Molecular Cloning, Characterization, Subcellular Localization and Dynamics of p23, the Mammalian KDEL Receptor

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Abstract. We have isolated a cDNA clone (mERD2) for the mammalian (bovine) homologue of the yeast *ERD2* gene, which codes for the yeast HDEL receptor. The deduced amino acid sequence bears extensive homology to its yeast counterpart and is almost identical to a previously described human sequence. The sequence predicts a very hydrophobic protein with multiple membrane spanning domains, as confirmed by analysis of the in vitro translation product. The protein encoded by mERD2 (p23) has widespread occurrence, being present in all the cell types examined. p23 was localized to the *cis*-side of the Golgi apparatus and to a spotty intermediate compartment which mediates ER to Golgi transport. A majority of the intracellular staining could be accumulated in the intermediate compartment by a low temperature (15°C) or brefeldin A. During recovery from these treatments, the spotty intermediate compartment staining of p23 was shifted to the perinuclear staining of the Golgi apparatus and tubular structures marked by p23 were observed. These tubular structures may serve to mediate transport between the intermediate compartment and the Golgi apparatus.

ESIDENT proteins in the lumen of the ER generally have a COOH-terminal retention signal which is either the tetrapeptide KDEL[1] or an analogous sequence (see Pelham, 1990, for review). Deletion of this sequence resulted in the secretion of the truncated protein and appendage of the sequence to a secreted protein resulted in its retention in the ER (Munro and Pelham, 1987). There is considerable evidence suggesting that ER retention of KDEL-containing proteins is achieved by their selective retrieval from the bulk cellular traffic in a post-ER compartment (Pelham, 1988). Thus, proteins bearing this retention signal may leave the ER after the bulk flow of vesicular traffic but are specifically retrieved by a "salvage" pathway (Pelham, 1990).

The mechanism for the selective retrieval of KDEL proteins has not been elucidated. However, it can be envisaged that it would involve the recognition of the retention signal by a receptor, which must then target the protein to a retrograde transport pathway back to the ER. In yeast, the product of the *ERD2* gene appears to function as such a receptor. The gene encodes a 26-kd integral membrane protein whose abundance determines the efficiency and capacity of the yeast HDEL (analogous to the mammalian KDEL) retention system (Semenza et al., 1990). Furthermore, the specificity of the retention system is also determined by the *ERD2* gene product (Lewis et al., 1990).

A putative mammalian KDEL receptor has been identified by monoclonal antibodies raised against antibodies to carboxy-terminal KDEL sequences of two resident, soluble ER proteins (Vaux et al., 1990). These anti-idiotypic antibodies recognized a 72-kd integral membrane protein which was localized to an intermediate compartment between the ER and the Golgi apparatus and showed some characteristics expected of a KDEL receptor. The striking difference between this protein and the *ERD2* gene product is however, difficult to reconcile if the only functional difference was the recognition of KDEL instead of HDEL. It was subsequently shown that mammalian (human) cells do express transcripts encoding a protein similar in sequence and size to the yeast *ERD2* product, an epitope-tagged form of which was localized to the Golgi apparatus by immunofluorescence (Lewis and Pelham, 1990).

In this report, we describe the cloning of a bovine cDNA for the putative mammalian KDEL receptor. Antibodies raised against a peptide encoded by a carboxy-terminal sequence of this gene recognized a 23-kd integral membrane protein (p23) which is found ubiquitously in all the cell types examined and is localized to the *cis* side of the Golgi apparatus and an intermediate compartment which mediates ER to Golgi transport. The subcellular dynamics of this protein are characteristic of that expected of a KDEL receptor and suggest the existence of tubular structures mediating the trans-

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1. *Abbreviations used in this paper*: BFA, brefeldin A; CGN, *cis*-Golgi-network; KDEL, -Lys-Asp-Glu-Leu; PLP, periodate-lysine-paraformaldehyde; VSV, vesicular stomatitis virus.

port from the intermediate compartment to the Golgi apparatus.

Materials and Methods

Molecular Cloning and Sequencing

Polymerase chain reactions, λgt11 library screening and dideoxy sequencing of DNA fragments generated by nested deletion using exonuclease III were carried out as described (Sambrook et al., 1989). In vitro transcription and translation were done using the Promega kit (Promega Corp., Madison, WI) as described previously (Hong and Doyle, 1990).

Cell Culture, Transfection, Transient Expression and Viral Infection

MDCK (strain II) cells were kindly provided by Dr. K. Simons (European Molecular Biology Laboratory). Other cell lines used were obtained from the American Type Culture Collection (Rockville, MD). They were maintained in DME supplemented with 10% FBS. Stable transfection of cells was achieved by the calcium-phosphate precipitation method. Transfectants were pooled and maintained in DME with 750 μg/ml G418. Expression of the transgenes driven by the RSV-SV40 promoter-enhancer system (Low et al., 1991) was induced by culturing the cells overnight in DME with 1 mM sodium butyrate. Transient expression of transgenes was achieved by liposome-mediated transfection of Cos cells (Lipofectin; Bethesda Research Labs, Gaithersburg, MD) as described by the manufacturer. Transfected cells were incubated for 16 h in DME supplemented with 2% FBS, transferred to coverslips and incubated for another 50 h. Transgene expression was induced for 3 h before immunofluorescence. The temperature sensitive vesicular stomatitis virus VSV mutant strain (ts045) was kindly provided by Dr. Kai Simons. Infection of normal rat kidney cells and temperature block was carried out essentially as described by Bonatti et al. (1989).

Antibodies

Monoclonal antibodies to dipeptidyl peptidase IV were generous gifts from Dr. D. L. Mendrick (Harvard Medical School, Boston, MA). Polyclonal rabbit antisera to p23 were generated by immunizing rabbits with a synthetic peptide (conjugated to keyhole limpet hemocyanin) comprising the 21 amino acids at the COOH terminus of the protein sequence predicted from the mERD2 cDNA and affinity purified with the same peptide bound to a solid support. Mouse monoclonal antibodies against VSV G were kindly provided by Dr. Thomas Kreis (EMBL).

Immunofluorescence Microscopy

Immunofluorescence microscopy was carried out using previously described procedures (Low et al., 1991). Cells grown on coverslips were fixed with 2.7% paraformaldehyde and permeabilized with 0.1% saponin before sequential incubation with primary antibodies and anti-IgG antibodies coupled to FITC. Immunofluorescence microscopy was carried out using a Zeiss microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with epifluorescence optics as previously described (Low et al., 1991).

Immunoelectron Microscopy

Immunoelectron microscopy was performed essentially as described by Brown and Farquhar (1989). Briefly, cells grown on 30-mm dishes were fixed with periodate-lysine-paraformaldehyde (PLP) fixative for 3 h at room temperature, permeabilized with 0.02% saponin for 15 min and then incubated with affinity purified antibodies against p23 for 2 h at room temperature. The cells were incubated sequentially with biotinylated anti-rabbit IgG antibody, avidin-HRP, fixed with 1.5% glutaraldehyde and then incubated with a 0.2% DAB solution. The color reaction was initiated by addition of H₂O₂ and allowed to proceed for 15–30 min. The cells were then fixed with reduced osmium tetraoxide, dehydrated by a graded series of ethanol and embedded for sectioning.

Results

Molecular Cloning and Sequencing of a Mammalian Homologue (mERD2) of the Yeast ERD2 Gene and Characterization of the Gene Product

Degenerate oligonucleotide mixtures corresponding to regions of homology between the reported yeast and human sequences (Lewis et al., 1990; Lewis and Pelham, 1990) were used to generate a DNA fragment from reverse-transcribed mouse liver mRNA by the polymerase chain reaction. The resulting fragment was then used to screen λgt11 libraries of rat, mouse and bovine liver. Positive clones were sequenced. A bovine clone containing a coding region with 99.5% amino acid sequence identity to the reported human sequence was obtained (Fig. 1 a). The deduced protein sequence contains no putative N-linked glycosylation sites. A hydropathy plot (Fig. 1 b) of the derived polypeptide sequence revealed that the protein is extremely hydrophobic and contains several putative transmembrane regions. These predictions were confirmed by analysis of the in vitro translated product (Fig. 1 c). In vitro translation of the derived mRNA using rabbit reticulocyte lysate resulted in a polypeptide with a relative wt of 23 kD (hence, the name p23). Translation in the presence of canine microsomes did not change the molecular weight (lanes 1 and 2), indicating that the product does not have a cleavable signal peptide. Treatment with proteinase K resulted in a slight reduction in size (lane 3). This suggests that only a small fraction of the protein is exposed on the cytoplasmic side within the cell. Proteinase K treatment in the presence of 0.1% Triton X-100 however, resulted in total degradation of the product (lane 4). This can be explained by the permeabilization of the membrane by the detergent, resulting in the exposure of luminal portions of the protein to the protease. The fact that the protein remains insoluble in a high pH wash (lanes 5 and 6) and its partitioning to the detergent phase of Triton X-114 (data not shown) further suggest that it is an integral membrane protein.

p23 is Present Ubiquitously in Mammalian Cells

Synthetic peptides corresponding to the hydrophilic regions of the protein were synthesized, conjugated to keyhole limpet hemocyanin and used to raise antibodies in both mice and rabbits. A rabbit antiserum raised against one particular peptide corresponding to 21 residues at the COOH terminal region of the protein showed high specificity in preliminary immunoblot and immunofluorescence analysis. Specific antibodies were affinity-purified and used for all subsequent experiments described in this report.

The sequence conservation between the predicted human and bovine sequence suggests that the protein is evolutionarily conserved. To see if the product of mERD2 indeed exists and to demonstrate its conservation at the protein level, immunoblot analysis was performed with six cell lines derived from five mammalian species (Fig. 1 d), all of which had a band of varying intensities with a relative molecular weight of 23 kD. MDCK cells transfected with mERD2 cloned into the high expression vector pRSN (Low et al., 1991) (lane 5) showed a marked increase in the protein level over the wild type (lane 4). The widespread occurrence of the gene product (p23) was confirmed by immunofluorescence microscopy.
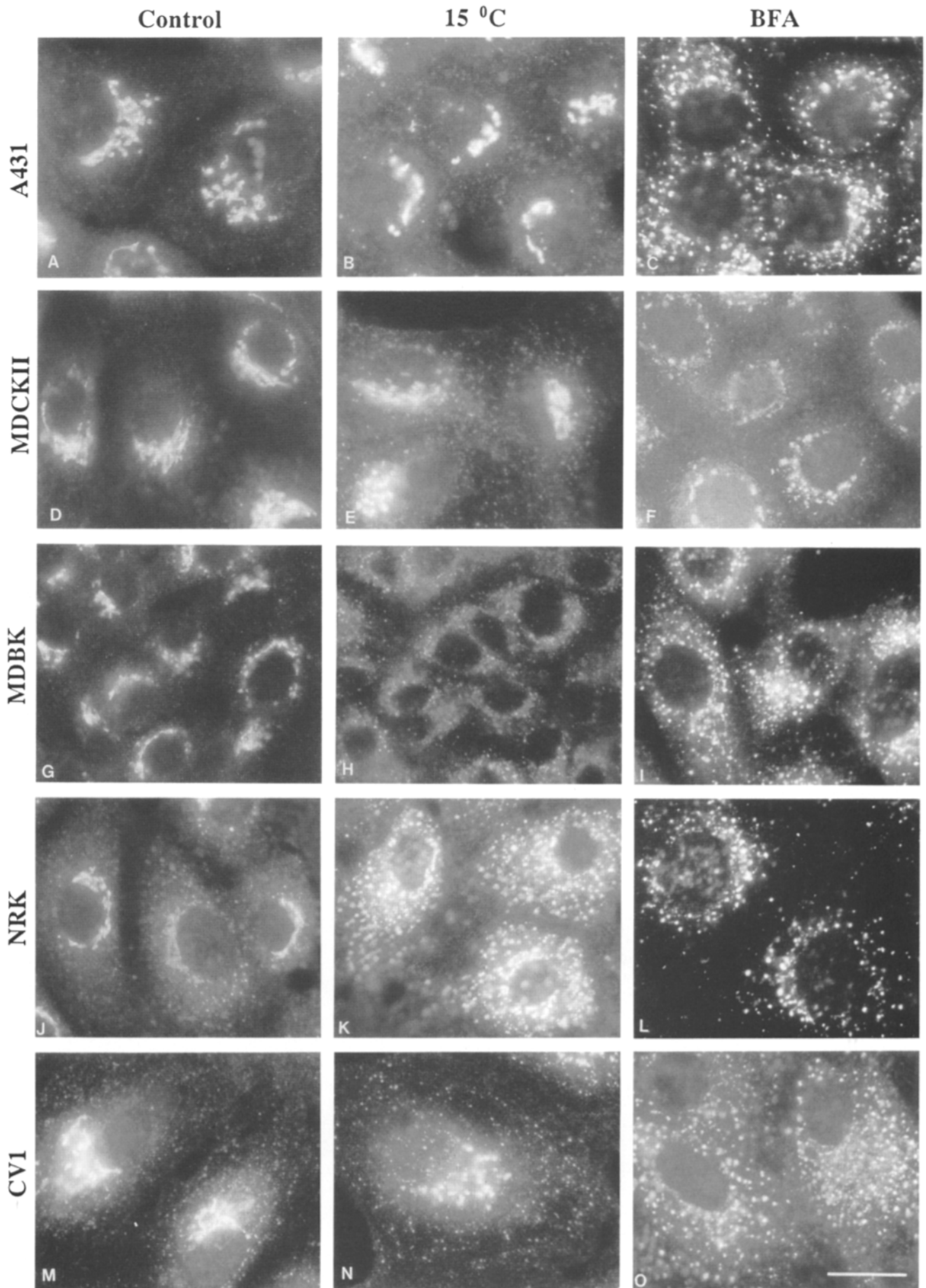
a

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                                                 50
MNLFRFLGDLSHLLAIILLLLKIWKSRSCAGISGKSQVLFAVVFTARYLD
.................................................
                                                100
LFTNYISLYNTCMKVVYIACSFTTVWLIYSKFKATYDGNHDTFRVEFLVI
................................................V
                                                150
PTAILAFLVNHDFTPLEILWTFSIYLESVAILPQLFMVSKTGEAETITSH
.................................................
                                                200
YLFALGVYRTLYLFNWIWRYHFEGFFDLIAIVAGLVQTVLYCDFFYLYIT
.................................................
         212
KVLKGKKLSLPA*
............
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b

c

d

Figure 1. (a) Amino acid sequence deduced from the cDNA sequence of mERD2. Also shown are residues of the previously reported human sequence (Lewis and Pelham, 1990) which differ from the bovine sequence. (b) Hydropathy plot of the mERD2 polypeptide using the Kyte-Doolittle algorithm. (c) Analysis of the in vitro translated product of mERD2. M, molecular weight markers; (lane 1) translation without canine pancreatic microsomes; (lane 2) translation with microsomes; (lane 3) proteinase K treatment; (lane 4) proteinase K treatment in 0.1% Triton X-100; (lane 5) supernatant of pH 11 sodium carbonate treatment; (lane 6) pellet of pH 11 sodium carbonate treatment. Translation for products in lanes 2-6 were carried out in the presence of canine pancreatic microsomes. (d) Immunoblot analysis of p23 in various cells and tissue samples. (M) molecular weight markers; (lane 1) rat liver total membrane; (lane 2) rat kidney total membrane; (lane 3) canine pancreatic microsomes; (lane 4) MDCK (II) cells; (lane 5) MDCK (II) cells transfected with mERD2; (lane 6) A431 cells; (lane 7) CV1 cells; (lane 8) MDBK cells; (lane 9) NRK cells; (lane 10) C6 (rat glioma) cells.

Subcellular Localization of p23

p23 was localized in several cell lines by immunofluorescence microscopy (Fig. 2). Three different types of intracellular staining were discernible, the relative intensities of which varied in the different cell types. All the cell types showed a bright perinuclear staining characteristic of the Golgi apparatus. In addition, the cells had bright spotty staining that was present throughout the cytoplasm. These spots were particularly apparent in NRK and CV1 cells. Lastly, weak ER staining could be seen in NRK and CV1 cells.

To determine more precisely the nature and location of the perinuclear and spotty stainings, cells were examined after low temperature (15°C) or brefeldin A (BFA) treatment. We were guided by previously reported findings that low temperature treatment had varying effects on proteins localized to different parts of the secretory pathway. It changes the perinuclear staining of a cis-Golgi protein, gp58 (Saraste and Svensson, 1991) and the intermediate compartment marker, p53, into spotty stainings with no apparent effect on the trans-Golgi protein, galactosyltransferase (Lippincott-Schwartz et al., 1990). BFA induces redistribution of Golgi markers like mannosidase II, galactosyltransferase (Lippincott-Schwartz et al., 1990) and sialyltransferase (Tang et al., 1992) but not gp58 (Saraste and Svensson, 1991) or p53 back to the ER.

As detailed in Fig. 2, a 15°C treatment has two somewhat different effects on the distribution of p23 in the cells examined. In all cell types, 15°C treatment increased the spotty staining to varying extents. This effect was most prominent in MDBK and NRK cells. The perinuclear staining was concomitantly reduced. For the other cell types, however, the perinuclear stainings were either unaffected (CV1) or became more condensed (A431 and MDCK). The changes in the staining pattern of p23 after 15°C treatment therefore resembled that of gp58 and p53, depending on the cell type examined.

Treatment of the cells with BFA had the general effect of dispersing the perinuclear staining into spotty stainings, similar but not identical to that seen in the 15°C treatment. All the cell types were apparently affected in very much the same way. The BFA effect on p23 was therefore different from its general effect on Golgi markers, but rather resembled that of the CGN markers, gp58 and p53.

Confirmation of the nature of the spotty stainings was attempted. It has been previously reported that incubation of cells infected with a temperature sensitive mutant of vesicular stomatitis virus (VSV) (Bonatti et al., 1989) and Semiliki Forest virus (Saraste and Kuismanen, 1985) at the nonpermissive temperature prevented the exit of newly synthesized membrane proteins from the ER. The VSV G protein accumulated at 15°C in spotty structures distinct from both the ER and the Golgi apparatus. These spotty structures were proposed to be an intermediate compartment involved in ER-Golgi transport (Bonatti et al., 1989). NRK cells grown on coverslips were infected with a temperature sensitive mutant of VSV (ts045) defective in ER to Golgi transport at a nonpermissive temperature. Infection was carried out at a nonpermissive temperature of 39.5°C and then shifted to 15°C. Double immunofluorescence labeling for both p23 and VSV G was performed before and after the 15°C shift. As shown in Fig. 3, VSV G was localized to the ER at 39.5°C whereas p23 remained as its normal perinuclear and spotty staining. In some cells expressing high levels of VSV G, the perinuclear staining of p23 was somewhat reduced. When the cells were shifted to 15°C for 2 h, the staining for VSV G

Figure 2. Immunofluorescence analysis of p23 in A431, MDCK(II), MDBK, NRK, and CV1 cells under normal growth conditions (control), after a 15°C or brefeldin A (BFA) treatment. Cells grown on coverslips were fixed and incubated with affinity-purified p23 antibody followed by goat anti-rabbit IgG conjugated to FITC (bar, 10 μm).
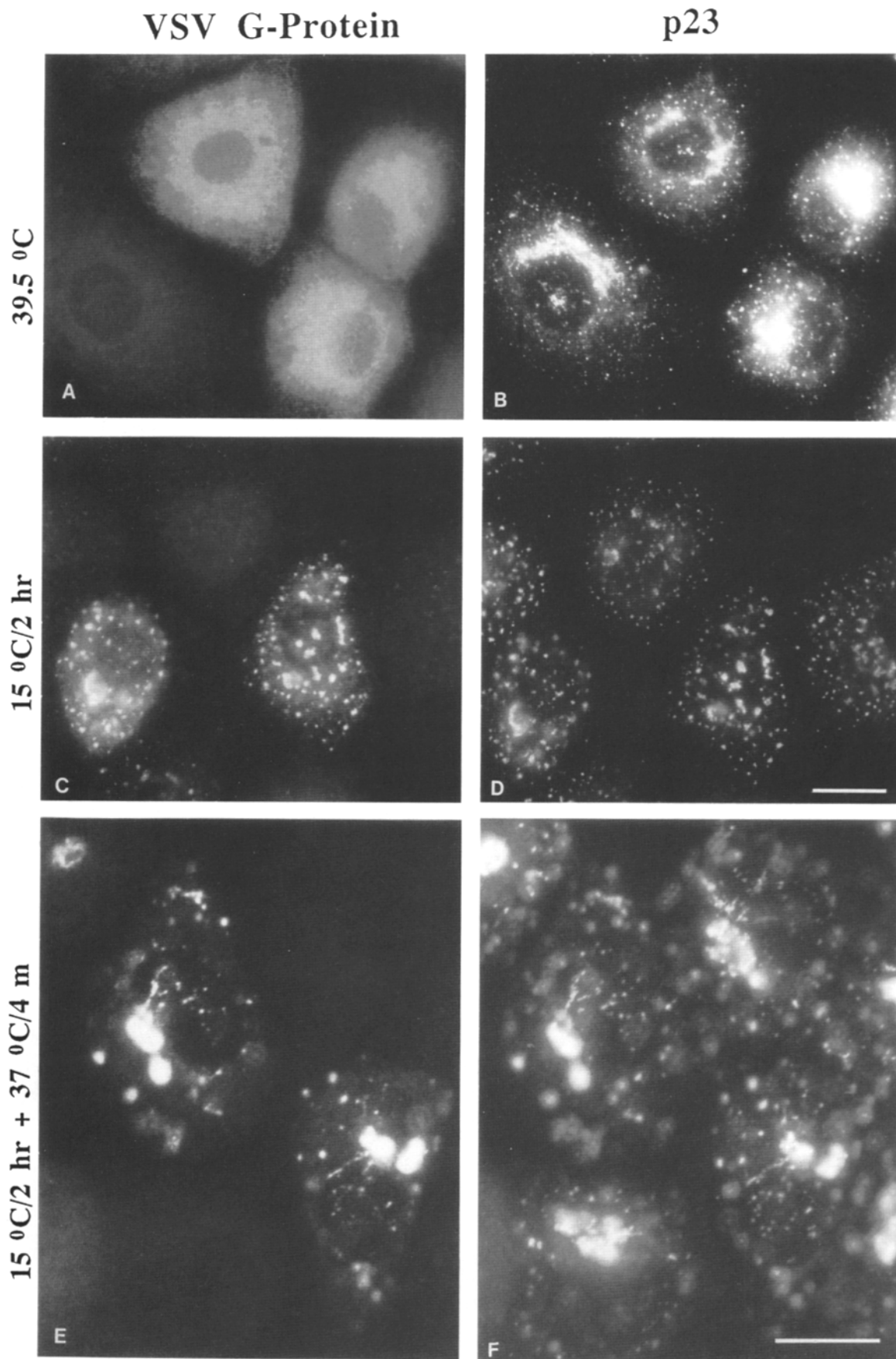
Figure 3. Double staining of p23 and VSV G protein in virus-infected NRK cells at the nonpermissive temperature (39.5°C), after a 2 h, 15°C treatment and after release (37°C for 4 min) from the 15°C block. NRK cells grown on coverslips were infected with VSV at the nonpermissive temperature for 2.5 h, subjected to the various treatments and then stained for both p23 and VSV G. Note that 39.5°C VSV G is localized to the ER (*A*). With the 15°C block, both p23 and VSV G stain the spotty intermediate compartment (*C, D*). Upon warming up to 37°C, the perinuclear as well as tubular structures stained by both antibodies colocalized well (*E, F*) (bar, 10 μm).
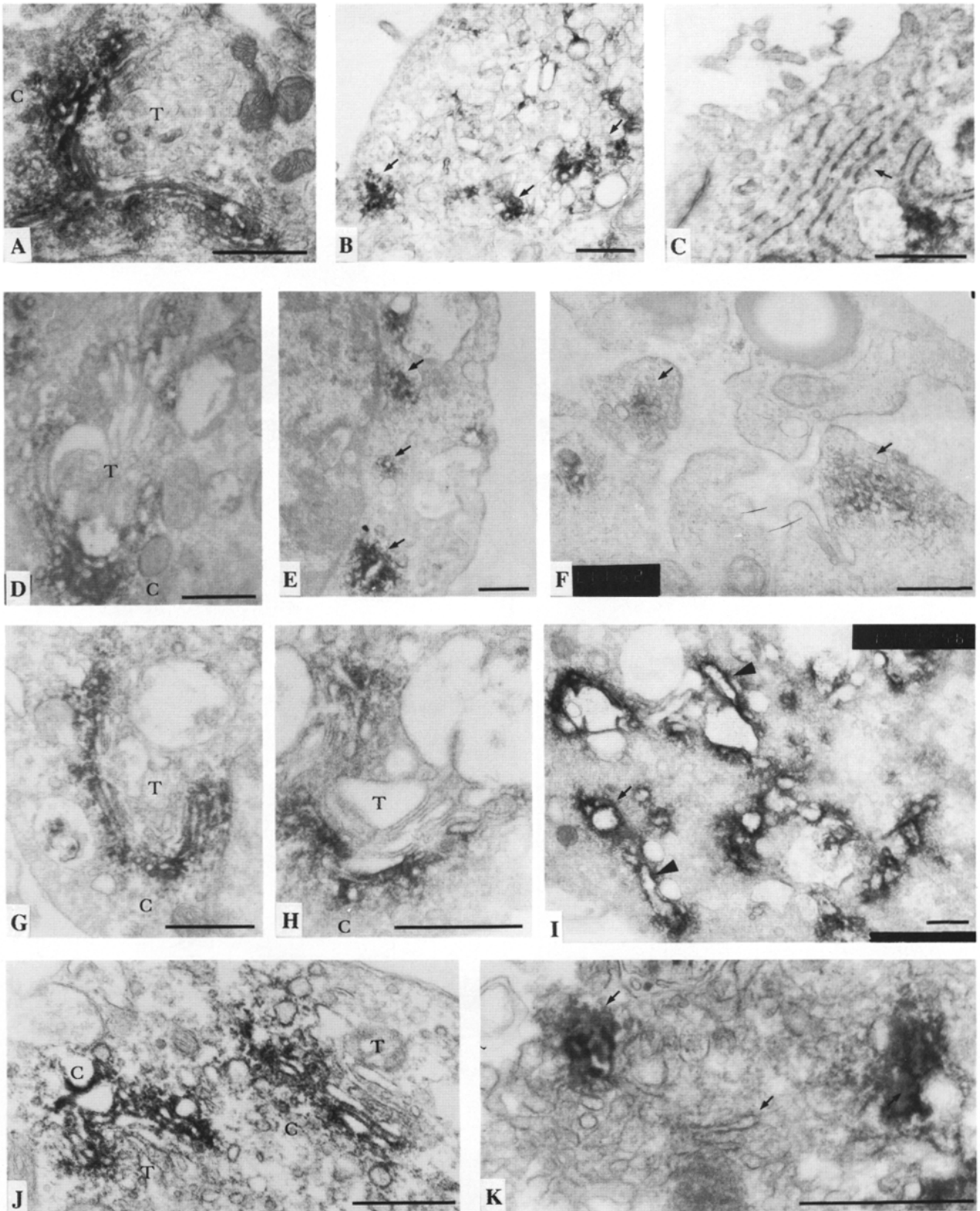
Figure 4. Immunoperoxidase staining of the various structures stained by p23 antibody examined under the electron microscope (70–90-nm sections for all frames and 200-nm sections for *I*, bars, 0.5 µm; *C*, *cis*-Golgi; *T*, *trans*-Golgi). Control NRK cells show all types (CGN-A, spotty-B and ER-C) of staining (*arrows*). Upon 15°C treatment, both CGN staining (*D*) and spotty staining (*E*) were apparent. The latter appeared to be vesicular-tubular structures which were also abundant in BFA treated cells (*F*). Upon recovery from both 15°C and BFA treatment, the spotty stainings were now found in the CGN (*G, H*). Stainings of tubular structures that was apparent during recovery from 15°C treatment are shown in *I* (*arrowheads*). Also shown are CGN stainings of MDCK II cells (*J*) and the tubular-vesicular staining of rat liver sections (*K*). DAB reactions were carried out for 30 min for *A, B, C, I,* and *K*, and 15 min for the rest.
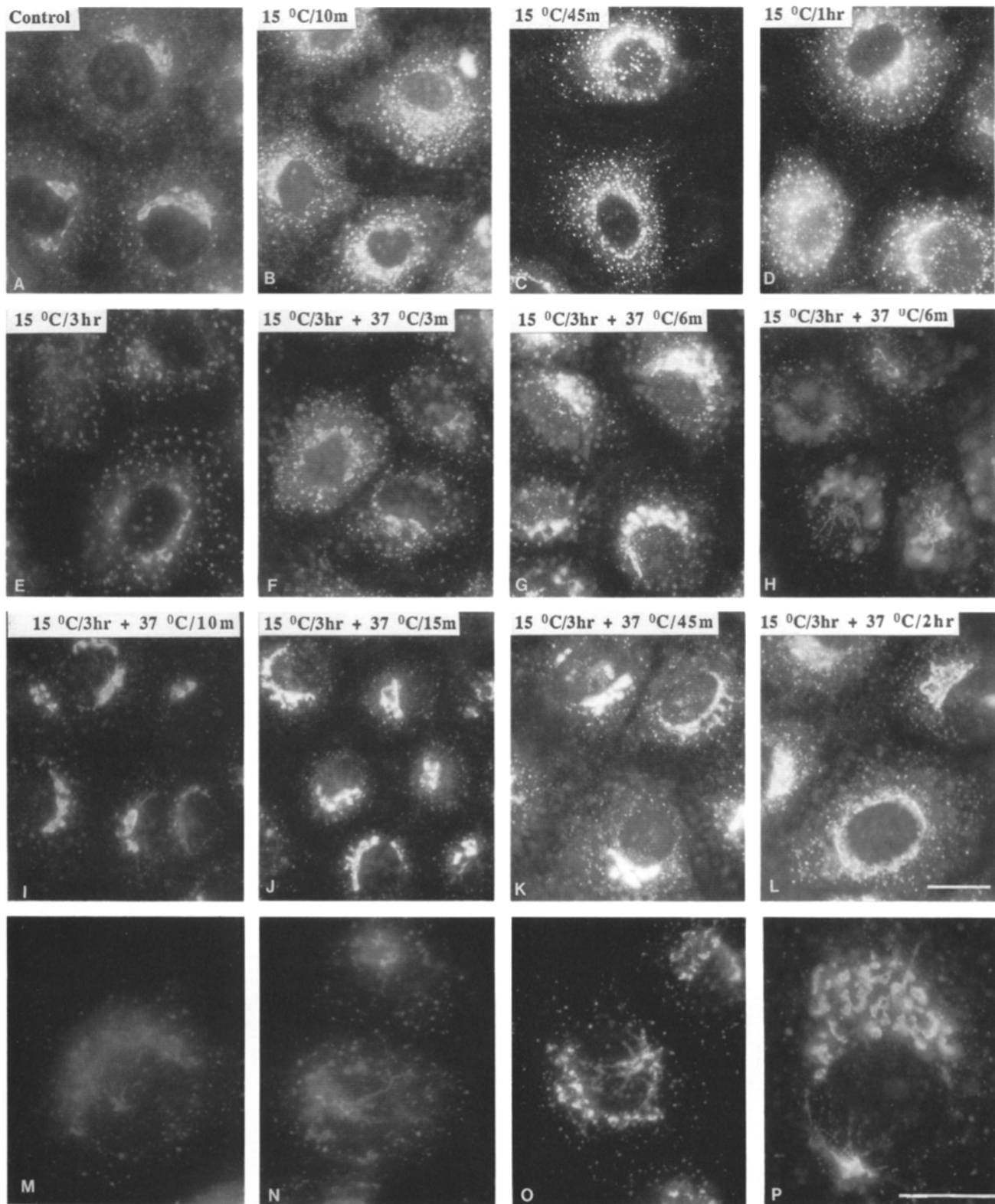
Figure 5. Time course analysis of NRK cells during 15°C treatment and recovery from the 15°C block by immunofluorescence. Note that ER staining appeared during the earlier time points of 15°C treatment (10 min, 45 min and 1 h) but disappeared after prolonged treatment (3 h). During recovery from the 15°C treatment, the perinuclear staining became most prominent at the 10 and 15 min time points. Tubular structures at various stages of development in single cells are shown at the bottom panel (*M, N, O, P*). *H* is focused to show the tubular structures at the 6 min time point (bar, 10 μm).

became spotty, although some weak ER staining was still discernible. These spots colocalized well with those of p23. These results confirmed that the spotty stainings of p23 are those of an intermediate compartment mediating transport from the ER to the Golgi apparatus.

Further information on the cellular localization of p23 came from immunoelectron microscopy (Fig. 4). As shown in Fig. 4, immunoperoxidase staining of p23 could be found throughout the Golgi stack, being more intense at the *cis*-cisternaes (the convex side of the Golgi crescent (Louvard et al., 1982; Saraste and Svensson, 1991; Wood et al., 1991; Yuan et al., 1987; Hsu et al., 1991)) in both NRK (*A, D, G, H*) and MDCK (*J*) cells. The spotty stainings appear to be vesicular-tubular structures at the ultrastructural level (*E, F, I, K*). Upon release from both 15°C (15 min) and BFA (45 min) block, the staining in these structures shifted back to the *cis*-side of the Golgi apparatus (*G, H*). ER staining was observed in both control NRK cells (*C*) and the liver tissue section (*K*). All three different types of staining were present in control NRK cells (*A-C*). In summary, p23 is localized predominantly in the *cis*-Golgi with detectable staining in the intermediate compartment and the ER.

The Subcellular Dynamics of p23

The increase in spotty staining when cells were subjected to 15°C and BFA treatment suggests that p23 migrates between the Golgi apparatus and the intermediate compartment. To establish the subcellular dynamics of p23, detailed time courses of cells cooling down from 37 to 15°C and the converse, cells being warmed up from 15 to 37°C, were followed by immunofluorescence. When NRK cells grown on coverslips were transferred from 37 to 15°C, the perinuclear staining diminished with time while spotty staining increased concomitantly. It was also apparent that during the early time points of cooling down, ER staining was enhanced. These reticular stainings were particularly prominent between 10–60 min of cooling down (Fig. 5). The ER staining however disappeared after a 3 h treatment. Depletion of perinuclear Golgi staining could be due to its shift into either the ER or the intermediate compartment. The fact that the ER staining only appeared transiently during the process, in contrast with the increased spotty staining which was much more prominent and persistent, would suggest that p23 was eventually shifted from the Golgi apparatus to the intermediate compartment, possibly via the ER. It is possible, however, that p23 was shifted directly to the intermediate compartment, and its appearance in the ER was via the intermediate compartment. To resolve this possibility, NRK cells incubated for 3 h at 15°C were shifted to 37°C and the time course of warming up was followed as before by immunofluorescence. Early in the warming up process, tubular structures began to emanate, apparently from the spotty stainings, extending towards the perinuclear staining region which increased in intensity with time. At the 3 and 6 min time points, more than 60% of the cells had these tubular structures (Fig. 5). By 10 and 15 min, the spotty stainings were greatly reduced and contrastingly, the perinuclear stainings were much more prominent than that in control cells. The p23 has clearly shifted back to the Golgi apparatus from the intermediate compartment via these tubular structures. It is interesting to note that during this shift, no reticular ER staining was ap-

parent. Therefore, p23 accumulated in the intermediate compartment by the 15°C treatment shifted directly back to the Golgi apparatus without going through the ER. This would support the view that the transient ER staining seen during cooling down came directly from the Golgi apparatus, and p23 was recycled back to the Golgi apparatus from the ER via the intermediate compartment. The p23 staining pattern of the cells approximated that of the control after prolonged warming up.

BFA treatment also shifted the perinuclear staining to a spotty one. To see if this process resembled that of the 15°C treatment, a time course of BFA treatment was followed (Fig. 6). It can be seen that the perinuclear staining was gradually dispersed into spots. However, no transient enhancement of ER staining was detected. When the cells were washed and then reincubated in medium without BFA, tubules were seen to emanate from the spots. These short tubules became longer with time and were most prominent ~10–20 min after being released from the BFA block (*E, F*). The complete shift back to perinuclear staining occurred at about 45 min after BFA was washed away (*G*). Prolonged incubation resulted in an approximation of the staining pattern seen in control cells (*H*). Although BFA is known to redistribute Golgi markers back to the ER, there is evidence suggesting the presence of post-ER compartments which remain physically and functionally distinct (Russ et al., 1991). The findings of Lippincott-Schwartz et al. (1990) are consistent with the idea that the intermediate compartment remains intact in BFA treated cells and that Golgi resident proteins continuously cycle between the ER and this compartment. It is interesting to note that reticular ER stainings were not apparent during the time course of BFA treatment. A likely scenario would be that BFA causes the fusion of the Golgi apparatus with the intermediate compartment. Golgi proteins are then recycled back to the ER via a retrograde default pathway, whereas p23 and other resident proteins of the intermediate compartment are not. Another interesting point to note is the difference in time required for the shift of p23 back to the Golgi apparatus after BFA and 15°C treatment. This may reflect the time required for BFA to be completely cleared from the cell before the normal rate of anterograde transport could be resumed.

The origin of the tubular processes observed during recovery from 15°C or BFA treatment remains obscure. These could either be generated during recovery or represent existing transport 'highways' utilized by p23 on its way back to the Golgi apparatus. The staining of these tubules colocalized with that of β-tubulin (Fig. 7), suggesting that microtubules may be involved. These tubular structures may represent connections between the intermediate compartment and the Golgi apparatus, by which p23 and other proteins, moving along the exocytotic pathway may utilize in their anterograde transport. This was confirmed by the colocalization of p23 with the VSV G protein to the tubular structures during recovery from a 15°C treatment (Fig. 3, *E* and *F*).

Overexpression of KDEL-Containing Proteins Alters the Steady-State Distribution of p23

The steady-state distribution of p23 has low levels of ER staining, the majority of which are in the Golgi apparatus and the intermediate compartment. To see if the steady-state
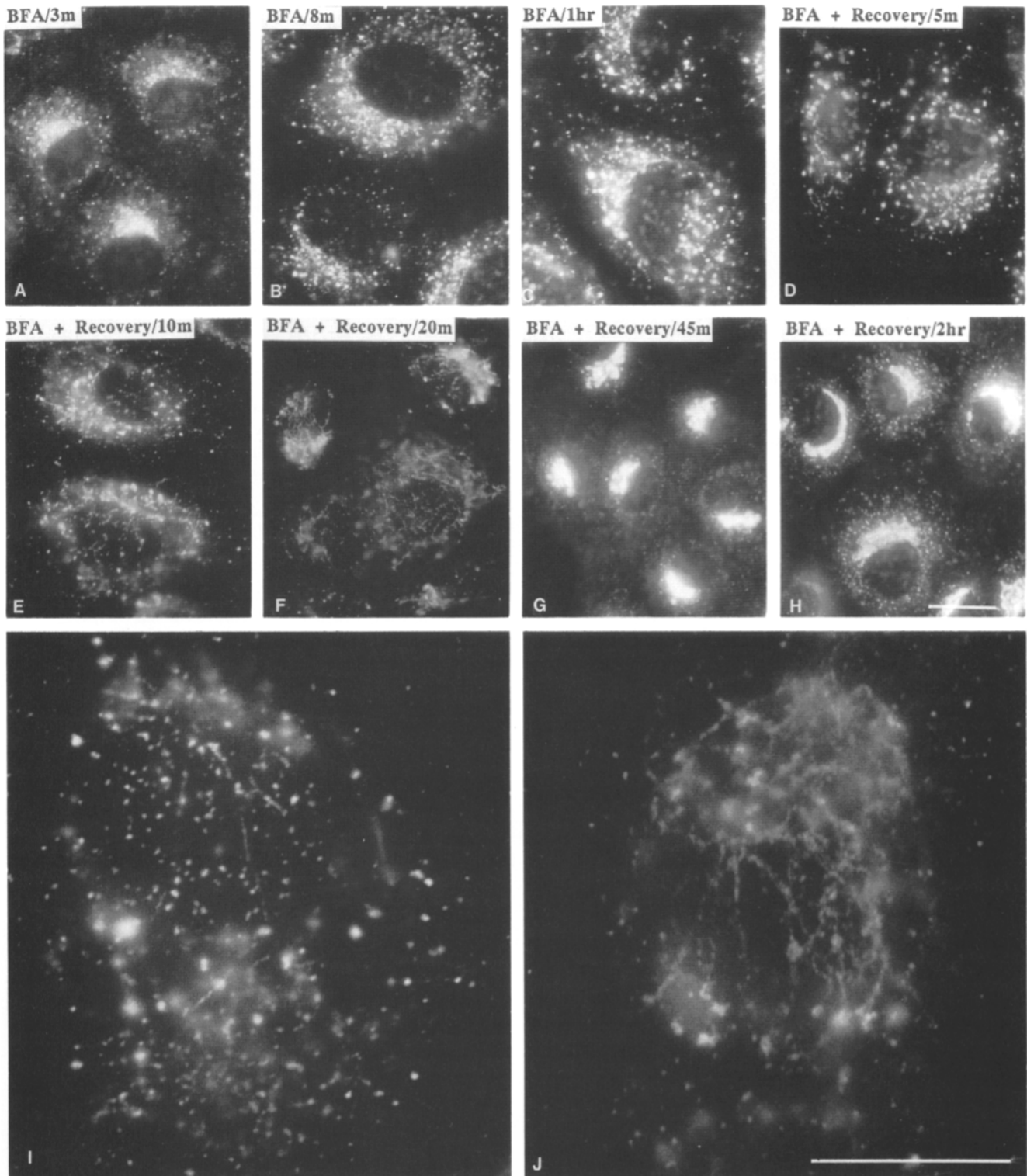
Figure 6. Time course analysis of NRK cells during BFA treatment and recovery from the BFA block by immunofluorescence. Note that ER staining is not apparent and the most prominent perinuclear staining occurs 45 min after BFA has been washed away. Tubular structures at various stages of progression in single cells are shown at the bottom panel (*I, J*) (bar, 10 μm).

distribution of p23 is altered by high expression of KDEL ligands, we studied the effects of ligand expression in three different cell systems. The KDEL ligands include a soluble protein, the α-subunit of human chorionicgonadotropin (α-hcg) appended with the KDEL sequence (Tang et al., unpublished data) and stably transfected into C6 cells; a type II

transmembrane surface protein dipeptidyl peptidase IV (D4) appended to the KDEL sequence (Tang et al., 1992) stably transfected into MDCK cells and transient expression of both these modified proteins in Cos cells. Previous studies have demonstrated that these modified proteins were selectively localized to the ER by the KDEL sequence. The cells

p23
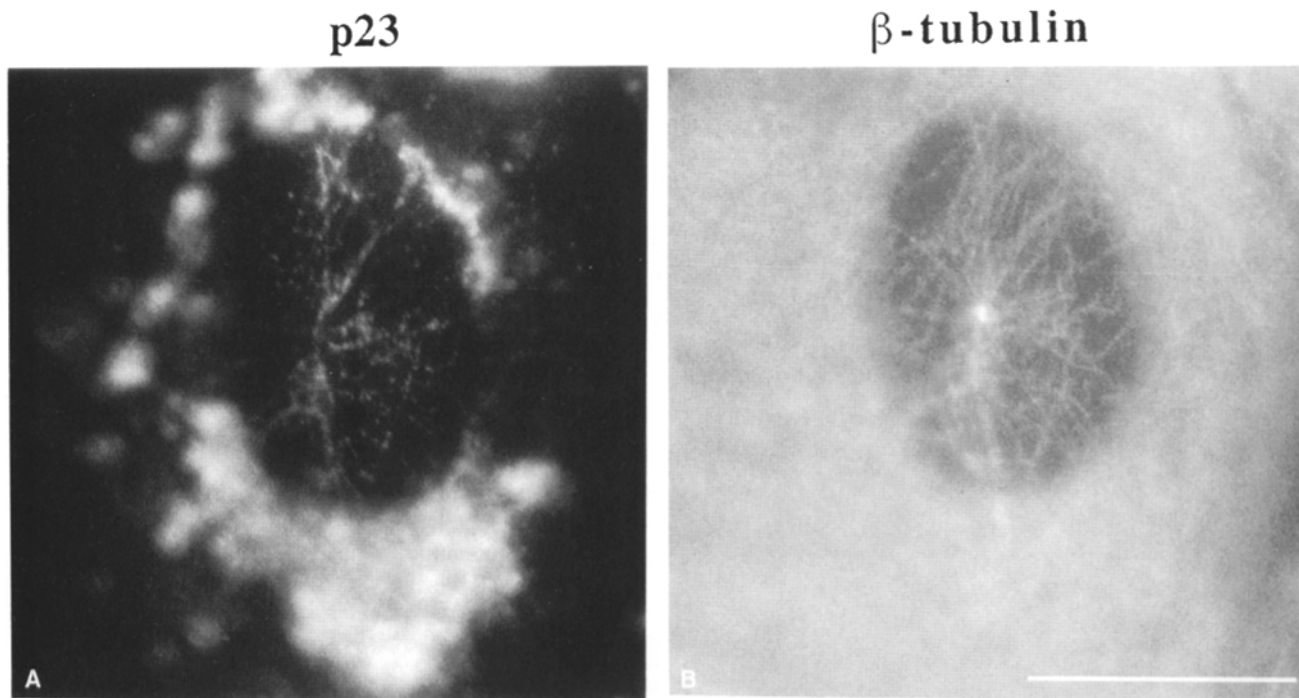
β-tubulin

Figure 7. Colocalization of β-tubulin and p23 tubular staining observed during recovery from a 15°C block (bar, 10 μm).

were double-labeled with p23 rabbit antibody and monoclonal antibodies against α-hcg or D4. In the cases of the stably transfected cells, expression of the KDEL ligands did not significantly alter the steady-state distribution of p23 (Fig. 8, *A, B, E,* and *F*). Particularly, no significant increase in ER staining was observed. To achieve a higher level of ligand expression, the proteins were transiently expressed in Cos cells. Under this condition, the steady-state staining of p23 in Cos cells was indeed altered. In all cells expressing the ligand, the perinuclear staining became diffused (Fig. 8, *C, D, G, H, I,* and *J*). There was also an increase in the spotty staining. It is not clear if this diffuse staining represents specialized regions of the ER. The possibility of it being a post-ER structure could not be ruled out. It is however obvious that sufficiently high level of ligand expression alters the steady-state distribution of the receptor. Similar observations were made in Cos cells overexpressing the lysozyme-KDEL ligand (Lewis, M. J., and H. R. B. Pelham, personal communication).

Overexpression of p23 Resulted in Its Redistribution Into the ER Together with a Golgi Marker

It was recently reported that overexpression of the myc-tagged form of the human p23 induced a BFA-like phenotype (Hsu et al., 1992). To rule out the possibility that this effect is due to epitope tagging and to demonstrate that the wild-type protein could induce the same effect, the bovine gene is transfected into Cos cells. Transfected cells transiently expressing p23 at high levels could be distinguished from untransfected cells based on their high immunofluorescence intensity and most prominently, their ER-like staining of p23 (Fig. 9, *A* and *C*). To see if the Golgi apparatus in these cells have been altered, we sought to double label the cells with anti-p23 antibody and a Golgi marker. As none of the Golgi-specific antibodies available to us gives good staining in Cos

cells, we used the *lens culinaris* lectin staining as a Golgi marker (Ridgeway et al., 1992; Hsu et al., 1992). The lectin binds to mannose-containing, fucosylated N-linked carbohydrate chains that terminate with galactose, N-acetylglucosamine or sialic acid. These types of chains are present in the Golgi, but not in the ER (Kornfeld and Kornfeld, 1985). Double labeling with the Golgi marker *lens culinaris* lectin revealed that the perinuclear Golgi staining has also been changed into an ER-like staining (Fig. 9, *B* and *D*). Overexpression of the wild-type p23 thus induced a BFA-like effect.

Discussion

Molecular Cloning and Characterization of a Bovine Homologue of Yeast ERD2

We described in this report the cloning of a bovine homologue of the yeast *ERD2* gene. The deduced amino acid sequence was found to be almost identical to a previously described human sequence (Lewis and Pelham, 1990) and bears high homology to its yeast counterpart (Semenza et al., 1990). The hydropathy plot of the predicted amino acid sequence suggests that the gene product is highly hydrophobic with several putative transmembrane domains. In vitro translation of the mRNA transcribed from the cDNA yielded a 23-kD product (p23) that was insoluble at high pH and partitions to the detergent phase upon Triton X-114 treatment. The small decrease in size associated with proteinase K treatment of the in vitro translation product suggests that only a small region of the polypeptide is cytoplasmically exposed. The protein has widespread occurrence as it is detected by both immunocytochemistry and immunoblot analysis in a broad range of cell types derived from several species, a fact that hints at its functional importance and evolutionary conservation.
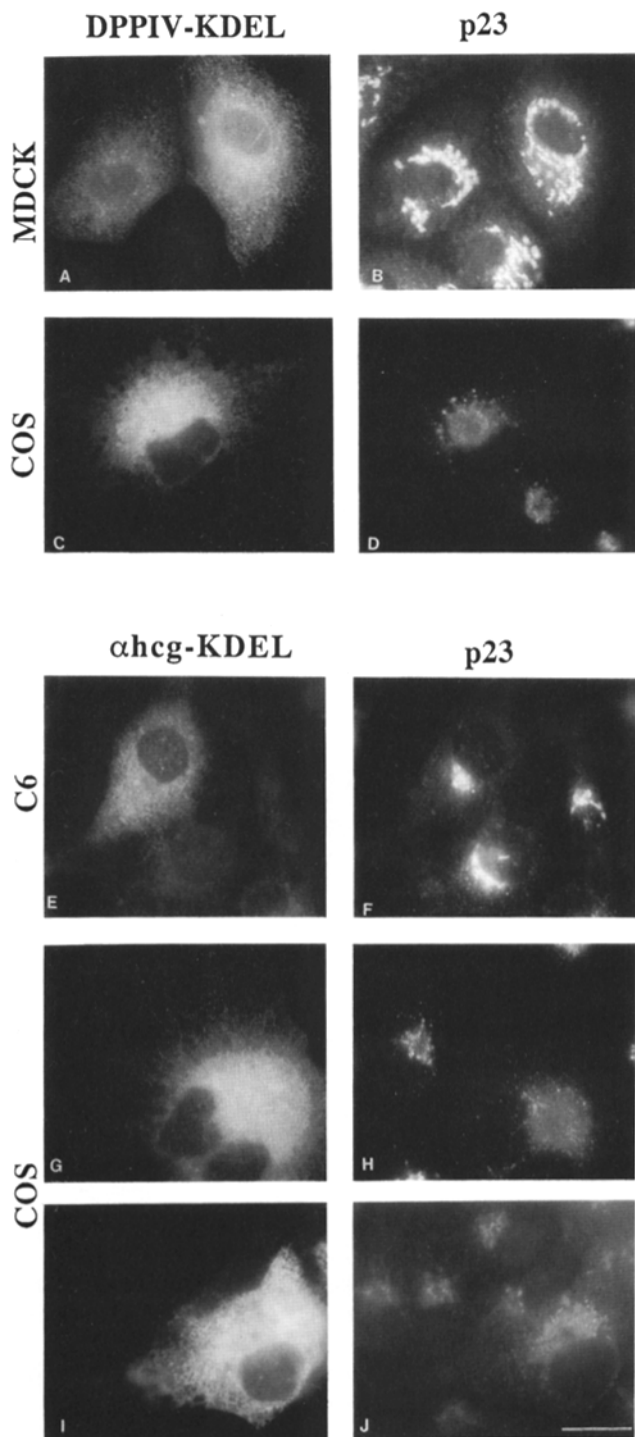
DPPIV-KDEL **p23**

αhcg-KDEL **p23**

Figure 8. Double immunofluorescence localization of p23 and KDEL ligands in cells overexpressing KDEL ligands, either in stably transfected cells (dipeptidyl peptidase IV-KDEL in MDCK cells (*A*, *B*) and α-chorionicgonadotrophin-KDEL in C6 cells (*E*, *F*) or transient expression of both DPPIV-KDEL (*C*, *D*) and α-hcg-KDEL (*G*, *H*, *I*, and *J*) in Cos cells (bar, 10 μm).

Predominant Golgi Staining of p23

It has been suggested that the retrieval of KDEL containing proteins occurs in a post-ER and pre- or early-Golgi compartment. The first possible site is the pre-Golgi compartment marked by a 53-kD protein (p53) (Schweizer et al.,

1988). Attachment of the KDEL sequence to the COOH terminus of a lysosomal/endosomal protein cathepsin D (cathepsin D-KDEL) resulted in its retention in the ER. The ER localized protein was however, modified by N-acetylglucosaminyl-1-phosphotransferase (Pelham, 1988), a *cis*-Golgi enzyme which does not cofractionate with p53 (Schweizer et al., unpublished results). The second possible compartment for retrieval of KDEL containing proteins is the *cis*-Golgi network (CGN). The term CGN was recently coined to describe cisternae and the associated vesicular-tubular structures on the *cis* side of the Golgi complex (Hsu et al., 1991). Modification of the ER localized cathepsin D-KDEL by the *cis*-Golgi N-acetylglucosaminyl-1-phosphotransferase would suggest that the CGN is the retrieval site. This possibility is further supported by the observation that the ER localized class I antigen of the major histocompatibility complex in a mutant cell line was able to recycle between the ER and the CGN (Hsu et al., 1991). Subcellular localization of p23 by immunofluorescence microscopy showed strong perinuclear staining characteristic of the Golgi apparatus. In addition, distinct spotty staining was observed throughout the cytoplasm. Immunoelectron microscopy showed that p23 is localized to the *cis* side of the Golgi complex, demonstrating that the perinuclear staining is due to p23 located in the *cis* side of the Golgi apparatus, consistent with the idea that the CGN is the major site of selective retrieval of KDEL proteins.

The Spotty Staining Corresponds to an Intermediate Compartment Mediating ER-Golgi Transport

We further investigated the subcellular localization of p23 by a low temperature (15°C) and BFA block. The 15°C block converted a large portion of the perinuclear staining into spots and vesicular structures. This staining pattern is different from that of a 72-kD putative mammalian KDEL receptor (Vaux et al., 1990) subjected to the same low temperature treatment. This staining pattern is however, similar to that of gp58, a protein localized to the *cis*-Golgi cisternae (Saraste et al., 1987) and p53 (Lippincott-Schwartz et al., 1990) after a 15°C treatment (Saraste and Svensson, 1991). Treatment with BFA resulted in the disruption of perinuclear staining of p23 into vesicular and spotty structures which are similar but not identical to those generated by a low temperature block. This staining pattern is clearly distinct from the classical reticular ER staining of Golgi proteins like mannosidase II and galactosyltransferase in BFA treated cells (Lippincott-Schwartz et al., 1990). Again, it is remarkably similar to the staining pattern of pg58 and p53 in BFA treated cells (Saraste and Svensson, 1991; Lippincott-Schwartz et al., 1990).

When cells recovered from a 15°C or BFA treatment, it was generally observed that the vesicular and spotty structures reduced in number with time. Concomitantly, extensive tubular structures were formed, seemingly connecting the spots and the perinuclear patch which became more prominent with time. At a particular time point during recovery, the perinuclear staining of p23 became most prominent and the spotty stainings were reduced to being barely detectable. With prolonged recovery, both the perinuclear and spotty staining patterns gradually approached the control level, which represents the steady-state distribution of both types of staining. No such extensive tubular structures were
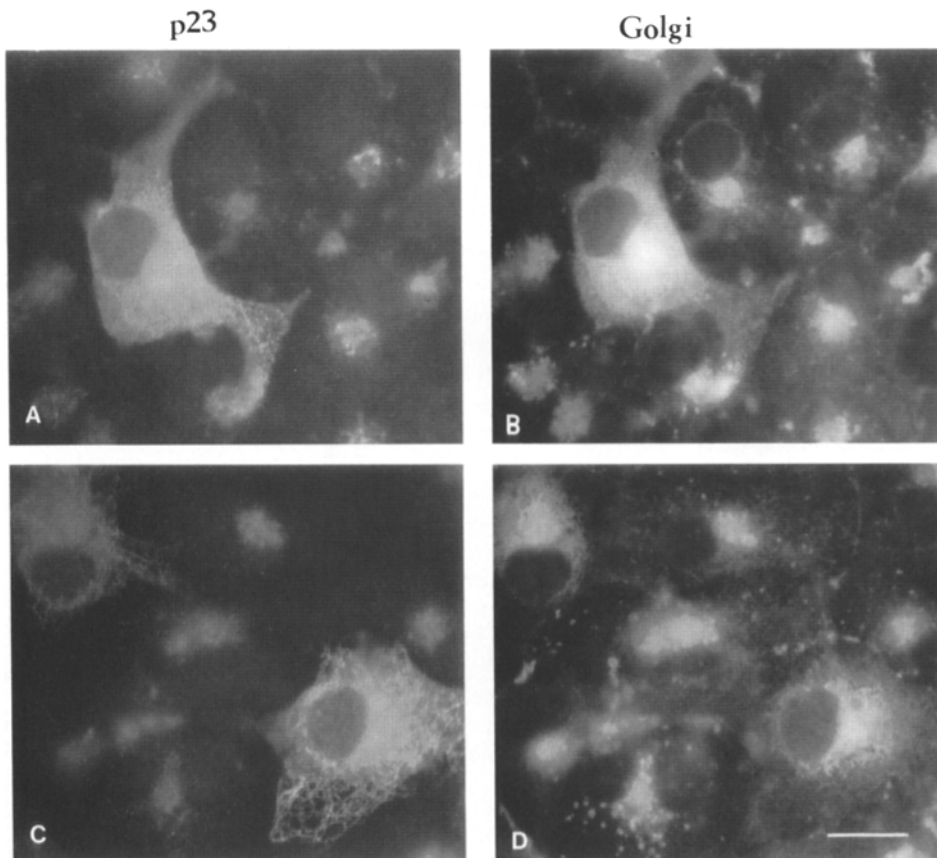
p23   Golgi

Figure 9. Effect of overexpression of p23 in Cos cells. Cells transfected with p23 cDNA were double labeled with antibody against p23 and *lens culinaris* lectin conjugated to rhodamine. Note the ER staining of p23 in cells overexpressing the receptor (*A* and *C*) and the corresponding ER staining of *lens culinaris* lectin in these cells (*B* and *D*). Bar, 10 μm.

observed during the time course of either 15°C or BFA treatment.

The morphological nature and the scattered distribution of the spots suggests that they belong to an intermediate compartment and p23 appears to cycle between these sites and the Golgi apparatus. At 15°C, recycling of p23 back to the Golgi apparatus appeared to be inhibited but transport of p23 from the Golgi apparatus to the spotty sites was not. The net result was an accumulation of the protein at these sites and a reduction in the Golgi (perinuclear) staining.

The observation of the spotty compartment raised several interesting questions, the most conspicuous of which concerns the nature and subcellular location of the compartment. The most likely candidate would be the intermediate compartment between the ER and Golgi apparatus marked by the vesicular stomatitis virus G protein and the Semiliki Forest virus E1 glycoprotein in 15°C arrested viral-infected cells (Bonatti et al., 1989; Saraste and Kuismanen, 1985). Both the p23 spotty staining observed at a 15°C arrest and the tubular structures formed when cells were shifted back to 37°C colocalized with that of VSV G. In detail time lapse monitoring of the reconstruction of the Golgi complex after removal of BFA from treated NRK cells, Alcalde et al. (1992) has shown that the Golgi markers shifted to the ER by BFA redistribute into discrete vesicular structures 10 min after BFA removal (Alcalde et al., 1992). These structures are distinct from the perinuclear morphology of the Golgi apparatus and strongly resemble the spotty stainings.

The nature of the intermediate compartment is not clear at present. The spots marked by p23 and VSV G at 15°C could represent specialized regions of the ER that are ac-

tively involved in transport, i.e., ER exit sites (Bonatti et al., 1989). Alternatively, they could be distinct post-ER and pre-Golgi structures.

Low Levels of ER Staining and Its Transient Enhancement During 15°C Incubation

Immunofluorescence microscopy demonstrated the existence of low levels of ER staining for p23 in NRK cells. This was confirmed by immunoelectron microscopy. The observation that ER staining is transiently enhanced during 15°C treatment suggests a transient shift in the steady state distribution of p23. This may result either from a relative increase in its rate of transport from the Golgi apparatus to the ER or a relative decrease in its rate of transport out of the ER (or both) at 15°C.

The Subcellular Dynamics of p23

Two points were immediately apparent from the results obtained. Firstly, p23 recycling from the Golgi apparatus back to the ER must proceed along a direct route, as p23 accumulated in the intermediate compartment goes to the Golgi apparatus but not the ER. Secondly, having being transported to the ER, the receptor is efficiently channeled into the anterograde transport back to the Golgi apparatus via the intermediate compartment. Transport from the intermediate compartment to the Golgi apparatus seems to proceed through a network of tubular structures possibly supported by microtubules. Transport processes mediated by tubular structures has been well documented in BFA treated cells. Within minutes of adding BFA to cells at 37°C, the Golgi

cisternae appear first to swell and then to extend long tubular processes out to the cell periphery (Lippincott-Schwartz et al., 1990). These tubules appear to be intermediates in the movement of Golgi membrane into the ER. In a cell free system where coated vesicle assembly is blocked by incubation with BFA, extensive tubule networks form and connect previously separate cisternae and stacks into a single topological unit, allowing the intermixing of contents of Golgi cisternae (Orci et al., 1991). Similarly, membrane tubules induced by BFA have been observed for the endosomes, lysosomes and the TGN (Lippincott-Schwartz et al., 1991; Wood et al., 1991; Hunziker et al., 1991). It has been recently proposed that tubules and coated vesicles may be alternative processes in membrane transport. The later being dependent upon assembly of cytosolic coat proteins at the point of budding whereas the former occur independently of coat proteins but could be regulated by specific coat protein assembly (Klausner et al., 1992). BFA inhibits anterograde transport by inhibiting the assembly of cytosolic proteins onto their target membranes. As a result, transport via coated vesicles is inhibited but transport processes involving tubulations are not. The obvious difference between the tubular structures reported here and those mentioned above is that they are apparent during recovery from rather than during BFA treatment. Observation of the existence of the tubular network was undoubtedly enhanced by the synchronized transport back to the Golgi apparatus by either the 15°C or the BFA.

An alternative way of recycling KDEL-containing proteins would be for the receptor to unload its ligand in the intermediate compartment. The receptor then gets recycled back to the Golgi apparatus after the anterograde flow and the ligand enters a default retrograde pathway back to the ER. This model is inconsistent with the low levels of ER staining at steady state and the transient increase in ER staining during the early time points of 15°C treatment. It is also inconsistent with the recent findings of Lewis and Pelham (1992). These authors provide the first evidence of interaction between the human KDEL receptor (hERD2) and KDEL ligands. Using an epitope-tagged form of hERD2, they showed that overexpression of the ligands causes a redistribution of the hERD2 from the Golgi apparatus to the ER. Similar but not identical observations were made with the wild type KDEL receptor in which the perinuclear staining simply looked more diffused with ligand overexpression.

The demonstration that a tubular network is involved during the transport from the spotty structures to the Golgi apparatus after release from 15°C or BFA treatment has important implications. Transport between the ER and Golgi apparatus has long been thought to be mediated by vesicles budding from the ER (Lodish et al., 1987). The ER is an extended structure throughout the cytoplasm whereas the Golgi apparatus is concentrated in a small area at the perinuclear region. It is therefore difficult to envisage how transport vesicles derived from the ER could be targeted to the Golgi apparatus with any efficiency. The existence of a tubular network which may serve as a connection between the ER and the Golgi apparatus would greatly facilitate transport between the two compartments.

References

Alcalde, J., P. Bonay, A. Roa, S. Vilaro, and I. V. Sandoval. 1992. Assembly and disassembly of the Golgi complex: two processes arranged in a cis-trans direction. *J. Cell Biol.* 116:69–83.

Bonatti, S., G. Migliaccio, and K. Simons. 1989. Palmitylation of viral membrane glycoproteins takes place after exit from the endoplasmic reticulum. *J. Biol. Chem.* 264:12590–12595.

Brown, W. J., and M. G. Farquhar. 1989. Immunoperoxidase methods for the localization of antigens in cultured cells and tissue sections by electron microscopy. *Methods Cell Biol.* 31:553–569.

Hong, W., and D. Doyle. 1990. Molecular dissection of the NH$_2$-terminal signal/anchor sequence of rat dipeptidyl-peptidase IV. *J. Cell Biol.* 111:323–328.

Hsu, V. W., L. C. Yuan, J. G. Nuchtern, J. Lippincott-Schwartz, G. J. Hammerling, and R. D. Klausner. 1991. A recycling pathway between the endoplasmic reticulum and the Golgi apparatus for retention of unassembled MHC class I molecules. *Nature (Lond.).* 352:441–444.

Hsu, V. W., N. Shah, and R. D. Klausner. 1992. A brefeldin A-like phenotype is induced by the overexpression of a human ERD-2-like protein, ELP-1. *Cell.* 69:625–635.

Hunziker, W., J. A. Whitney, and I. Mellman. 1991. Selective inhibition of transcytosis by brefeldin A in MDCK cells. *Cell.* 67:617–628.

Klausner, R. D., J. G. Donaldson, and J. Lippincott-Schwartz. 1992. Brefeldin A: Insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* 116:1071–1080.

Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 54:631–664.

Lewis, M. J., and H. R. B. Pelham. 1990. A human homologue of the yeast HDEL receptor. *Nature (Lond.).* 348:162–163.

Lewis, M. J., and H. R. B. Pelham. 1992. Ligand-induced redistribution of a human KDEL receptor from the Golgi complex to the endoplasmic reticulum. *Cell.* 68:353–364.

Lewis, M. J., D. J. Sweet, and H. R. B. Pelham. 1990. The *ERD2* gene determines the specificity of the luminal ER protein retention system. *Cell.* 61:1359–1363.

Lippincott-Schwartz, J., J. G. Donaldson, A. Schweizer, E. G. Berger, H. Hauri, L. C. Yuan, and R. D. Klausner. 1990. Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. *Cell.* 60:821–836.

Lippincott-Schwartz, J., L. Yuan, C. Tipper, M. Amherdt, L. Orci, and R. Klausner. 1991. Brefeldin-A's effects on endosomes, lysosomes and the TGN suggests a general mechanism for regulating organelle structure and membrane traffic. *Cell.* 67:601–616.

Lodish, H. F., N. Kong, S. Hirani, and J. Rasmussen. 1987. A vesicular intermediate in the transport of hepatoma secretory proteins from the rough endoplasmic reticulum to the Golgi complex. *J. Cell Biol.* 104:221–230.

Louvard, D., H. Reggio, and G. Warren. 1982. Antibodies to the Golgi complex and the rough endoplasmic reticulum. *J. Cell Biol.* 92:92–107.

Low, S. H., S. H. Wong, B. L. Tang, and W. Hong. 1991. Involvement of both vectorial and transcytotic pathways in the preferential apical cell surface localization of rat dipeptidyl peptidase IV in transfected LLC-PK1 cells. *J. Biol. Chem.* 266:19710–19716.

Munro, S., and H. R. M. Pelham. 1987. A C-terminal signal prevents secretion of luminal ER proteins. *Cell.* 48:899–907.

Orci, L., M. Tagaya, M. Amherdt, A. Perrelet, J. G. Donaldson, J. Lippincott-Schwartz, R. Klausner, and J. Rothman. 1991. Brefeldin A, a drug that blocks secretion, prevents the assembly of non-clathrin-coated buds on Golgi cisternae. *Cell.* 64:1183–1195.

Pelham, H. R. B. 1988. Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:913–918.

Pelham, H. R. B. 1990. The retention signal for soluble proteins of the endoplasmic reticulum. *Trends Biochem. Sci.* 15:483–486.

Ridgway, N. D., P. A. Dawson, Y. K. Ho, M. S. Brown, and J. L. Goldstein. 1992. Translocation of oxysterol binding protein to Golgi apparatus triggered by ligand binding. *J. Cell Biol.* 116:307–319.

Russ, G., J. R. Bennink, T. Bachi, and J. W. Yewdell. 1991. Influenza virus hemagglutinin trimers and monomers maintain distinct biochemical modifications and intracellular distribution in brefeldin A-treated cells. *Cell Regulation.* 2:549–563.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Saraste, J., and E. Kuismanen. 1985. Pre- and post-Golgi vacuoles operate in the transport of Semiliki Forest virus membrane glycoproteins to the cell surface. *Cell.* 38:535–549.

Saraste, J., G. E. Palade, and M. G. Farquhar. 1987. Antibodies to rat pancreas Golgi subfractions: identification of a 58 kD *cis*-Golgi protein. *J. Cell Biol.* 105:2021–2029.

Saraste, J., and K. Svensson. 1991. Distribution of the intermediate elements operating in ER to Golgi transport. *J. Cell Sci.* 100:415–430.

Schweizer, A., J. A. M. Fransen, T. Bachi, L. Ginsel, and H. Hauri. 1988. Identification, by a monoclonal antibody, of a 53-kD protein associated with a tubulovesicular compartment at the *cis*-side of the Golgi apparatus. *J. Cell Biol.* 107:1643–1653.

Schweizer, A., K. Matter, C. M. Ketcham, and H. Hauri. 1991. The isolated ER-Golgi intermediate compartment exhibits properties that are different from ER and *cis*-Golgi. *J. Cell Biol.* 113:45–54.

Semenza, J. C., K. G. Hardwick, N. Dean, and H. R. B. Pelham. 1990. *ERD2*, a yeast gene required for the receptor-mediated retrieval of luminal ER proteins from the secretory pathway. *Cell.* 61:1349–1357.

Tang, B. L., S. H. Wong, S. H. Low, and W. Hong. 1992. Retention of a type II surface membrane protein in the endoplasmic reticulum by the KDEL sequence. *J. Biol. Chem.* 267:7072–7076.

Tang, B. L., S. H. Wong, X. Qi, V. N. Subramaniam, and W. Hong. 1992. Golgi localized β-galactoside α2,6-sialyltransferase in transfected CHO cells is redistributed into the endoplasmic reticulum by brefeldin A. *Eur. J. Cell Biol.* In press.

Vaux, D., J. Tooze, and S. Fuller. 1990. Identification by anti-idiotype antibodies of an intracellular membrane protein that recognizes a mammalian endoplasmic reticulum retention signal. *Nature (Lond.).* 345:495–502.

Wood, S. A., J. E. Park, and W. J. Brown. 1991. Brefeldin A causes a microtubule-mediated fusion of the *trans*-Golgi network and early endosomes. *Cell.* 67:591–600.

Yuan, L., J. G. Barriocanal, J. S. Bonifacino, and I. G. Sandoval. 1987. Two integral membrane proteins located in the *cis*-middle and *trans*-part of the Golgi system acquired sialylated N-linked carbohydrates and display different turnovers and sensitivity to cAMP-dependent phosphorylation. *J. Cell Biol.* 105:215–227.