

## CaBP1, a calcium binding protein of the thioredoxin family, is a resident KDEL protein of the ER and not of the intermediate compartment

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### SUMMARY

A cDNA encoding rat CaBP1 has been isolated and sequenced. The deduced polypeptide chain consists of 440 amino acids including two internal thioredoxin-like domains and a C-terminal KDEL retention/retrieval signal. Regarding the high degree of identity to the hamster protein P5, CaBP1 is considered to be the homologous rat protein. Previous work has suggested that CaBP1 is a resident luminal protein of the intermediate compartment (Schweizer, A., Peter, F., Nguyen Van, P., Söling, H. D. and Hauri, H. P. (1993) *Eur. J. Cell Biol.* 60, 366-370). Our conclusion that CaBP1 is a resident protein of the endoplasmic reticulum and not of the intermediate compartment is based on three different approaches: subcellular fractionation, indirect immunofluorescence and overexpression of CaBP1. Subcellular fractionation of Vero cells in a velocity controlled step gradient led to copurification of CaBP1-containing vesicles and several marker proteins for the ER including calreticulin and  $\alpha$ -SSRP. The intermediate com-

partment, as defined by a monoclonal antibody against the marker protein p53 (ERGIC-53), could be separated from these ER markers. Double immunofluorescence analysed by laser scanning microscopy showed no significant colocalization between CaBP1 and p53, but between CaBP1 and calreticulin. In additional experiments, Vero cells were infected with VSV tsO45. At 15°C the VSV-G protein accumulated in punctuate structures representing the intermediate compartment, while CaBP1 maintained its original reticular localization. Even after high-level overexpression in COS cells, CaBP1 was not detected in the intermediate compartment, but was efficiently retained in the ER as judged by light microscopy.

Key words: calcium binding protein (CaBP1), endoplasmic reticulum, intermediate compartment, p53/ERGIC-53, KDEL-retention signal

### INTRODUCTION

CaBP1 is a luminal calcium binding protein, which was first isolated from rat liver microsomes (Nguyen Van et al., 1989). Subcellular fractionation of rat hepatocytes indicated that CaBP1 might belong to the intermediate compartment (IC) between ER and Golgi apparatus (Peter et al., 1992). The IC or 15°C compartment was first described as a system of vacuoles located between the ER and the *cis*-Golgi that newly synthesized membrane and secretory proteins could enter but not leave at 15°C (Saraste and Kuismanen, 1984). Among other systems, the glycoprotein of the temperature-sensitive mutant tsO45 of vesicular stomatitis virus (VSV tsO45) has been used extensively for studies of ER to Golgi transport (reviewed by Balch, 1989; Bergmann, 1989). In Vero cells the IC is defined by a monoclonal antibody against a 53 kDa transmembrane protein (p53 or ERGIC-53) (Schweizer et al., 1988). At 15°C VSV tsO45-infected cells accumulated viral glycoprotein (VSV-G) and p53 in the same structures located between the ER and the Golgi apparatus (Schweizer et al., 1990). An isolation procedure for the IC (Schweizer et al.,

1991) led to a 40-fold enrichment of both p53 and CaBP1 compared to the homogenate. In addition, immunofluorescence microscopy seemed to show an overlapping distribution of CaBP1 and p53 (Schweizer et al., 1993a).

CaBP1 catalyses the renaturation of denatured reduced model proteins *in vitro* (Rupp et al., 1994) and binds calcium with high affinity (Nguyen Van et al., 1989). Since protein folding and calcium storage have been generally associated with the rough ER, the presence of CaBP1 in the IC would have interesting implications for the organization of the early secretory pathway. Besides the obvious involvement of the IC in vesicular transport between ER and Golgi, little is known about the functionality of this compartment. It is considered to be the site of reversible palmitoylation (Bonatti et al., 1989; Mundy and Warren, 1992; Schweizer et al., 1993b) and O-linked glycosylation (Tooze et al., 1988), and might be identical to a pre-Golgi degradation compartment (Tsao et al., 1992).

The retrieval of luminal proteins carrying a C-terminal KDEL sequence from post-ER locations has been shown to depend on the product of the ERD2 gene, the KDEL receptor

(Lewis and Pelham, 1992). Although the KDEL receptor has been found to accumulate in the IC together with the VSV tsO45-G protein at 15°C (Tang et al., 1993), at steady state it seems to be preferentially concentrated not in the intermediate compartment but rather in the Golgi complex (Lewis and Pelham, 1992). The presence in the IC of CaBP1, which carries a KDEL sequence at the C terminus (Peter et al., 1992), would indicate a complex retrieval/retention pattern that would allow different sorting of KDEL proteins. To date, all other KDEL proteins, including BiP, calreticulin and PDI (reviewed by Pelham, 1989), have been shown to be resident in the endoplasmic reticulum.

Regarding the question of whether the IC is a transient transport intermediate (Saraste and Svensson, 1991) or a permanent structure (Hauri and Schweizer, 1992), the presence of a resident luminal protein like CaBP1 would strongly suggest the latter possibility.

Failing to isolate the intermediate compartment biochemically using CaBP1 as a marker protein (unpublished data), we then re-examined the subcellular localization of CaBP1. In this report, we provide evidence that CaBP1 is not present in the intermediate compartment but is in the ER. First, we were able to separate p53 and CaBP1-containing vesicles biochemically by gradient centrifugation. Second, double immunofluorescence shows no detectable colocalization of p53 and CaBP1. Using a different approach, VSV tsO45-infected cells were incubated at 39.5°C and 15°C. The viral glycoprotein accumulated at 39.5°C in the ER and was codistributed with CaBP1, but after applying the 15°C block VSV-G moved into the intermediate compartment whereas CaBP1 remained in the ER. Third, even after overexpression, CaBP1 was retained in the ER and did not reach the p53 compartment in detectable amounts.

## MATERIALS AND METHODS

### Antibodies

Antibodies against rat liver CaBP1, rat liver calreticulin and the KDEL peptide were produced in rabbits according to standard procedures. Mouse monoclonal antibodies against p53 (mAb G1/93) (Schweizer et al., 1988) and p63 (mAb G1/296) (Schweizer et al., 1993b) were from Dr H. P. Hauri (Biocenter of the University of Basel, Basel, Switzerland). The goat antibody against canine calreticulin (used for the double immunofluorescence with CaBP1) was from Dr M. Michalak (University of Alberta, Edmonton, Canada). Antibodies against  $\alpha$ -SSRP were a gift from Dr T. A. Rapoport (Max-Delbrück-Center for Molecular Medicine, Berlin-Buch, Germany). Monoclonal antibody P5D4 against the C-terminal domain of VSV-G protein (Kreis, 1986) was supplied by Dr W. E. Balch (Scripps Research Institute, San Diego, CA, USA).

### cDNA cloning

Peptides and CaBP1 protein were sequenced by automated Edman degradation on a 477A Protein Sequencer (Applied Biosystems, Weiterstadt, Germany). Oligonucleotides were synthesized on a 381 A DNA Synthesizer. Restriction enzymes, M13mp18 and alkaline phosphatase from calf intestine were purchased from Pharmacia (Freiburg, Germany), pGEM4Z from Promega (Madison, Wisconsin, USA), *Escherichia coli* SURE and the  $\lambda$ ZAPII rat liver cDNA library from Stratagene (Heidelberg, Germany), T4 ligase from Gibco (Berlin, Germany) and the rTth DNA polymerase from Perkin Elmer Cetus (Weiterstadt, Germany).

CaBP1 was purified according to the method of Nguyen Van et al. (1989). Two degenerate oligonucleotides were constructed, corresponding to the N-terminal sequence of CaBP1 (LYSSDDVIELTP), as determined by direct sequencing of the purified protein, and to a peptide obtained by V8 protease digestion (LENALGIGGFGYPAM).

cDNA cloning was performed according to standard procedures (Sambrook et al., 1989). One positive clone (Kam 36.2) was obtained by screening a rat liver  $\lambda$ ZAPII expression library with a polyclonal rabbit anti-rat CaBP1 antibody and the two <sup>32</sup>P-labelled oligonucleotides. The 1.1 kbp insert of Kam 36.2 was subcloned into M13mp18 and sequenced by the dideoxy termination procedure (Sanger et al., 1977). It contained 3' sequences, coding for the C-terminal KDEL sequence followed by a stop codon but not the N-terminus of the rat protein. This information revealed a high homology to the previously published sequence of the hamster protein P5 (Chaudhuri et al., 1992). As we were interested in expressing the CaBP1 protein in COS cells, we decided to use nine amino acids of the signal sequence of the hamster P5 protein, including the starter methionine, to construct a full-length cDNA for CaBP1 with reverse PCR. Total rat liver RNA was prepared according to the method of Chomczynski and Sacchi (1987). First-strand synthesis and PCR were performed with rTth polymerase according to standard methods given by the supplier. The following primers were used: sense-primer B33 (5'-GAC TAT GGA TCC GAA TTC CCT CGG CCC GCC ATG GCT CGC CTT GGG TTC GGT CTG GTG-3') containing an *Eco*RI restriction site, the GC-rich Kozak sequence (Kozak, 1986) and the start codon; antisense-primer B 45 (5'-TCA ACG CGT AAG CTT TCA CAA CTC GTC CTT CTC TAG GTC-3'), which includes the stop codon and a *Hind*III restriction site. For partial sequences we used the additional primers B37 (sense), corresponding to bases 657-679, and B39 (antisense), corresponding to bases 557-581. The reverse PCR products were subcloned into pGEM 4Z for propagation, into M13mp18 for single-strand sequencing and into the mammalian expression vector pCMV2 (Anderson et al., 1989). The sequencing results were confirmed by sequencing the cDNA of two independent reverse PCR reactions using different total rat liver RNA preparations.

### Turbidimetric assay of the insulin disulphide reduction

CaBP1 was purified according to the method of Nguyen Van et al. (1989) except that after the Mono Q step a S 200 gel filtration column was used. The assay was performed with purified PDI (Lambert and Freedman, 1983) or CaBP1 as described (Holmgren 1979), except that reduced glutathione (GSH) instead of DTT (DL-threo-1,4-dimercapto-2,3-butandiol) was used. Briefly, 0.14 mM insulin was mixed with 0.28  $\mu$ M protein and the reaction was started by addition of 5.0 mM GSH. The insulin disulphide reduction was detected by measuring the extinction change at 578 nm.

### Subcellular fractionation

All steps of the fractionation procedure were carried out at 4°C. Vero cells were grown to confluency in 145 cm<sup>2</sup> Petri dishes (Greiner, Nürtingen, Germany) and washed with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.5). After adding 3 ml of PBS/dish, cells were collected with a rubber policeman and sedimented for 10 minutes at 70 g. The cell pellet was resuspended in HOP (homogenisation buffer: 130 mM NaCl, 50 mM Tris-HCl, pH 7.5, 30 mM KCl, 3 mM EDTA) and centrifuged again at 70 g for 10 minutes. After an additional wash with HOP, cells were gently resuspended in 0.5 ml HOP/100 mg wet weight and homogenized by passing through a syringe (5× 22G/0.7 mm, 5× 24G/0.55 mm, 2× 27G/0.4 mm). The homogenate was centrifuged for 10 minutes at 1,000 g and the supernatant recentrifuged under the same conditions. The resulting postnuclear supernatant was brought to 6 ml with HOP and layered on top of a step gradient (6 ml 30%, 4 ml 20%, 4 ml 15%, 4 ml 10%, 4 ml 7.5%, 4 ml 5%, 4 ml 2.5% (w/v) Nycodenz (Nycomed, Oslo, Norway) solutions in

HOP). After centrifugation in a SW28 rotor (Beckmann, Munich, Germany) for 15 minutes at 33,000  $g_{av}$ , nine fractions were collected from top to bottom.

#### Gradient characterization

Rotenone-insensitive NADH cytochrome *c* reductase (Sottocasa et al., 1967) for the ER, UDP-galactosyltransferase (Verdon and Berger, 1983) for the Golgi, alkaline phosphodiesterase (Aronson and Trouster, 1974) for the plasma membrane, and protein (Bradford, 1976) were measured according to standard procedures.

For analysis of p63 and luminal ER proteins (CaBP1, calreticulin and KDEL proteins), samples of each fraction were diluted with the same volume of HOP and centrifuged at 100,000  $g$  for 2 hours in a TLA45 rotor (Beckmann, Munich, Germany). The resulting pellets were analysed using 10% SDS-PAGE (Laemmli, 1970) and blotting onto nitrocellulose. The blots with CaBP1, calreticulin, the KDEL proteins and p63 were blocked with PBS-5% dry milk and incubated with primary antibodies (1:1000) followed by detection with peroxidase-coupled secondary antibodies using 4-chloro-1-naphthol and *N,N*-diethyl-*p*-phenylenediamine as substrates. The p63 blot was developed using the BM Chemiluminescence Western Blotting Reagents (Boehringer Mannheim, Mannheim, Germany).

The distributions of p53, and for direct comparison CaBP1 again, were examined by dotting 2.5  $\mu$ l each of the fractions of the Nycodenz gradient onto nitrocellulose (total fractions for p53 and sedimented fractions for CaBP1). After air-drying, the dot blots were processed as described above and developed with the BM Chemiluminescence Western Blotting Reagents (Boehringer Mannheim, Mannheim, Germany).

#### Immunofluorescence

Vero cells grown on coverslips were fixed with 3% paraformaldehyde in PBS at 4°C for 20 minutes and permeabilized with ice-cold acetone (2 minutes 50%, 3-4 minutes 90%, 2 minutes 50%) or methanol (-20°C, 2 minutes). Antibody incubations were for 1 hour at room temperature in 0.2% gelatine/PBS (20  $\mu$ l droplet incubation for p53 and p63). Secondary antibodies were coupled to fluorescein or tetramethylrhodamine (Dianova, Hamburg, Germany). Coverslips were mounted in Mowiol and viewed using a Zeiss Axioskop microscope equipped with a Pan-Neofluar  $\times$ 100/1.30 objective.

The red/green overlay images of CaBP1 vs p53 were obtained with a laser scan microscope (LSM 10, Zeiss, Oberkochen, Germany).

#### Viral infection

Vero cells were infected with vesicular stomatitis virus strain tsO45 (kindly provided by Dr K. Simons, EMBL, Heidelberg, Germany) at a multiplicity of 20-50 plaque forming units/cell for 45 minutes at 32°C in  $\alpha$ -MEM (Gibco, Berlin, Germany), 20 mM HEPES, pH 7.2. After 3 hours of incubation at 39.5°C in  $\alpha$ -MEM/5% fetal calf serum (FCS), cycloheximide (200  $\mu$ M) was added and the cells were transferred to 15°C.

#### Cell culture and transfection

Vero cells (African green monkey kidney cells, ATCC CCL-81) were grown in 250 ml culture flasks (Greiner, Nürtingen, Germany) in Dulbecco's minimal essential medium (DMEM, Biochrom, Berlin, Germany) supplemented with 10% FCS (Vitromex, Vilshofen, Germany) under standard tissue culture conditions. Vero cells used for the subcellular fractionation were obtained directly from Dr H. P. Hauri (Biocenter of the University of Basel, Basel, Switzerland).

COS cells (ATCC CRL-1650) were grown to 50% confluency in DMEM/10% FCS on coverslips. The transfections were carried out with plasmid pCMV2-CaBP1 DNA by the calcium phosphate method (Sambrook et al., 1989). The plasmid pCMV2 was a generous gift from Dr G. Thiel (Institute for Genetics, Cologne, Germany). After 24 hours the cells were processed for immunofluorescence.

## RESULTS

### Cloning and sequencing of a cDNA for CaBP1

The coding sequence of the cDNA of CaBP1 consists of 1320 bp and 440 amino acids, corresponding to a molecular mass of 49 kDa. The sequence includes the starter methionine, a hydrophobic ER import signal sequence, two thioredoxin-like motifs and the C-terminal KDEL retrieval signal (Fig. 1). The first nine of the 19 amino acids of the signal sequence correspond to the hamster P5 protein. However, as this sequence is cleaved off immediately after synthesis, it is without consequence for the structure and function of the mature protein. The first amino acid of the purified CaBP1 is Leu19 as determined by direct sequencing. Regarding the striking homology between CaBP1 and P5 (95% identity), we believe that they represent the same protein expressed in different species.

### PDI-like activity of CaBP1 in the insulin reduction assay

PDI and thioredoxin contain an enzymatic activity for protein disulphide reduction (Holmgren, 1979). To demonstrate the biochemical activity of the thioredoxin-like domains present in CaBP1, the reduction of insulin in the presence of GSH and CaBP1 was investigated. The reaction was followed by measuring the extinction at 578 nm caused by the reduced insoluble B-chain of insulin.

CaBP1 shows a protein disulphide reduction activity (Fig. 2) significantly above the background of spontaneous reduction without protein, which started only after about 100 minutes. The reduction of insulin in the presence of PDI was considerably faster than with CaBP1. The rate of insulin reduction using an ER protein without thioredoxin-like motif (grp94) was almost identical to the control reaction containing buffer instead of protein (not shown).

### Subcellular fractionation of Vero cells

Postnuclear supernatants of Vero cells were fractionated on a velocity controlled Nycodenz step gradient. Separation between the Golgi marker UDP-galactosyltransferase and the ER enzyme rotenone-insensitive NADH cytochrome *c* reductase was best at 33,000  $g$  for 15 minutes in a gradient volume of 36 ml. Under these conditions, sedimentation rate rather than density determines the separation pattern. Another advantage of this procedure - as compared to equilibrium density centrifugation - is that cytosolic proteins do not enter the Nycodenz-containing solution but stay on top of the gradient. This was verified by measuring lactate dehydrogenase activity, which was found to be restricted to fractions 1 and 2 (not shown).

The step gradient was designed specifically to ensure that the interface between different Nycodenz concentrations was recovered in the middle of each fraction. Fractions 7 and 8, which contain the highest activity of rotenone-insensitive NADH cytochrome *c* reductase (ER), therefore correspond to the 15/20% and 20/30% Nycodenz interfaces (Fig. 3A). Immunoblotting was used to identify the distribution of two additional marker proteins for the ER in the gradient:  $\alpha$ -SSRP, which is a transmembrane protein associated with the cotranslational translocation event (Görlich et al., 1990) and therefore considered to be a marker for the rough ER, and calreticulin, which is considered to be a luminal calcium storage protein of

CaBP1	<u>MARLGFGLVSCTFFLAVSALYSSSDVIELTPSNFNREVIQSDSLWLVEF</u>	50
P5	.....A.G.....N.....	50
CaBP1	YAPNCGGHCQRLTPEWKKAASALKDVVKVGAVNADKHQSLGGQYGVQGFPT	100
P5	.....T.....D.....	100
CaBP1	IKIFGANKNKPEDYQGGRTGEAIVDAALSALRQLVKDRLGGRSGGYSSGK	150
P5	.....S.....	150
CaBP1	QGRGDSKSKDVVELTDDTFDKNVLDSEVWVVEFYAPNCGGHCNLEPEW	200
P5	.....I.....D.....	200
CaBP1	AAAATEVKEQTKGKVKLAAVDATVNQVLASRYGIKGFPTIKIFQKGESPV	250
P5	·T.....N.....R.....A··	250
CaBP1	DYDGGRTSRSDIVSRALDLFSDNAPPELLEIINEDIAKKTCEEHQLCVVA	300
P5	.....V··M.....	300
CaBP1	VLPHILDTGATGRNSYLEVLLKLADKYKKMWGLWTEAGAQYELENALG	350
P5	.....-A.....I.....S.....	349
CaBP1	IGGGYPAMAAINARKMKFALLKGSFSEQGINEFLRELSFGRGSTAPVGG	400
P5	.....R.....GA.....	399
CaBP1	<u>GSFPNITPREPWDGKDGELPVEDDIDLSDELDDLEKDEL</u>	440
P5	····A··A····R····	439

Fig. 1. Amino acid sequence of the cDNA of CaBP1 and comparison with the Chinese hamster protein P5. The N-terminal signal sequence and the C-terminal retention/retrieval motif are underlined. The thioredoxin-like motives are shaded.

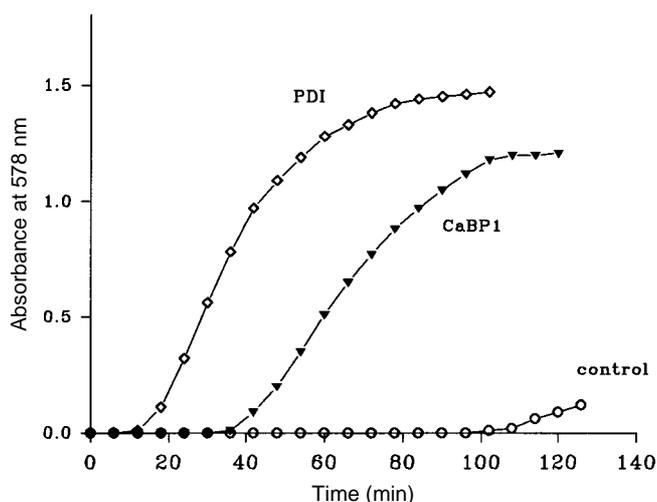


Fig. 2. Turbidimetric assay of insulin disulphide reduction: 0.14 mM insulin was mixed with 0.28  $\mu$ M PDI, 0.28  $\mu$ M CaBP1 or buffer and the reaction was started by addition of 5.0 mM GSH. The catalytic effect of CaBP1 is appreciable, but not as pronounced as with PDI.

the ER (reviewed by Michalak et al., 1992). Taking the three markers together, fractions 6 to 8 are enriched in ER vesicles (Fig. 3A,B). In addition, probing with an antibody against the tetrapeptide KDEL revealed two prominent proteins with apparent molecular masses of 60 and 80 kDa, which we believe to be identical with the abundant ER-resident proteins protein disulphide isomerase (PDI) and immunoglobulin heavy-chain binding protein (BiP/grp78). Regarding the obvious codistribution of CaBP1 with the various ER marker proteins, samples of fractions 7 and 8 were diluted with buffer and subjected to

an equilibrium density centrifugation. Again, CaBP1 could not be separated from the ER proteins (not shown).

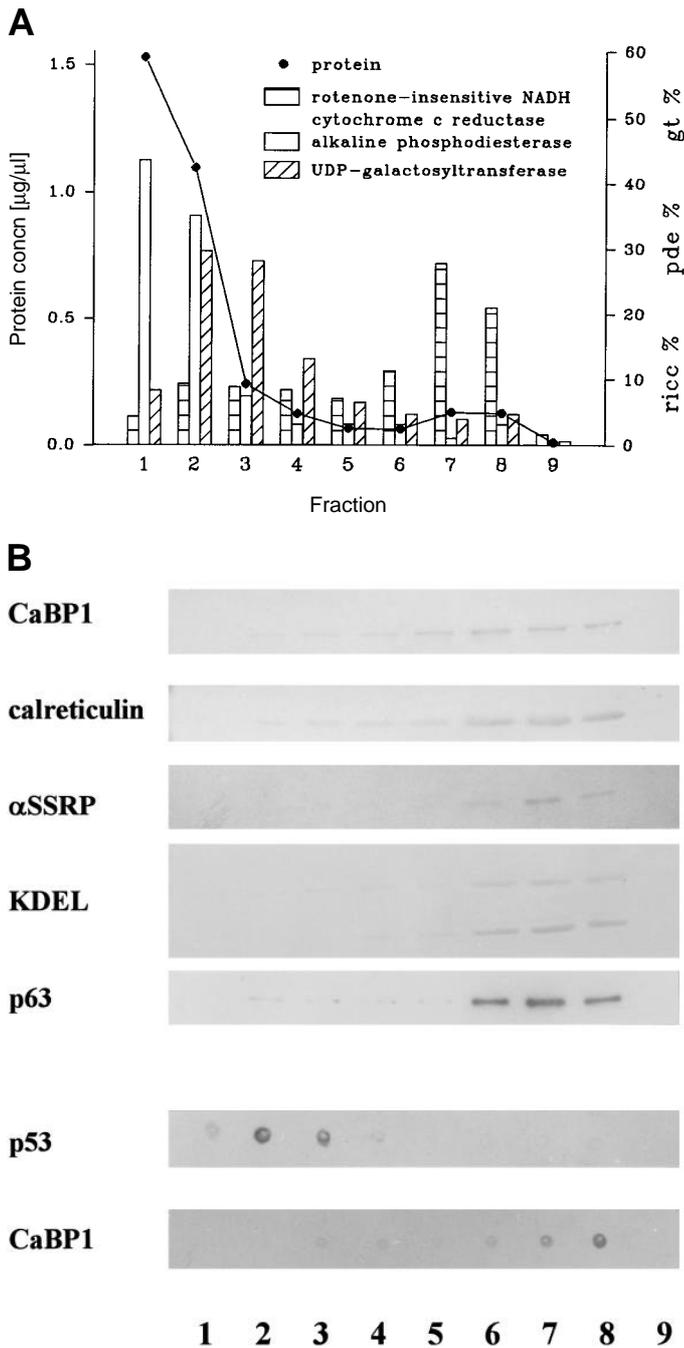
The distribution of p53 was analysed using a dot-blot procedure because the monoclonal antibody does not recognize the SDS-denatured protein (Schweizer et al., 1991). The localization of p53 (predominantly fraction 2) is strikingly different from CaBP1 (fractions 6 to 8) and is distributed under these conditions with the Golgi marker UDP-galactosyltransferase (Fig. 3). For better comparison, a dot-blot using an anti-CaBP1 antibody is also shown. Interestingly, the pattern of p63, which was reported to be a resident protein of the intermediate compartment (Schweizer et al., 1993b), shows an enrichment in the ER fractions and is clearly separated from p53, an established marker for the intermediate compartment (Fig. 3B).

Subcellular fractionation using a variety of methods (equilibrium density, flotation gradient, velocity controlled centrifugation and combined approaches) and different cell types (CHO, CHO 15B, NRK, COS) invariably led to a copurification of CaBP1 and the different ER marker proteins (results not shown). In our hands, Percoll gradient centrifugation (Schweizer et al., 1991) did not lead to a sufficient separation between ER and Golgi vesicles (not shown).

Regarding previous subcellular fractionation experiments, we now believe that the reason for the difference in distribution between CaBP1 and other KDEL proteins as reported by Peter et al. (1992) was due to the presence of artificially released luminal ER proteins.

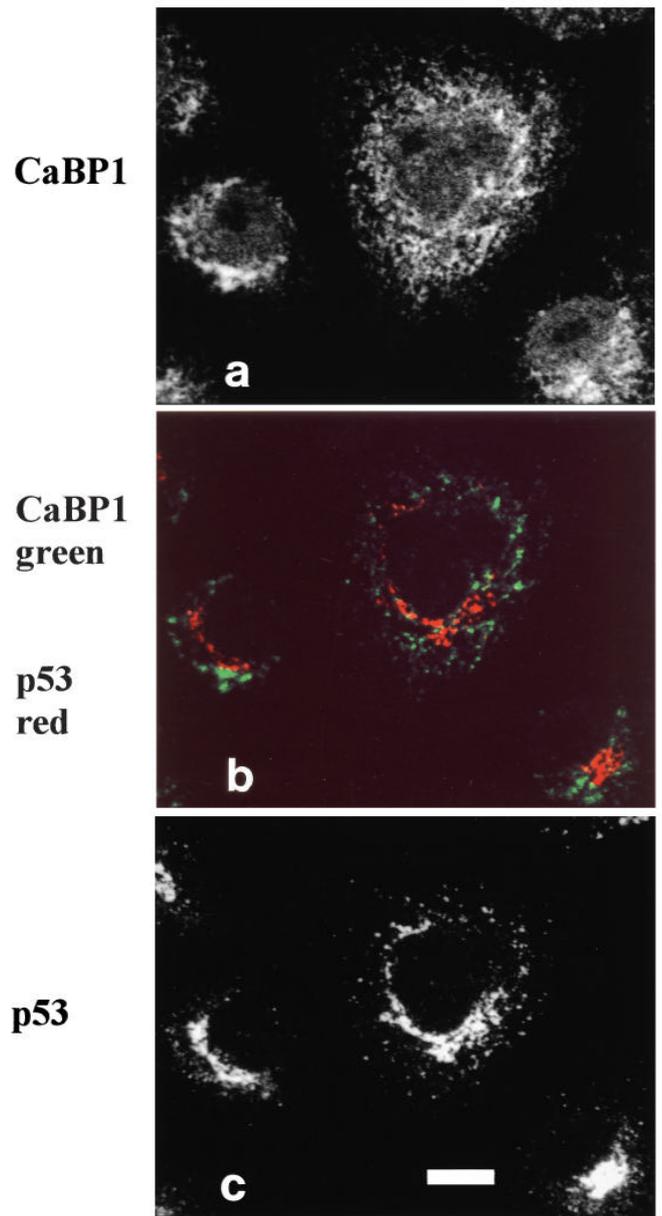
#### Double immunofluorescence of Vero cells

The distributions of CaBP1 and p53 in Vero cells were visualized by indirect double immunofluorescence using a monospecific polyclonal rabbit antiserum against CaBP1 and the monoclonal mouse antibody G1/93, which is specific for the intermediate compartment marker protein p53. Permeabiliza-



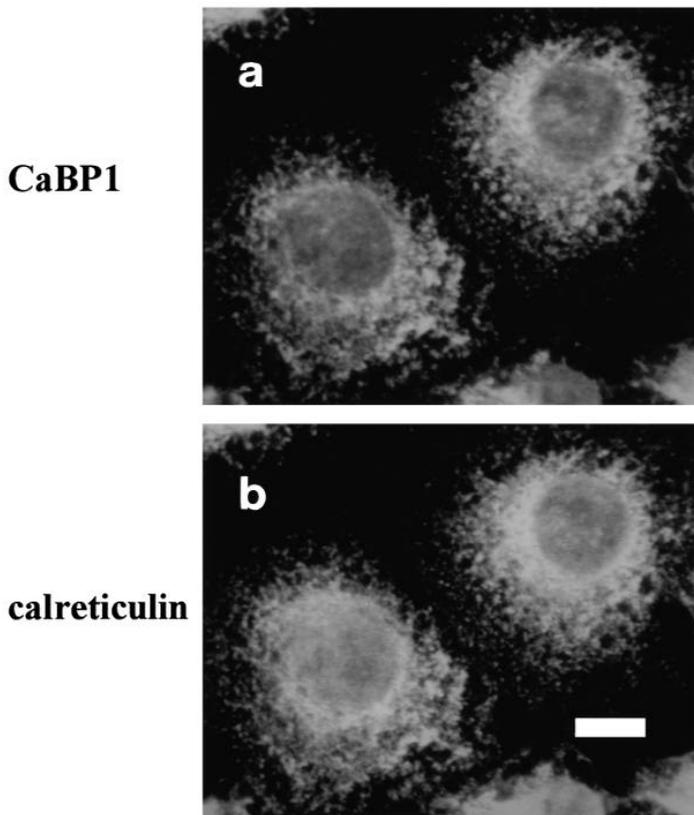
**Fig. 3.** Distribution of CaBP1, p53 and marker proteins on velocity controlled Nycodenz step gradients. Postnuclear supernatants from Vero cells were fractionated on 2.5% to 30% (w/v) Nycodenz gradients by centrifugation for 15 minutes at 33,000 *g*. The gradient was divided into nine fractions starting at the top. Enzymatic activities (A) of rotenone-insensitive NADH cytochrome *c* reductase (ricc), alkaline phosphodiesterase (pde) and UDP-galactosyltransferase (gt) were calculated as % of total activity present in the gradient. For details of immunoblot analysis (B) see Materials and Methods.

tion was performed with acetone or methanol, since in our hands saponin gave very poor staining of luminal ER proteins. p53 appeared as a bright perinuclear staining and punctate peripheral elements (Fig. 4c), which is in agreement with pre-



**Fig. 4.** Double immunofluorescence of Vero cells with antibodies against CaBP1 (a) and the intermediate compartment marker protein p53 (c). The images were obtained with a laser scan microscope and combined to give a coloured overlay (b; CaBP1, green; p53, red). Yellow colour would indicate a colocalization. Note that even without resorting to the confocal modus the two-dimensional picture does not show any significant colocalization between CaBP1 and p53. Bar, 10  $\mu\text{m}$ .

viously published results (Schweizer et al., 1988). On the other hand, CaBP1 showed an extended reticular pattern throughout the cytoplasm (Fig. 4a). The images were obtained with a laser scan microscope and combined to give a coloured overlay. The pattern of CaBP1 was coded in green, that of p53 in red (Fig. 4b). There is no significant colocalization detectable, rather it seems that the two proteins exclude each other. The distribution of CaBP1 looked very much like that of an ER protein, including an ER exclusion area with strongly reduced staining



**Fig. 5.** Double immunofluorescence of CaBP1 (a) and the ER marker calreticulin (b) in Vero cells. The images were obtained with a laser scan microscope. Bar, 10  $\mu$ m.

(Alcade et al., 1992) that is occupied by the Golgi apparatus and the p53 compartment.

Using an antibody raised in goat against the ER marker calreticulin, double immunofluorescence revealed a very similar distribution for CaBP1 (Fig. 5a) and calreticulin (Fig. 5b).

A new protein termed p63 has been described recently as another member of the intermediate compartment (Schweizer et al., 1993b). As the distribution of p63 in Vero cells gave a rather ER-like staining pattern (Fig. 6d), double immunofluorescence with the rough ER marker  $\alpha$ -SSRP was performed. Apart from the staining of the nucleus with  $\alpha$ -SSRP antiserum, both structures look very similar (Fig. 6c,d). In contrast, p53 and  $\alpha$ -SSRP did not overlap significantly (Fig. 6a,b). The difference in their distributions resembled that for p53 and CaBP1. Although anti-CaBP1 stained the same structures as anti-p63 (not shown), it seems to us that at the level of light microscopy a distinction between the ER and the p63 compartment is not possible.

#### Infection of Vero cells with VSV tsO45

The intermediate or 15°C compartment was first described as a system of vacuoles between the ER and the *cis*-Golgi, which newly synthesized viral membrane proteins can enter but not leave at 15°C (Saraste and Kuismanen, 1984). As the establishment of p53 as an intermediate compartment marker was based on the colocalization of p53 and the viral glycoprotein VSV-G at 15°C (Schweizer et al., 1990), we compared the dis-

tribution of CaBP1 with that of VSV-G protein at different temperatures (Fig. 7).

Strain tsO45 of vesicular stomatitis virus (VSV) contains a temperature-sensitive mutant of the membrane glycoprotein VSV-G, which accumulates in the ER at the restrictive temperature (39.5°C). At the permissive temperature (32°C) VSV-G is transported along the secretory pathway to the plasma membrane; however, at 15°C VSV-G is still able to leave the ER, but accumulates in the intermediate compartment.

After incubation of VSV tsO45-infected Vero cells for 3 hours at 39.5°C, double immunofluorescence showed a very similar pattern of ER-retained VSV-G and CaBP1 (Fig. 7a,b). However, after transferring the cells to 15°C for 1 hour, VSV-G left the ER and accumulated in the punctuate structures of the intermediate compartment, whereas the distribution of CaBP1 did not change significantly (Fig. 7c,d). Examination of VSV tsO45-infected NRK and CHO 15B cells gave the same results (not shown).

#### Overexpression of CaBP1 in COS cells

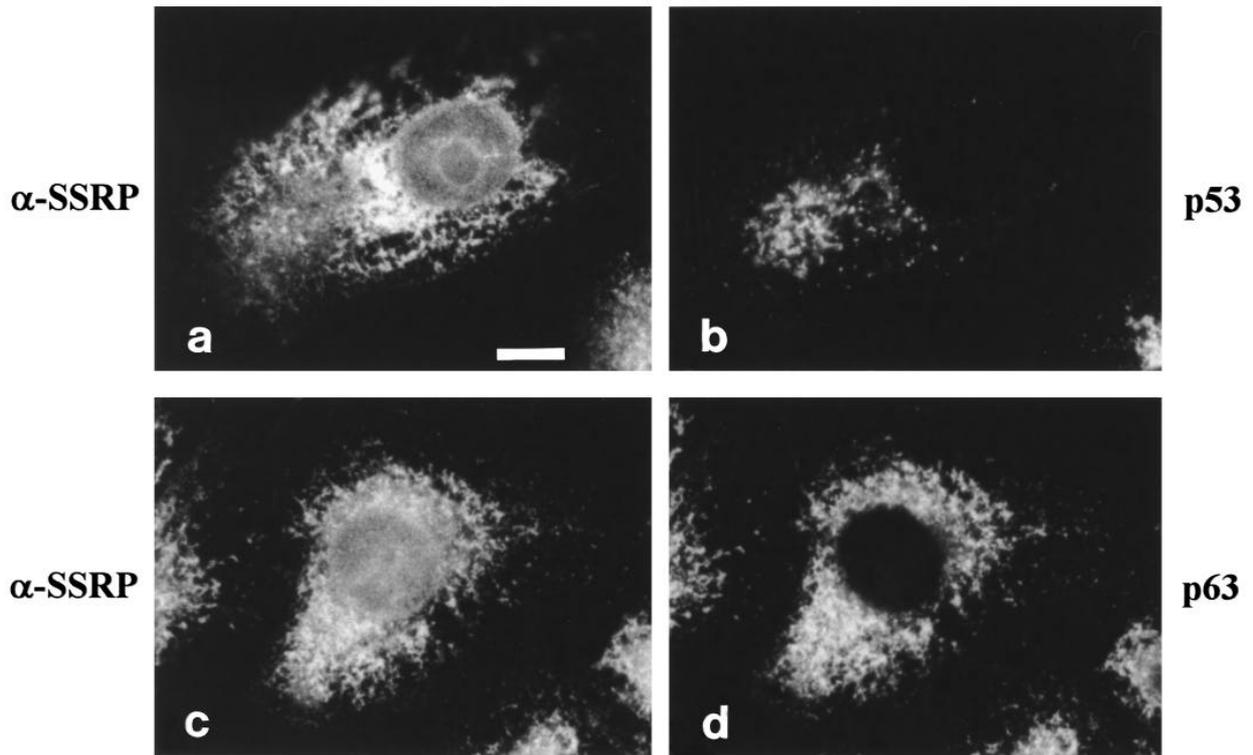
For transient expression of CaBP1 we used the pCMV2 vector with the promoter-enhancer region of the major immediate early gene of the human cytomegalovirus (Anderson et al., 1989). The Kozak sequence (CGG CCC GCC) was added just before the start codon of the CaBP1 cDNA (Kozak, 1986). In our hands the addition of this sequence resulted consistently in a higher expression level (data not shown).

In order to examine whether at least a small amount of CaBP1 might be present in the intermediate compartment, COS cells were transfected with CaBP1-pCMV2 DNA. We reasoned that if CaBP1 were really present in other organelles in addition to the ER, high overexpression might lead to its detection in these compartments. However, this was not the case. The transfected cells showed a reticular staining typical for ER with anti-CaBP1 serum (Fig. 8, green). The bulk of p53 (red) is resident in a perinuclear structure resembling the Golgi apparatus with only weak ER staining. The distribution of p53 seemed to be slightly variable as in some cells the distinct perinuclear structure was lost.

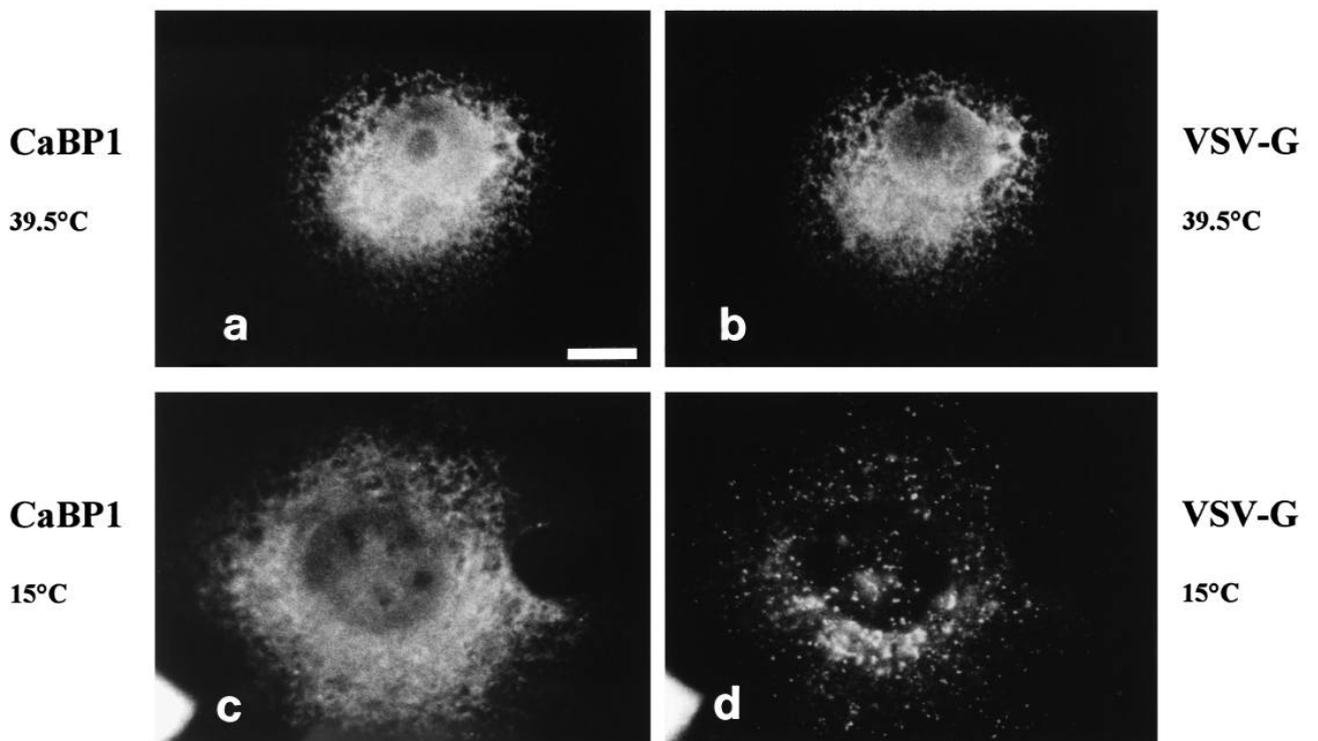
#### DISCUSSION

In this study we report the primary sequence and subcellular localization of CaBP1, a calcium binding protein of rat liver microsomes. The amino acid sequence deduced from the isolated cDNA is highly homologous (95% identity) to a previously described protein termed P5 cloned from a hydroxy-urea-resistant hamster cell line (Chaudhuri et al., 1992). Two internal thioredoxin-like motives are present in the CaBP1/P5 sequence. The suggestion that they are biochemically active is based on the ability of purified CaBP1 to catalyse the reduction of insulin (this study) and the refolding of denatured reduced model proteins such as ribonuclease AIII and the F<sub>ab</sub> fragment of a monoclonal anti-human creatine phosphokinase antibody (Rupp et al., 1994).

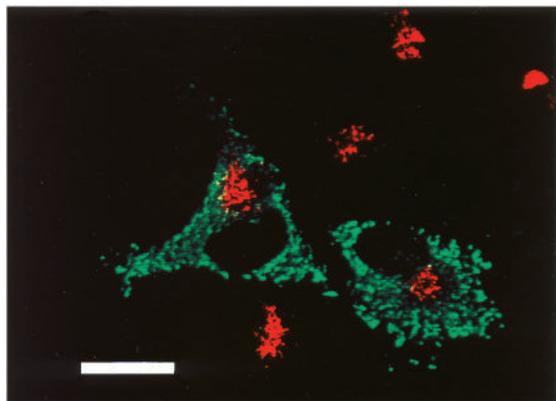
It seems that there are at least three members of the PDI family present in the ER: protein disulphide isomerase itself (reviewed by Freedman, 1989), ERp72/CaBP2 (Mazzarella et al., 1990; Nguyen Van et al., 1993) and CaBP1/P5 (Rupp et al., 1994; Chaudhuri et al., 1992). It remains to be shown



**Fig. 6.** Double immunofluorescence with antibodies against the rough ER marker  $\alpha$ -SSRP (a, c) and monoclonal antibodies against p53 (b) as well as p63 (d). Note that p53 is mostly present in an area with weak ER staining. Bar, 10  $\mu$ m.



**Fig. 7.** Vero cells were infected with VSV tsO45 and kept for 3 hours at the restrictive temperature (a,b). After addition of cycloheximide, cells were further incubated for 1 hour at 15°C (c,d). VSV-G protein (b, d) was accumulated in the ER (b; 39.5°C) and the intermediate compartment, respectively (d; 15°C). The distribution of CaBP1 (a, c) remained unaffected by the temperature shift. Bar, 10  $\mu$ m.



**Fig. 8.** Double immunofluorescence staining for CaBP1 and p53 in COS cells overexpressing CaBP1. The antiserum against CaBP1 was used at a dilution that permitted the recognition of transfected cells. The images were obtained with a laser scan microscope and combined to give a coloured overlay (CaBP1, green; p53, red). There is no apparent colocalization with p53 (two transfected cells are seen). Bar, 20  $\mu$ m.

whether this variety is due to different substrate affinities or preferred localization in different subcompartments of the ER.

The subcellular localization of CaBP1 was established by both biochemical and morphological approaches. Biochemical investigation of Vero cells using a sedimentation rate controlled gradient showed that CaBP1 was clearly separated from the intermediate compartment marker protein p53 but was recovered in the same fractions as several ER markers. At present we are not able to say why CaBP1 and p53 cofractionated in a gradient system consisting of two equilibrium density centrifugation steps (Schweizer et al., 1991, 1993a). In that type of gradient, the distribution of CaBP1 differed from rough ER markers such as PDI, BiP and ribophorin. We can only speculate that the homogenization procedures used may have led to different subpopulations of ER vesicles. While a successful separation of two proteins strongly argues for their presence in different (sub-) compartments, a cofractionation can always be the result of inadequate techniques. Therefore, we still cannot exclude the possibility that CaBP1 might be resident in a subcompartment of the ER that is not shared by all ER markers. However, we are convinced that the separation achieved between CaBP1 and p53 can only be the result of their presence in different compartments of the cell.

Morphological analysis by indirect double immunofluorescence using a laser scan microscope confirmed that CaBP1 and p53 are present in different compartments with no apparent overlapping structures. This is in contrast to previously published results (Schweizer et al., 1993a). On the other hand, CaBP1 and the ER marker calreticulin colocalized completely - at least at the level of light microscopy. As p53 was established as a marker for the intermediate compartment by colocalization with VSV-G protein at 15°C (Schweizer et al., 1990), we applied the same conditions to the analysis of the localization of CaBP1. Again, significant amounts of CaBP1 could not be detected in the intermediate compartment. In this context it is worth noting that both p53 and the KDEL receptor (Tang et al., 1993) colocalize with VSV-G at 15°C but show only a partial overlap in uninfected cells at 37°C (unpublished

results). This suggests that a protein that colocalizes with VSV-G at 15°C is not necessarily an adequate marker for the intermediate compartment at 37°C. This holds true especially if one regards the 15°C compartment as an accumulation of transport intermediates that might occur only in small amounts in uninfected cells at 37°C (Saraste and Svensson, 1991).

Recently, another protein termed p63 has been suggested to be a resident member of the intermediate compartment (Schweizer et al., 1993b). However, in our hands the subcellular fractionation of Vero cells showed separation of p63 from p53 and cofractionation with several ER markers. Moreover, double immunofluorescence with the rough ER marker  $\alpha$ -SSRP revealed very similar distributions of  $\alpha$ -SSRP and p63 that were quite distinct from that of p53. Further studies are needed to elucidate the exact subcellular localization of p63.

Overexpression of CaBP1 cDNA in COS cells resulted in an enhanced ER staining pattern but, even under these conditions, a significant colocalization with p53 was not observed.

Our conclusion that the ER protein CaBP1 is in a different compartment from p53 does not exclude the possibility of membrane continuity between both structures (Krijnse-Locker et al., 1994). Since the methods used here measure the steady state distribution of CaBP1 rather than its distribution kinetics, the possibility cannot be excluded that a small amount of CaBP1 may leak out of the ER into later compartments, including the intermediate compartment from where it could be retrieved following interactions with the KDEL receptor.

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