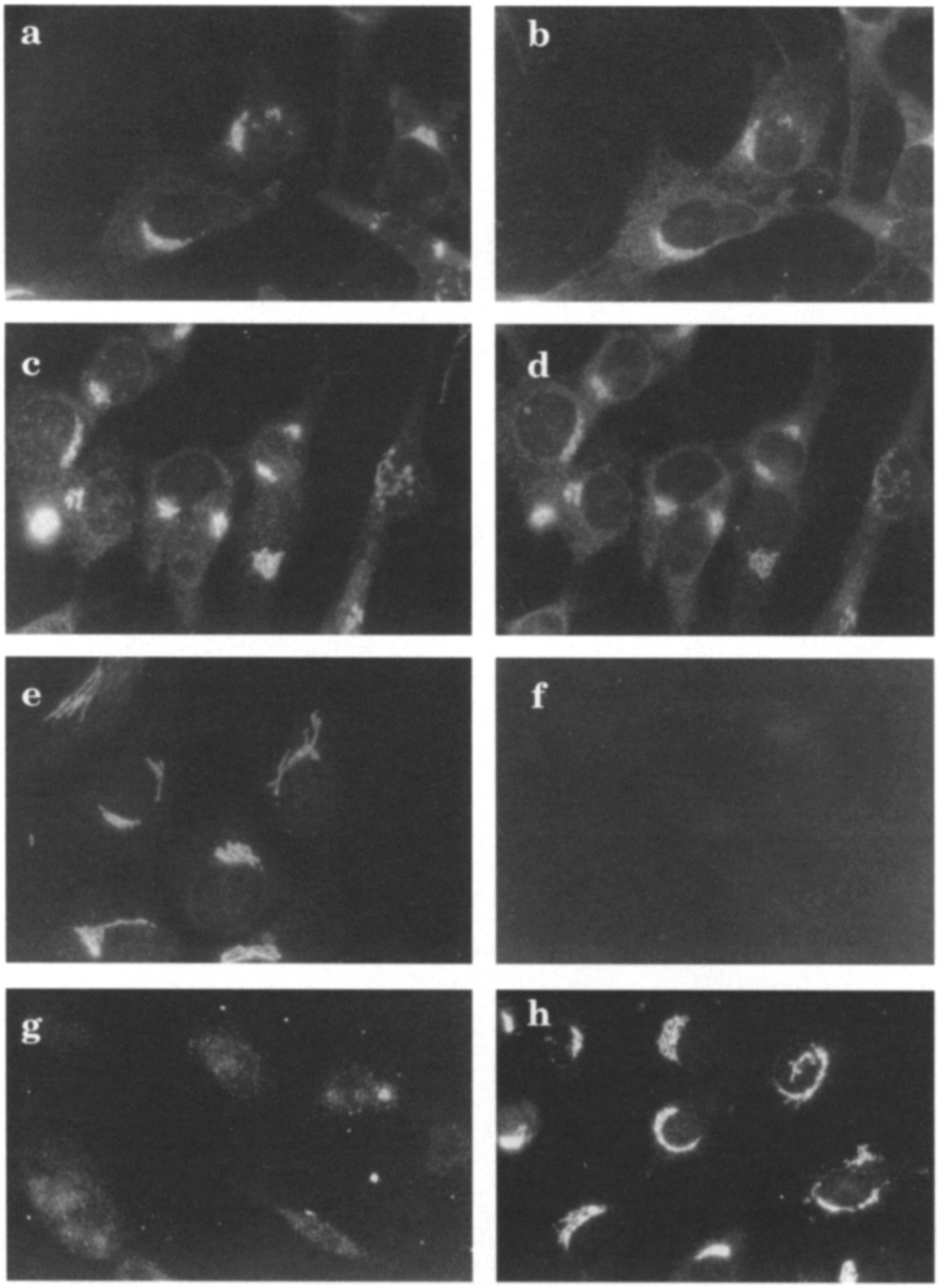

Figure 8. Cab45 antibodies give rise to a Golgi-like staining pattern in 3T3-cells. *a* and *b* show a double-labeling experiment with affinity purified anti-Cab45 antibody (visualized with a secondary LRSC-conjugated antibody (*a*) and an anti-Golgi antibody (FITC-conjugated secondary antibody (*b*). As both primary antibodies are rabbit IgG, an extra blocking step was introduced in this experiment as well as in all others in which two rabbit antibodies were used: This blocking step was a saturation with 100 μg/ml of affinity purified goat anti–rabbit IgG for 2 h after the incubation with LRSC-conjugated antibody. *c* and *d* show an analogous double labeling experiment (*c* anti-Cab45; *d* anti-Golgi) with cells that have been treated with 300 μM cycloheximide for 8 h. *e* shows another representative micrograph of cells stained for Cab45 after 8 h in the presence of cycloheximide. Note the condensed Golgi stacks in *c–e*, a phenomenon typically observed in cells that have undergone prolonged exposure to cycloheximide. *f* shows staining with pre-immune IgG's. The bottom two panels show an experiment with anti-Cab45 incubated in the presence of the immune peptide (*g*) or of another peptide from a different region of the protein (*h*) and visualized with a secondary LRSC-conjugated antibody.

ERGIC-53 accumulates in larger size vesicles (*f*), while, as in the experiment in Fig. 9, Cab45 redistributes into a myriad of small vesicles characteristic of the ER (*e*). The patterns of Cab45 in Cos cells before and after brefeldin A treatment appear slightly different from those in 3T3 cells (compare with Fig. 9, *a* and *c*), probably due to differences in the morphology of the organelles in the two cell types. The differential susceptibility to brefeldin A suggest that Cab45 and ERGIC-53 localize to distinct intracellular compartments.

Taken together, the immunofluorescence experiments (Figs. 8–11) establish that Cab45 resides in the Golgi compartment, and not in the ER or ERGIC. This is consistent with the results of the EndoH digestion studies (Fig. 7), which establish that Cab45 contains an N-linked oligosaccharide characteristic of a post-*medial* Golgi compartment.

Discussion

We have identified a novel calcium-binding protein that localizes to the Golgi compartment. It belongs to a new family of calcium-binding proteins residing within the secretory pathway. Members of this family share similar overall topology, have the same number of EF-hands, and are likely to be related functionally.

Unlike the two other members of the family, Cab45 does not contain an ER-retention signal that is found at the extreme carboxy terminus of resident ER proteins. Conventionally, this signal is the tetrapeptide K D E L

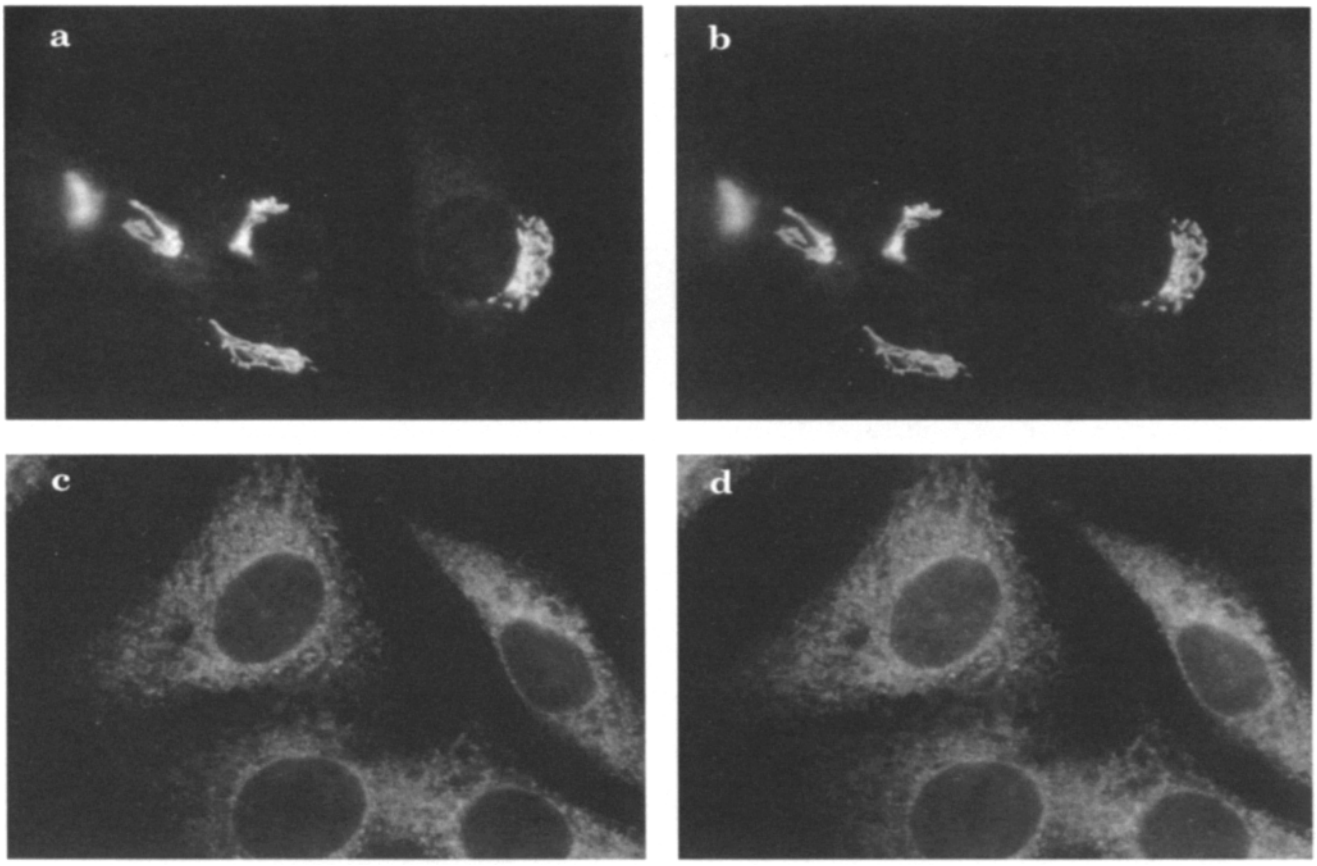

Figure 9. Cab45 co-localizes with the Golgi enzyme α-mannosidase II and resides in a brefeldin A–sensitive compartment. *a* and *b* show a double-labeling experiment using 3T3 cells with affinity-purified anti-Cab45 antibody (visualized with a secondary Cy3-conjugated antibody; *a*) and an anti-α-mannosidase II antibody (FITC-conjugated secondary antibody; *b*). *c* and *d* are similar to *a* and *b*, respectively, except the cells were treated for 3 h with 5 μg/ml brefeldin A. Upon treatment with brefeldin A, the typical Golgi pattern collapses into a diffuse punctate pattern characteristic of the ER, as seen both with α-mannosidase II (*d*) and Cab45 (*c*).

(Munro and Pelham, 1987). While a number of changes can be made at the -4 and -3 positions without abolishing ER retention activity, a very small number of conservative changes are tolerated in the -2 and the carboxy-terminal residue -1 (Andres et al., 1990, 1991). In particular, the lysine residue in -4 can be replaced by histidine without loss of activity. Even though the histidine at the -4 position was originally identified in a yeast ER retention signal, Erc55 was the first mammalian protein shown to terminate with -H D E L rather than -K D E L (Weis et al., 1994). The only mutation tolerated at the COOH-terminal -1 position is leucine to isoleucine, whereas substitution with a valine results in secretion rather than ER retention (Andres et al., 1991). Cab45 contains the carboxy-terminal tetrapeptide -H E E F. The residues in -4, -3, and -2 positions are found at the corresponding positions in some resident ER proteins. However, the phenylalanine residue at the carboxy terminus suggests that Cab45 should not localize to the ER. By a number of approaches we have demonstrated that Cab45 indeed does not localize to the ER, but rather to the Golgi.

Cab45 contains a single consensus sequence for N-linked glycosylation. We have shown that the Cab45 indeed undergoes N-linked glycosylation and thus is localized to the secretory pathway. As proteins undergo core-glycosylation in the ER, they become sensitive to the glycosidase EndoH. Upon leaving the ER, further modifications render the carbohydrates resistant to EndoH. Resistance is acquired after the action of Golgi mannosidase II in the medial Golgi. We have shown that Cab45 quickly loses sensitivity to EndoH, consistent with rapid processing and exiting from the ER to a post-medial Golgi compartment. Cab45 does remain sensitive to PNGaseF, a glycosidase that is not affected by further carbohydrate modifications occurring in the Golgi. Very little Cab45 is found in the tissue culture medium, consistent with the notion that Cab45 is retained in a post ER compartment and is not a secreted protein.

Staining of 3T3-fibroblasts with anti-Cab antibodies coincides with the staining observed with antibodies raised against a purified Golgi compartment (Louvard et al., 1982) as well as with antibodies specific for the medial Golgi marker enzyme α-mannosidase II. The staining profile of Cab45 does not overlap with the staining observed with antibodies raised against a purified ER compartment (Wada et al., 1991) or a specific resident ER protein, PDI. Even after exposure of the cells to cycloheximide for as long as eight hours, the intensity of immunofluorescence obtained with anti-Cab45 antibodies did not decrease significantly, suggesting that Cab45 protein is neither secreted nor degraded.

A major concern is potential localization of Cab45 to

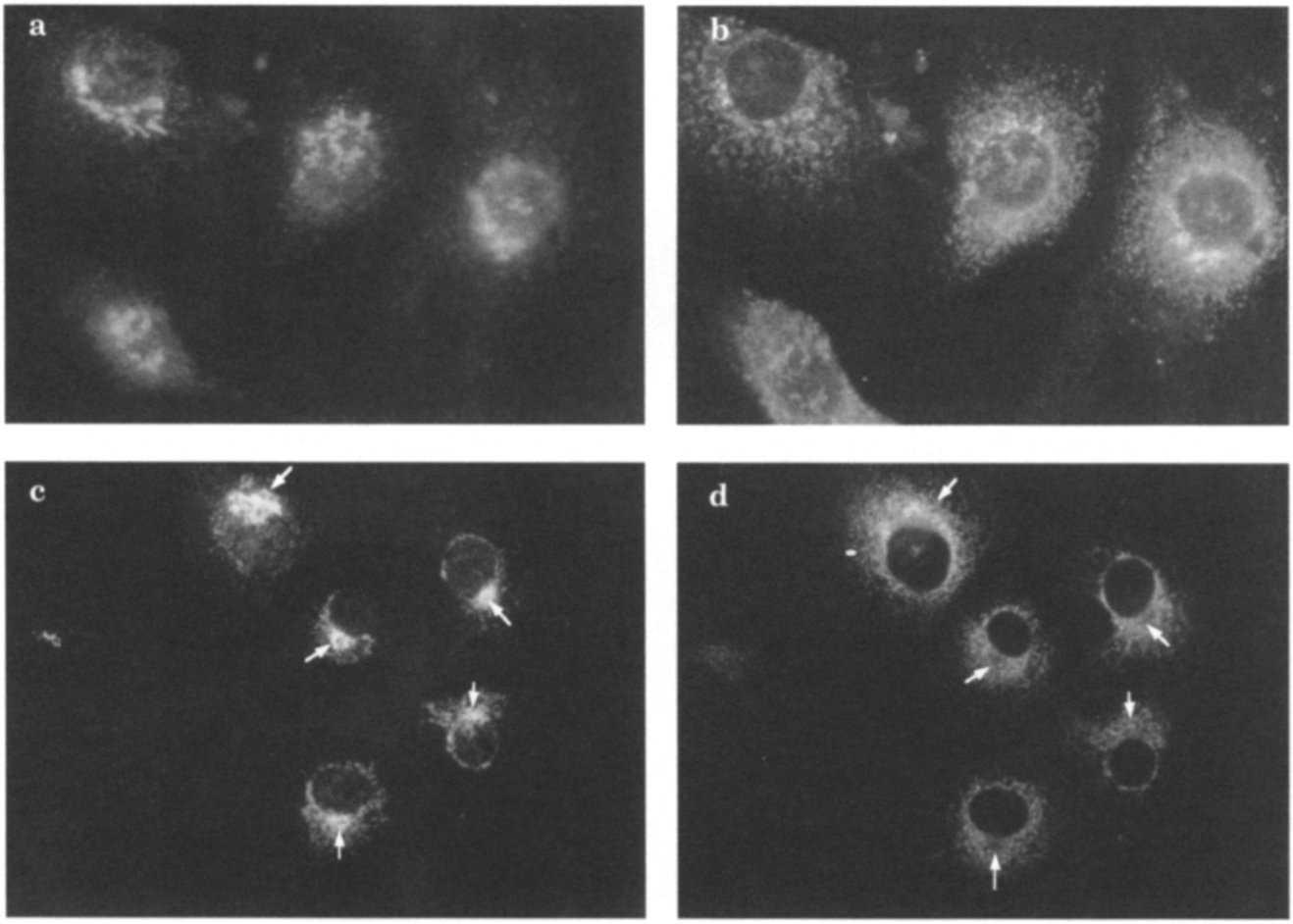

Figure 10. Cab45 does not co-localize with ER proteins. *a* and *b* show a double-labeling experiment using 3T3 cells and an affinity purified anti-Cab45 antibody (visualized with a secondary LRSC-conjugated antibody; *a*) and an anti-ER antibody (FITC-conjugated secondary antibody; *b*). *c* and *d* show an analogous double labeling experiment (*c*, anti-Cab45; *d*, anti-PDI) using Cos7 cells transfected with pcDNA1-Cab45b. Cells were analyzed 48 h after transfection. Arrows indicate areas labeled specifically with the anti-Cab45 antibody and specifically not labeled with the anti-PDI antibody.

the ER/Golgi intermediate compartment. Superficially, the distribution of a corresponding marker protein (ERGIC-53) is reminiscent of a Golgi pattern. However, three independent lines of evidence lead us to conclude that Cab45 does not reside in the ER/Golgi intermediate compartment. First, double-labeling experiments between Cab45 and ERGIC-53 show only partial, but not complete overlap. Second, Cab45 and ERGIC-53 display different susceptibilities to brefeldin A. While ERGIC-53 remains concentrated in large vesicular structures after brefeldin A treatment, Cab45 and also the medial Golgi enzyme α-mannosidase II redistribute into a disperse punctate pattern corresponding to an ER localization. Third, Itin et al. (Itin et al., 1995) recently introduced an N-glycosylation site into ERGIC-53. By all criteria tested this modified ERGIC-53 molecule functioned identically to the wild-type protein. In a pulse–chase experiment in Cos7 cells, <10% of this modified ERGIC-53 population acquired EndoH resistance within 1 h of chase, and even after 2 h of chase, <15% of the ERGIC-53 proteins had become EndoH resistant. This is in contrast to the situation with Cab45, where virtually all newly made proteins acquire EndoH resistance after only 20 min of chase. However, at the level of resolution of immunofluorescence, it is not possible to determine where within the Golgi compartment Cab45 is localized.

Cab45 is the first soluble protein reported to localize to the Golgi. Independent of its biological function, Cab45 is therefore an attractive candidate to test the hypothesis that there is a retention mechanism for soluble proteins in the Golgi compartment. It will be interesting to see whether a potential retention signal lies within a linear stretch of amino acids or whether a more complex tertiary structure is involved. The fact that Cab45 has a carboxy terminus reminiscent of an ER-retention signal but yet sufficiently distant to abolish interaction with the KDEL receptor is intriguing. It could hint to a mechanism for Golgi retention related to ER retention of soluble proteins, involving a Golgi receptor homologous to its ER counterpart. This receptor in turn could be a membrane-attached resident Golgi protein that irreversibly retains Cab45 in the Golgi. Alternatively, the receptor could be most abundant in the *trans*-Golgi network and be responsible for retrieval of Cab45 back into the Golgi, by analogy to the KDEL receptor which is most abundant in the ER-to-Golgi intermediate compartment and the *cis*-Golgi and whose pri-

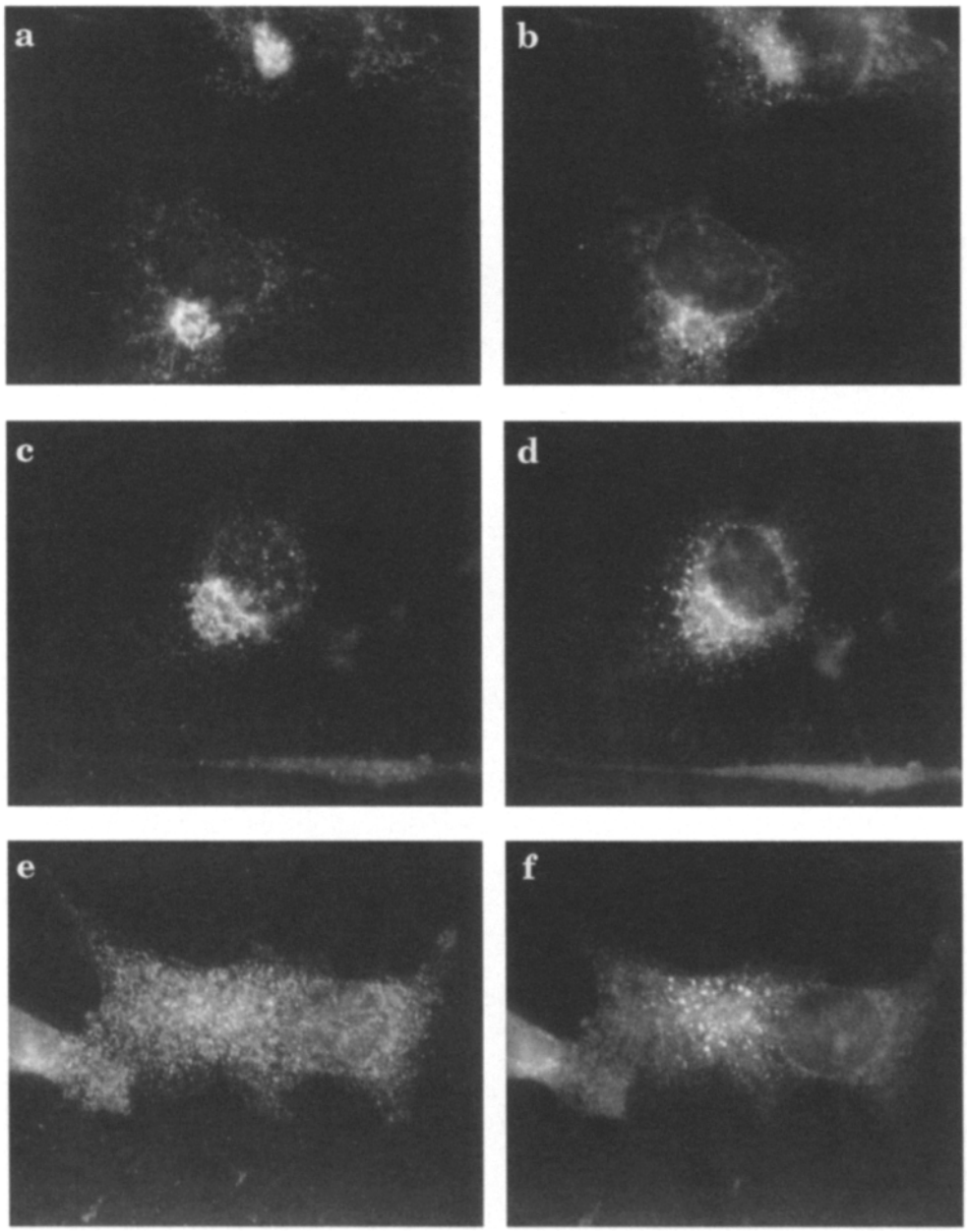

Figure 11. Cab45 and ERGIC-53 do not localize to the same compartment. Cos7 cells (transfected with pcDNA1-Cab45b) were labeled 48 h after transfection with either anti-Cab45 (*a*, *c*, and *e*; visualized with a secondary Cy3-conjugated antibody) or anti-ERGIC-53 (*b*, *d*, and *f*; visualized with a secondary FITC-conjugated antibody). Labeling was performed on either untreated cells (*a–d*) or cells pretreated with 5 μg/ml brefeldin A for 3 h (*e* and *f*).

mary function is thought to be retrieval of KDEL-containing proteins back to the ER.

It is difficult to speculate about the biological function of Cab45. No functions have so far been assigned to Reticulocalbin or Erc55, but as all three proteins are structurally closely related, we assume that they are also functionally similar. As Cab45 localizes to a different compartment than Reticulocalbin and Erc55, it is tempting to consider the possibility that there are functional requirements for this category of protein along the entire secretory pathway. This would make unlikely a direct involvement of these family members in an ER-specific process, such as protein folding. Similarly, Weis et al. exclude a direct involvement of Erc55 in ER–protein folding based on its relatively low abundance.

Cab45 will be instrumental both for understanding the functions of this protein family, as well as a valuable tool for dissecting the mechanisms of soluble protein retention in the Golgi.

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