

# Transport into and out of the Golgi Complex Studied by Transfecting Cells with cDNAs Encoding Horseradish Peroxidase

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**Abstract.** We have developed a novel technique with which to investigate the morphological basis of exocytotic traffic. We have used expression of HRP from cDNA in a variety of cells in combination with peroxidase cytochemistry to outline traffic into and out of the Golgi apparatus at the electron microscopic level with very high sensitivity. A secretory form of the peroxidase (ssHRP) is active from the beginning of the secretory pathway and the activity is efficiently cleared from cells.

Investigation of the morphological elements involved in the itinerary of soluble ER proteins using ssHRP

tagged with the ER retention motif (ssHRP<sup>KDEL</sup>) shows that it progresses through the Golgi stack no further than the *cis*-most element. Traffic between the RER and the Golgi stack as outlined by ssHRP<sup>KDEL</sup> occurs via vesicular carriers as well as by tubular elements.

ssHRP has also been used to investigate the *trans* side of the Golgi complex, where incubation at reduced temperatures outlines the *trans*-Golgi network with HRP reaction product. Tracing the endosomal compartment with transferrin receptor in double-labeling experiments with ssHRP fails to show any overlap between these two compartments.

**I**N recent years the secretory pathway as originally outlined by Palade and his co-workers (reviewed by Palade, 1975) has been extensively elaborated upon. In particular, the Golgi complex is now thought to have well-developed tubular networks co-extensive with its *cis*- (Schweizer et al., 1988; Hsu et al., 1991) and *trans*- (Griffiths and Simons, 1986; Luzio et al., 1990; Geuze and Morré, 1991) cisternae. The relationships between this organelle and the endoplasmic reticulum (as indicated by KDEL processing; Pelham, 1991) and the endocytic pathway (as indicated by the glycosylation of proteins recycling from the plasma membrane; Snider and Rogers, 1985; Green and Kelly, 1992) now need to be re-evaluated in detail (Mellman and Simons, 1992). In this re-evaluation the continuities induced by the fungal metabolite brefeldin A (Lippincott-Schwartz et al., 1989; Wood et al., 1991), the extensive interconnections being demonstrated in living cells by high-resolution microscopy (Cooper et al., 1990; Hopkins et al., 1991), and the domains defined by various cytoplasmic coatings (Weidman et al., 1993) will also need to be taken into account.

Previous morphological studies of the exocytic pathway have been constrained by the availability of methods sensitive

enough to detect the low levels of endogenous protein existing within its various compartments. This is especially true for proteins in the earlier, pre-Golgi elements of the endoplasmic reticulum and transitional cisternae. For many studies this problem has been successfully circumvented by introducing exogenous protein into the secretory pathway by viral infection (Saraste and Kuismanen, 1984; Griffiths and Simons, 1986; Hedman et al., 1987). For the detailed analysis required for future work, however, probes which generate an amplified reporter signal and can be used at levels of expression which correspond more closely to endogenous trafficking proteins will be required.

Our approach to meeting these requirements has been to introduce chimeric proteins containing HRP into exocytic pathways by cDNA expression. The enzyme activity of this probe allows considerable signal amplification, an advantage widely exploited in its use as an exogenous, fluid phase marker in the endocytic pathway (Graham and Karnovsky, 1966; Tooze and Hollinshead, 1991) and which has also allowed it to be identified as an endogenous constituent within the exocytic pathway of cell types synthesizing peroxidase (Bainton and Farquhar, 1970; Herzog and Miller, 1972). An important advantage of the HRP reaction product, when generated in the soluble phase within intracellular compartments, is that it remains contained within the lumen of the cisterna or the vesicle in which the enzymes resides. In thick, unstained sections this usually allows even the most attenuated connections between compartments to be detected. A further, widely appreciated advantage of HRP as

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a morphological probe is its ability to remain enzymically active after being conjugated to a variety of protein ligands (Hopkins, 1985). The cDNA expression approach therefore also has the potential of being extended to chimeric proteins in which HRP can be incorporated within integral membrane proteins.

In this report we have evaluated the use of HRP as a probe for the exocytotic pathway, and have focused, in particular, on entry into the *cis* and exit from the trans cisternae of the Golgi complex. We show that HRP is active from the earliest part of the secretory pathway and that it can be efficiently secreted from cells, thus acting as an excellent tracer for the exocytic pathway. Using a form of HRP carrying the ER-retention motif KDEL (Lys, Asp, Glu, Leu), we identify the *cis*-Golgi elements responsible for processing KDEL-bearing proteins, and identify vesicles and cisternal elements involved in both anterograde and retrograde traffic between the ER and the Golgi complex. Finally, we also show that the tubular elements of the *trans*-Golgi which are distributed throughout the pericentriolar area are separate from the closely adjacent tubules of the recycling endocytotic pathway.

## Materials and Methods

### Cell Culture

Cos7 (CRL 1651; American Type Culture Collection, Rockville, MD) and HEp2 (CCL 23; ATCC) cells were grown in DME (Life Technologies Ltd., Paisley, Scotland) containing 10% FCS. CaCo-2 cells (HTB 37; ATCC) were grown in DME containing 20% FCS. FCS batches were screened for low endogenous peroxidase activity before use.

### Transfections

Cos7 and HEp2 cells were transiently transfected by electroporation (Chu et al., 1987) using an electroporator (Bio-Rad, Hertfordshire, UK). The day before transfection cells were seeded to ~75% confluence with minimal cell-cell contact. On the day of transfection each 9-cm dish of cells was trypsinized and washed in DME containing 10% FCS, followed by HeBs (20 mM Hepes, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na<sub>2</sub>HPO<sub>4</sub>, 6 mM D-glucose) and finally resuspended in 250  $\mu$ l HeBs. 10  $\mu$ g of DNA (pSR $\alpha$ .ss.HRP or pSR $\alpha$ .ss.HRP<sup>KDEL</sup>) was added and the DNA/cell mix was electroporated (one pulse) in 0.4-cm cuvettes (Bio-Rad) at 300 V, 125 microfarads, and infinite ohms. The cells were then reseeded into the same size dishes and assayed for expression or processed for EM after 3 d in nonselective medium.

Stable lines of HEp2 and CaCo2 cells were produced by lipofection (Felgner et al., 1987; Chang and Brenner, 1988). The day before transfection cells were seeded to ~75% confluence with minimal cell-cell contact. On the day of transfection 10  $\mu$ g DNA (pSR $\alpha$ .ss.HRP or pSR $\alpha$ .ss.HRP<sup>KDEL</sup>) was diluted to 250  $\mu$ l in OPTI-MEM and 100  $\mu$ l lipofectin was diluted to 250  $\mu$ l in OPTI-MEM (GIBCO BRL, Life Technol. Inc., Gaithersburg, MD). The DNA and lipofectin were mixed and the complex allowed to form at room temperature for 5 to 10 min. The mixture was then pipetted onto the cells (pre-washed with OPTI-MEM to remove traces of serum), incubated at 37°C for 20 min followed by the addition of 2 ml serum-free DME, and the incubation then continued for another 5 h. DME containing FCS was then added and cells cultured for 1 (HEp2) or 3 d (CaCo2 cells), followed by trypsinization, and plating into six 96-well plates in the presence of selective agent (0.5 mg/ml G418; GIBCO BRL [Life Technologies Inc.] for HEp2 or 1 mg/ml for CaCo2 cells).

### DNA Constructs

DNA manipulations were carried out by standard procedures, using reagents according to manufacturer's instructions.

**Construction of pSR $\alpha$ .ssHRP.** A construct encoding HRP isoenzyme c was artificially constructed by overlapping oligonucleotides (Ortlepp et

al., 1989) on the basis of its published amino acid sequence (Welinder, 1979). This construct (pSA247) was kindly provided by Amersham Intl. (Amersham, UK). To enable entry into the secretory pathway, the signal sequence from human growth hormone (Hall et al., 1990) was added. The signal sequence (sshGH) was removed from the polylinker site of pMTL22p (Chambers et al., 1988) where it had been inserted as a BamHI/HindIII fragment to produce pEKSP. This construct was kindly provided by H. J. Gilbert (University of Newcastle upon Tyne, Newcastle, UK). pEKSP was linearized with HindIII and the ends were blunted with the Klenow fragment of DNA polymerase. The DNA encoding HRP was excised from pSA247 by EcoRV/HpaI digestion and ligated into the blunt-ended HindIII site, downstream of sshGH to yield pEKSP.HRP. The fragment encoding an in-frame fusion of the sshGH with the mature HRP was removed from pEKSP.HRP by EcoRV/SphI digestion. The 3' overhang of the SphI site was removed by T4 DNA polymerase and the resultant fragment ligated into the pSR $\alpha$  plasmid (Takebe et al., 1988; a kind gift from DNAX, Palo Alto, CA) which had been linearized by BamHI and blunted with the Klenow fragment of DNA polymerase to give sshGH fused to the second amino acid (leucine) of mature HRP.

**Construction of pSR $\alpha$ .ssHRP<sup>KDEL</sup>.** HRP was generated from BBG10 (R and D Systems, Oxford, UK) by PCR amplification using the oligonucleotides: 5' G GAA TTC CAG TTA ACG CCG ACT TTC 3' and 5' C CCC AAG CTT AGA GTT GAC 3' such that the resulting fragment was flanked by EcoRI and HindIII sites.

The *c-myc* KDEL sequence was obtained from the sequence, 5'TTG GAG CAA AAG CTC ATT TCT GAA GAG GAC TTG AAG GAC GAA CTT TAA GCT 3' by PCR amplification using the oligonucleotides: 5' C CCC AAG CTT GAG CAA AAG CTC ATT TCT GAA GAG GAC 3' and 5' C CCG CTC GAG TTA AAG TTC GTC CTT CAA 3' such that the resulting fragment was flanked by HindIII and XhoI sites.

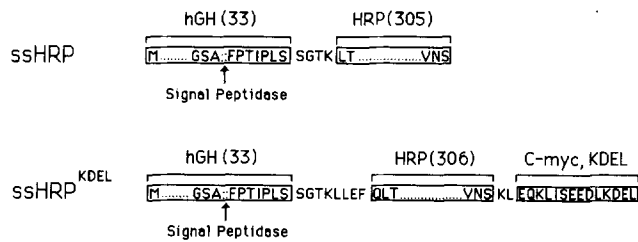
The HRP and *c-myc* KDEL fragments bearing the relevant cohesive ends were ligated together and the product electrophoresed into LGT agarose (FMC Corp. BioProducts, Rockland, ME): The appropriate ligated product was then ligated into pSR $\alpha$ .ss (produced by cloning the signal sequence from pEKSP as a StuI/EcoRV fragment, into pSR $\alpha$  at the XhoI site which had been blunt-ended using the Klenow fragment of DNA polymerase) which had been precleaved with EcoRI and XhoI and the ends dephosphorylated using calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, IN).

### HRP Assay

To measure cell-associated HRP activity, transfected or control cells grown on 9-cm dishes were rinsed three times in PBS containing 0.5% BSA and suspended in 1 ml of 50 mM Tris/Cl (pH 2.5). Cells were then freeze/thawed three times in a methanol/dry ice bath and centrifuged in a microfuge for 5 min at 13,000 rpm. Media samples were clarified by centrifugation (3,000 rpm, 10 min) and then treated as for cell samples. Reactions were carried out in 50 mM Tris/Cl (pH 2.5) containing 0.1% *o*-phenylene diamine (Sigma, Poole, UK) and 0.02% H<sub>2</sub>O<sub>2</sub> (Sigma) at 37°C in the dark. The reaction was stopped after 30 min by the addition of 1 M H<sub>2</sub>SO<sub>4</sub> and the absorbance read at OD<sub>450</sub>. As controls, buffer alone, mock- and HRP-transfected cells incubated in the absence of H<sub>2</sub>O<sub>2</sub> were tested.

### Electron Microscopy

Cells were plated out on 3-cm petri dishes, cultured for 3 d in DME 10% FCS, and fixed directly or incubated for various times at 20°C or 15°C before fixation. Where reduced temperature incubations in a waterbath were carried out the medium was supplemented with 20 mM Hepes, pH 7.4. In some experiments cycloheximide (100  $\mu$ g/ml) or colchicine (2  $\mu$ M) were added. In all experiments cells were rinsed with serum-free DME before fixation. In some cases, for the last hour at 20°C the medium was supplemented with anti-transferrin receptor antibody (B<sub>3/25</sub>) gold complexes prepared as described previously (Hopkins and Trowbridge, 1983). Fixation was carried out in Karnovsky fixative (2% paraformaldehyde, 2.5% glutaraldehyde in 100 mM sodium cacodylate, pH 7.5; Karnovsky, M. J. 1965. *J. Cell Biol.* 27:137a). They were incubated with H<sub>2</sub>O<sub>2</sub> and diamino-benzidine prepared as described by Graham and Karnovsky (1966), rinsed, osmicated, dehydrated, and embedded by standard procedures (Hopkins and Trowbridge, 1983). Sections were cut at either 70 nm, stained with lead citrate, and uranyl acetate or cut 1- $\mu$ m thick, stabilized with a thin film of evaporated carbon, and viewed unstained in a Phillips CM12 transmission electron microscope.



**Figure 1.** Structure of ssHRP and ssHRP<sup>KDEL</sup>. This figure shows the elements of the two constructs used in this study. Boxed sequences are the main components; sequences outside the boxes are amino acids introduced as a consequence of the cloning strategy used. The predicted site of signal peptidase cleavage is shown. *hGH*, human growth hormone; *c-myc*, the epitope from *c-myc* recognized by antibody 9E10 that we used as a spacer; KDEL, the retention motif. Numbers of residues in elements shown as partial sequences are in brackets after the name. Note the single residue missing from the amino terminus of HRP in ssHRP.

## Results

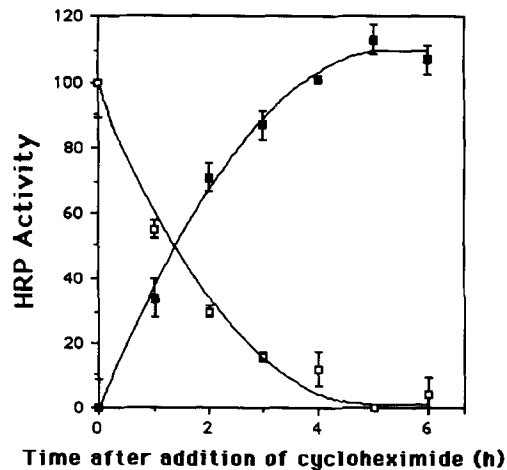
### HRP Can Be Efficiently Secreted from Animal Cells

We obtained a cDNA (pSA247) encoding mature HRP. To ensure entry of this enzyme into the secretory pathway we constructed a chimeric cDNA whereby the signal sequence from human growth hormone was attached to the amino terminus of HRP (Fig. 1 and Materials and Methods). This chimeric cDNA, "ssHRP", was then cloned into the expression vector pSR $\alpha$  (Takebe et al., 1988) to create pSR $\alpha$ .ssHRP and transfected into Cos7 cells. Enzyme assays (not shown) indicated that a level of HRP activity 400-fold above background resulted from this transient expression.

We then attempted to establish stable lines of HEp2 cells and CaCo-2 cells expressing ssHRP. This was successful although few of the cell lines we established survived beyond 10 passages. The onset of mortality seemed to correlate with the level of peroxidase expression, implying that prolonged exposure to the enzyme could not, for some reason, be tolerated. Despite this problem, 10 passages provide sufficient cells for most studies and, as shown by electron microscopy, there is no evidence of degenerative change during these earlier passages.

To test if the ssHRP was entering the exocytic pathway, we investigated whether the HRP activity was efficiently secreted from Cos7 cells. Our experiments were designed to determine whether ssHRP was cleared from the cell by assaying for enzyme activity in the medium in the absence of continuing protein synthesis. Significant pools of activity remaining within the cell would suggest that the protein was being diverted and sequestered away from the constitutive secretory pathway and would complicate the interpretation of EM experiments.

Cos7 cells were transfected to produce transient expression of ssHRP. They were then rinsed and incubated with cycloheximide for varying lengths of time and at each point enzyme activity in the cells and medium was determined. As Fig. 2 shows, HRP activity is secreted from the cells with a  $t_{1/2}$  of  $\sim 80$  min, while intracellular activity is reduced to background levels within 4–5 h. All of the intracellular activity can eventually be recovered in the medium suggesting



**Figure 2.** Secretion of HRP activity from Cos7 cells. Cos7 cells were transfected with pSR $\alpha$ /ssHRP and left for 36 h for plasmid replication to increase expression. Cells were then washed three times in DME/FCS supplemented with 0.2% BSA, and then incubated in the same medium for varying times in the presence of cycloheximide (100  $\mu$ g/ml). HRP activity (mean  $\pm$  range,  $n = 3$ ) in both medium (■) and cells (□) was assayed as described (Materials and Methods).

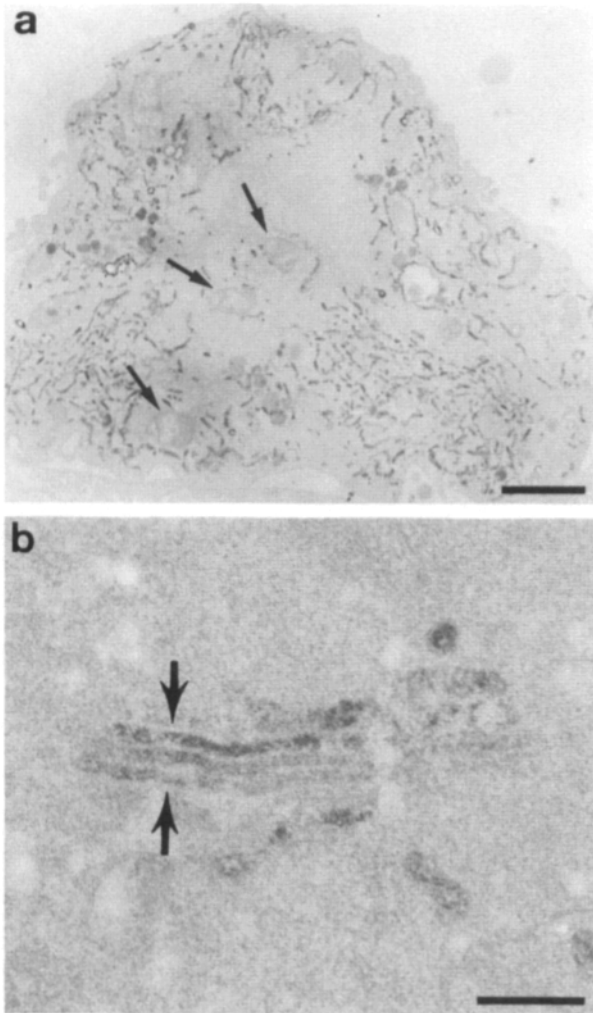
that intracellular degradation is negligible. Parallel experiments with HEp2 cells transfected with pSR $\alpha$ /ssHRP gave similar results (not shown).

### Morphology of the Secretory Pathway between the RER and the Golgi Stack

Having shown that intracellular HRP activity is present within the secretory pathway of transfected cells, we carried out EM peroxidase cytochemistry. Cos7 cells expressing high levels of ssHRP show reaction product throughout the nuclear envelope and the cisternae of the RER. Golgi cisternae are also strongly stained but mitochondria, endocytotic elements and the plasma membrane are entirely negative (Fig. 3 *a*). In stably transfected CaCo-2 cells ssHRP reaction product is most strongly displayed in flattened Golgi cisternae (Fig. 3 *b*). Where the section plane cuts across the flattened Golgi stack a gradient of reaction product, (concentration increasing towards the *trans* side) is often seen. Small (50-nm diam) ssHRP-containing vesicles, which presumably represent constitutive secretory vesicles, are also found in the *trans*-Golgi area.

In HEp2 cells expressing ssHRP incubated at 37°C, HRP activity was barely detectable by electron microscopy. We therefore reduced the incubation temperature to 20°C, inducing an increase in the level of intracellular HRP. The increase in HRP activity which occurs in HEp2 cells incubated at 20°C arises because the release of activity is inhibited; on returning to 37°C in the presence of cycloheximide this HRP activity is secreted with a  $t_{1/2}$  of 20 min and the enzyme is cleared from the cell within 120 min.

Fig. 4 shows a Golgi area of a HEp2 cell expressing ssHRP incubated for 4 h at 20°C in which HRP reaction product is detectable within the *trans*-most cisternae of the Golgi stack



**Figure 3.** EM cytochemistry of peroxidase in *Cos7* and *Caco-2* cells. (a) *Cos7* cells were transiently transfected with pSR $\alpha$ /ssHRP and left for 72 h before fixation and processing for EM. The section shows HRP reaction product distributed evenly throughout the ER; arrows indicate mitochondria and lysosomal elements which are negative. (b) Expression of HRP in *Caco-2* cells stably transfected with pSR $\alpha$ .ssHRP. The intensity of DAB staining is stronger in cisternae on the *trans* side of the Golgi stack which is bracketed by arrows. Bars, 0.2  $\mu$ m.

and in associated vacuoles. At this low level of intracellular activity (compare with Fig. 10) the distribution of HRP reaction product is discrete and clearly delineates the limiting membrane of the compartment which contains it. It is also worth noting that the membranes themselves and any associated cytoplasmic coatings are clearly displayed.

We believe the increase in reaction product toward the *trans* side of the Golgi stack arises because 20°C blocks exit from the TGN (Griffiths and Simons, 1986) but that the process(es) which concentrate secretory product continue to operate within compartments proximal to this block. Because cells are fixed and washed before the DAB reaction is carried out, and local gradients of small molecules and ions will be dissipated during this processing, the intensity of the staining resulting from HRP activity is not affected by local

physiological conditions (such as pH). The staining intensity of luminal content should, therefore, directly reflect the amount of enzyme present within the compartment in which it is contained.

To examine the transfer of soluble phase tracer between the RER and the Golgi stack in more detail we constructed an HRP chimera which carried a KDEL retention signal at the COOH terminus; "ssHRP<sup>KDEL</sup>" (Fig. 1). The extent to which ssHRP and ssHRP<sup>KDEL</sup> are retained within HEP2 cells during a 24-h incubation was compared (Fig. 5). It is evident, as expected (Munro and Pelham, 1987), that the presence of the KDEL tetrapeptide causes an intracellular accumulation of the tracer. However, a significant amount of ssHRP<sup>KDEL</sup> was released into the medium in some of these experiments, suggesting that the capacity of the KDEL retention system can be overloaded in HEP2 cells at relatively modest levels of expression.

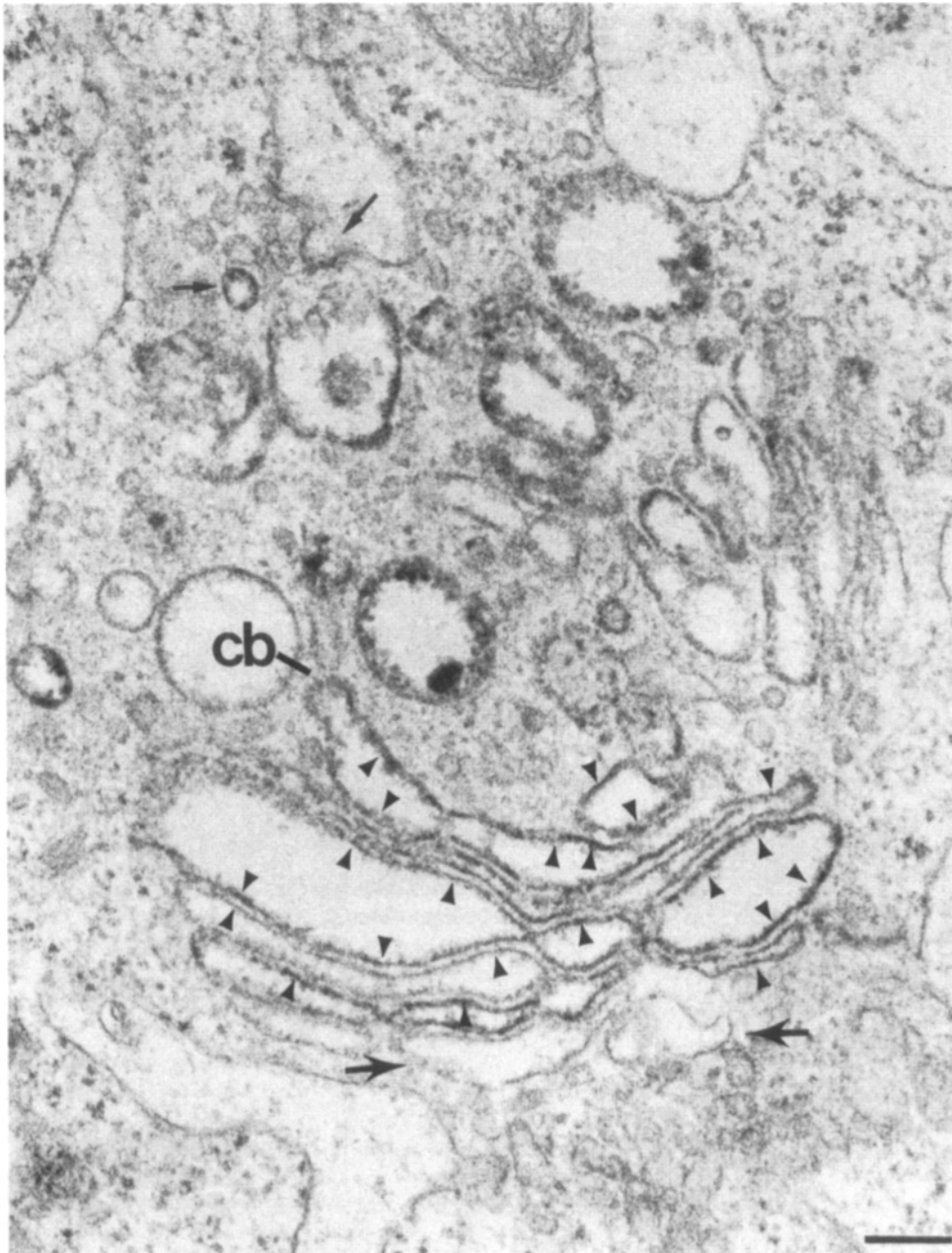
EM cytochemistry of HEP2 cells transfected with pSR $\alpha$ .ssHRP<sup>KDEL</sup> and incubated at 37°C showed HRP reaction product distributed evenly throughout the endoplasmic reticulum and nuclear envelope. In addition to RER cisternae the *cis*-most cisternae of the Golgi stacks also contained DAB reaction product (Fig. 6). Within the *cis*-most cisternae it is sometimes restricted to short stretches of the flattened cisternae or to their bulbous termini (Fig. 7, a and b). In thin sections there are also frequent profiles to suggest continuity between the Golgi stack and adjacent cisternae which may belong to the ER (Fig. 7 a).

In addition to the flattened cisternae of the RER there were also HRP-positive 50-nm-diam vesicles which often seemed to be in continuity with the RER or the nuclear envelope (Fig. 7 d). Sometimes these vesicular elements appeared to be connected to one another by narrow tubules so that they resembled small bunches of grapes. Thin sections suggested that a proportion of these structures are free vesicles (Fig. 7 c) and thick sections up to 10 times their diameter (not shown) showed that some were, indeed, unconnected to their neighbors. The diaminobenzidine (DAB)<sup>1</sup> reaction product within the 50-nm-diam vesicles filled the lumen and was often considerably more electron opaque than the DAB reaction product distributed in the adjacent RER cisternae.

The only other class of DAB-positive structures in cells transfected with ssHRP<sup>KDEL</sup> were 80–100-nm-diam vesicles (Fig. 6). These larger vesicles did not have as consistent a size or as distinctively spherical a shape as the 50-nm vesicles, often they were ovoid in form. They were grouped in clusters and scattered amongst the cisternae of the ER throughout the cytoplasm as well as in the vicinity of the *cis*-Golgi complex. Within the clusters adjacent to the RER some profiles showed direct continuity between the 80-nm vesicles containing dense DAB reaction product and ER cisternae (Fig. 6 c). In the Golgi area these vesicles were sometimes single and clearly adjacent to the *cis*-Golgi complex (Fig. 7 a), on other occasions they had an elongated, sinuous profile and were indistinguishable from *cis*-most Golgi cisternal elements (Fig. 6, a and b).

The intensities of DAB staining in these various elements are characteristic. In the RER and 50-nm vesicles and associated tubules, a moderate level of staining is usually seen.

1. Abbreviation used in this paper: DAB, diaminobenzidine.



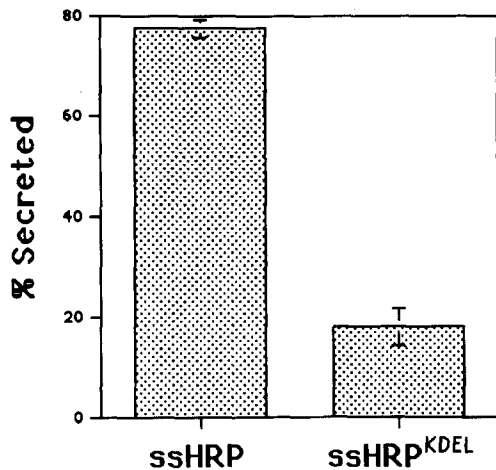
**Figure 4.** EM of HEP2 cell expressing ssHRP at reduced temperature. HEP2 cells stably expressing ssHRP were incubated at 20°C for 4 h and then processed for EM peroxidase cytochemistry. This section shows DAB reaction product distributed as a discrete precipitate of moderate electron opacity on the luminal side of the trans Golgi cisternal membranes (*arrowheads*). The *cis*-most Golgi cisternae (*large arrows*) are negative as are most ER cisternae. Small arrows indicate DAB staining of an ER transitional element and an adjacent 50 nm vesicle. A coated bud (*cb*) on a *trans*-Golgi cisterna is indicated. Bar, 0.2  $\mu$ m.

In the *cis*-Golgi and the 80-nm vesicles a noticeably higher level of staining is the norm, although this staining is less evenly distributed, often concentrated at the perimeter membrane.

Previous work on the processing of KDEL-bearing cathepsin (Pelham, 1988) has shown that temperatures in the region of 14.5–15°C inhibit the processing of its oligosaccharides characteristic of delivery to the Golgi stack. When HEP2 cells transfected with ssHRP<sup>KDEL</sup> are incubated at 15°C the distribution of the tracer is altered (Fig. 7 *e*). Within 2 h reaction product becomes barely detectable in the RER, compact clouds of 50-nm-diam vesicles scattered throughout the cytoplasm appear, and the 80–100-nm-diam vesicles which are such a prominent feature at 37°C are much reduced in number. Within the groups of 50-nm vesicles only a minority stain with DAB (Fig. 7 *e*).

With longer incubations at 15°C the form of the Golgi complex changes dramatically; the flattened, stacked cisternae curl to form compact, concentric whorls and the cytoplasm in the vicinity of these cisternal whorls becomes filled with vesicles of various sizes (Fig. 8). Thin section profiles suggest that the vesicles are derived from the breakdown of the Golgi stack during the 15°C incubation. It is of interest, therefore, that within these clouds of vesicles the ssHRP<sup>KDEL</sup> vesicles remain separate and identifiable even after an incubation of 20 h at 15°C (Fig. 8).

Previous studies on the post-Golgi stack processing of KDEL-bearing ligands suggest that they are carried back to the endoplasmic reticulum bound to the erd2 receptor (Lewis and Pelham, 1992; Wilson et al., 1993). The effects of both nocodazole and griseofulvin suggest that this transfer requires the presence of an intact microtubular cytoskeleton



**Figure 5.** Release of HRP activity from HEP2 cells expressing ssHRP and ssHRP<sup>KDEL</sup>. HEP2 cells stably expressing ssHRP or ssHRP<sup>KDEL</sup> were incubated for 24 h in serum-free medium. HRP activity ( $n = 3 \pm \text{SD}$ ) in the medium and in cell lysates was assayed as described (Materials and Methods), and the percentage secreted as a fraction of the total HRP activity expressed was calculated.

(Lippincott-Schwartz et al., 1990) and so we examined the distribution of ssHRP<sup>KDEL</sup> in cells pretreated with colchicine for 1 h at 37°C. In these cells DAB reaction product is distributed throughout the RER and *cis*-most Golgi cisterna (Fig. 9). However, the most obvious difference from untreated cells is in the increased number of 80–100-nm vesicles, especially in the vicinity of residual Golgi elements. A similar increase in the number of 80–100-nm-diam vesicles is evident in cells undergoing mitosis (not shown). In mitotic cells, clearly defined Golgi complexes were not observed and the 80–100-nm-diam ssHRP<sup>KDEL</sup> vesicles accumulate in large groups throughout the cytoplasm.

### The Morphology of the Secretory Pathway beyond the Golgi Stack

At 20°C ssHRP accumulates in the Golgi stack. In addition to the flattened stacks, HRP reaction product is also distributed in a variety of vacuoles, branching 60-nm-diam tubules and flattened saccules in the Golgi area. These HRP-positive tubules lie on the concave side of the stack and presumably represent elements of the *trans* Golgi reticulum. By cutting thick (1  $\mu\text{m}$ ) sections and viewing them unstained (not shown) it is clear that many of these HRP-positive elements are part of an extensive tubular reticulum distributed throughout the pericentriolar area.

In HEP2 cells there is a well-developed endocytotic compartment in the pericentriolar area which also consists of branching, 60-nm-diam tubules (Hopkins and Trowbridge, 1983; Hopkins, 1985). For further detailed analysis of this part of the pathway it was, therefore, of interest to determine the spatial relationship between these two tubular systems. This was examined by incubating HEP2 cells expressing ssHRP at 20°C for 60 min with anti-transferrin receptor antibody (B<sub>3/25</sub>)-gold complexes. As has been shown in previous studies (Hopkins and Trowbridge, 1983; Hopkins et al.,

1991) this gold tracer outlines the endocytotic pathway and, in particular, identifies the tubules of the recycling pathway located in the pericentriolar area (Hopkins et al., 1994). Electron microscope cytochemistry for HRP shows both the ssHRP and the gold tracers distributed in tubular/vesicular elements in the *trans*-Golgi/pericentriolar area. While the labeled vesicles often lie very close to each other and thick sections suggest that the labeled tubules are frequently intertwined, neither double-labeled tubules nor vesicles were observed (Fig. 10 *a*). However, at 20°C, transport of ssHRP beyond the *trans*-Golgi towards the cell surface is prevented and in subsequent experiments the 20°C incubation was therefore followed by a chase at 37°C (in the continuing presence of B<sub>3/25</sub> gold, but with further HRP synthesis prevented by the addition of cycloheximide). With the transfer to 37°C, HRP activity rapidly drained from the Golgi complex whilst the pericentriolar elements of the endocytotic pathway remained extensively labeled with gold. Under these conditions double-labeled elements were still not observed (Fig. 10, *b* and *c*). We conclude, therefore, that the *trans*-Golgi reticulum and the tubules of the endocytotic pathway in the pericentriolar area are separate systems. The transfer between endosomes and the *trans*-Golgi which has been reported previously (e.g., Snider and Rogers, 1985; Geen and Kelly, 1992) probably depends upon a selective, receptor-mediated, concentrative step and this might well be difficult to detect with a fluid phase tracer like ssHRP.

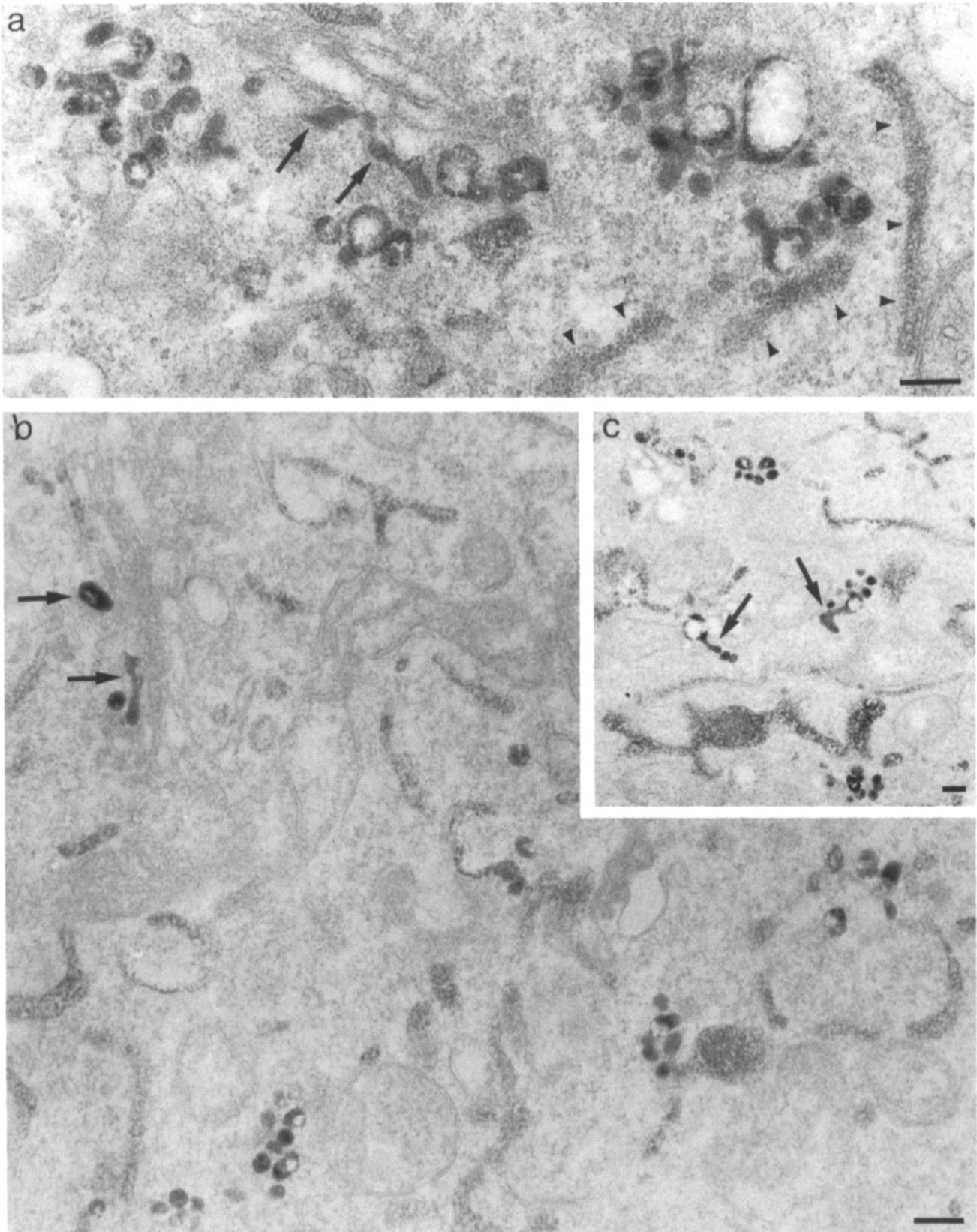
### Discussion

In this paper we show that introducing HRP into the RER allows constitutive pathways into and out of the Golgi stack to be examined in detail. The newly synthesized HRP is enzymically active from the time it is translocated into the lumen of the RER and it is potentially possible, therefore, to trace the entire exocytotic pathway. The kinetics with which ssHRP is cleared from the HEP2 and *cos7* cells are similar to those observed for other proteins traveling in the soluble phase (e.g., Lodish et al., 1983). This, together with EM cytochemistry which shows no indication of HRP in pathways other than the exocytotic route (lysosomes are, for example, negative at all times) clearly suggests that the ssHRP is a reliable fluid phase marker for the exocytic pathway.

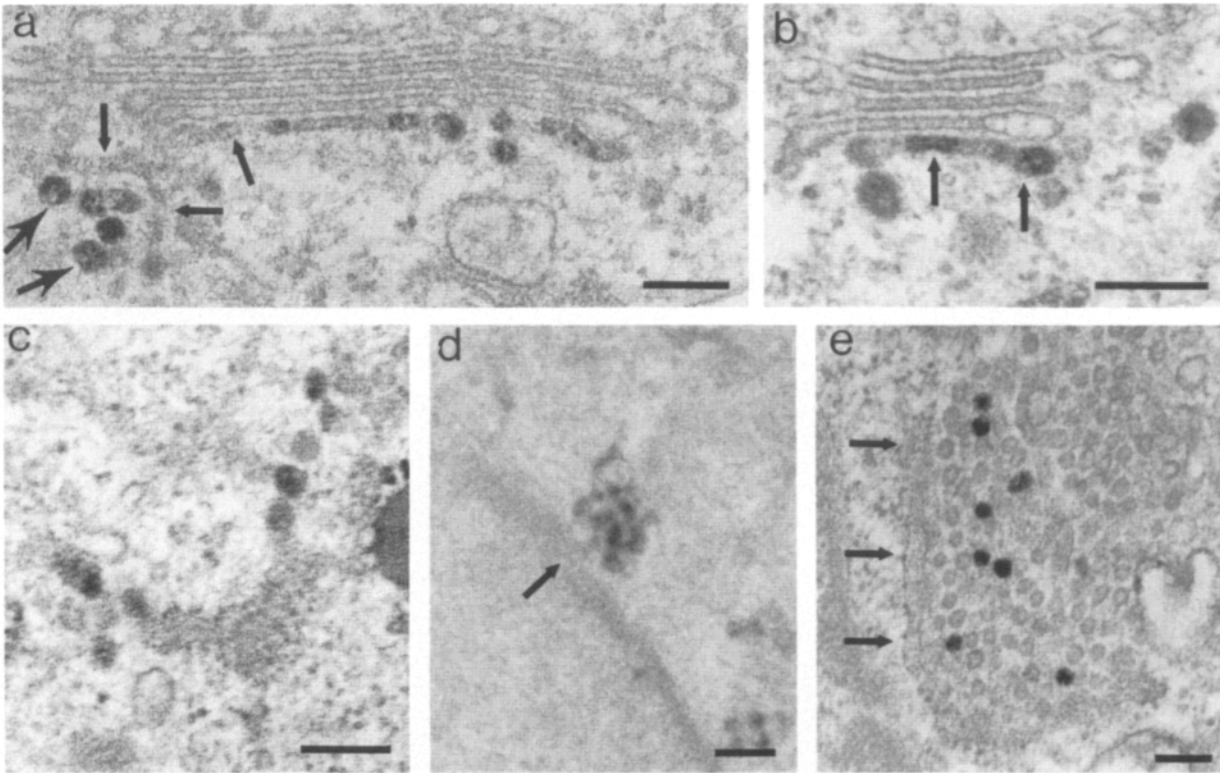
In previous electron microscopical studies of KDEL-bearing proteins a variety of localizations have been obtained. In plants (Napier et al., 1992), and Purkinje cells (Yamamoto et al., 1991) immunogold electron microscopy identified KDEL proteins only in the ER, whereas in the exocrine pancreas (Takemoto et al., 1992) these proteins were found distributed throughout the entire secretory pathway. Using ssHRP<sup>KDEL</sup>, we find reaction product within the ER, transport vesicles, and the *cis*-Golgi at 37°C. Our data thus conform with biochemical observations which show that KDEL-bearing proteins are transported from the RER to the Golgi complex before retrieval (Pelham, 1988), and with the distribution of *erd2* as seen by immunofluorescence (Lewis and Pelham, 1992) and EM (Tang et al., 1993).

Our data emphasize the extent to which KDEL-tagged proteins can traffic through a variety of different membrane-bound compartments. Moreover, the staining levels we ob-

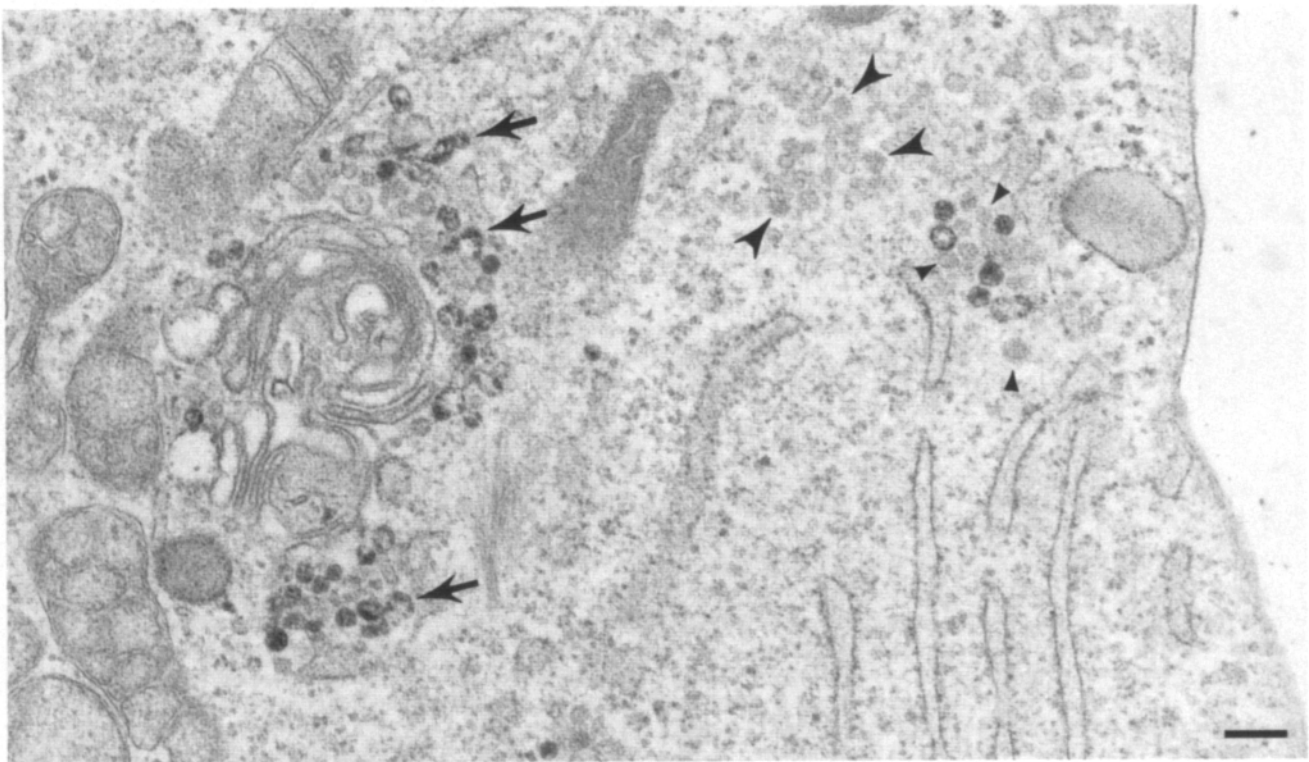




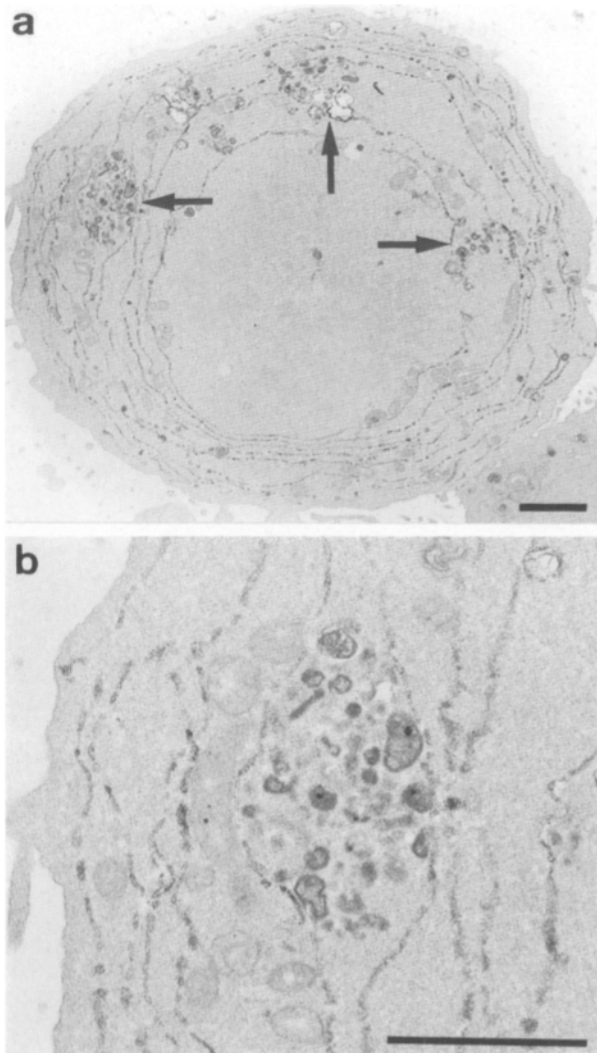
**Figure 6.** EM of Hep2 cells expressing ssHRP<sup>KDEL</sup> at 37°C. Hep2 cells stably expressing ssHRP<sup>KDEL</sup> were processed for EM peroxidase cytochemistry. (a) *cis*-Golgi area showing sinuous elements (arrows) and 80–100-nm diam vesicles containing DAB reaction product. The medial cisternae of the stack are negative while the ER cisternae (arrowheads) stain with moderate intensity. (b) General view showing DAB-positive ER cisternae widely distributed throughout the cytoplasm, also scattered groups of 80–100-nm vesicles which stain more strongly, as do elements on the *cis* side of the Golgi stack (arrows). (c) Low power view showing DAB-positive ER cisternae and 80–100-nm-diam vesicles and profiles (arrows) indicating direct connections between cisternae and vesicles. Bars, 0.2 μm.



**Figure 7.** HEP2 cells expressing ssHRP<sup>KDEL</sup>. HEP 2 cells stably expressing ssHRP<sup>KDEL</sup> were incubated at 37°C (*a-d*) or at 15°C for 4 h (*e*) before fixation and processing for EM peroxidase cytochemistry. (*a*) View of a Golgi stack showing DAB reaction product labeling the *cis*-most Golgi cisterna as well as the adjacent RER cisternae with which it may be connected (*small arrows*). Large arrows indicate 80–100-nm-diam vesicles. (*b*) View of a Golgi stack within the *cis*-most cisterna of which DAB reaction product identifies one domain within the central flattened region and another within a bulbous rim (*arrows*). (*c*) An RER cisterna cut in grazing section, displaying poly-ribosome arrays and associated, DAB-positive 50-nm-diam vesicles. (*d*) A thick (<1 μm) section showing an interconnected group of 50-nm vesicles with a connection to the nuclear envelope (*arrow*). (*e*) A view of the effects of reduced temperature showing a cloud of 50-nm vesicles lying adjacent to the RER (*arrows*). Only a minority of the vesicles are labeled with the DAB reaction product which, typically, fills the lumen. Bars, 0.2 μm.







**Figure 9.** The effect of colchicine on a HEP2 cell expressing ssHRP<sup>KDEL</sup>. HEP2 cells stably expressing ssHRP<sup>KDEL</sup> were treated with colchicine at 2  $\mu$ M for 1 h before fixation and processing for EM. (a) Low power view shows that DAB reaction product is distributed throughout the ER and within 80–100-nm-diam vesicles which are grouped in clusters throughout the cytoplasm (arrows). Note that this cell has a morphology typical of mitotic cells which have not been colchicine treated. (b) Enlargement of area shown in a revealing details of accumulated DAB-positive vesicles. Bars, 2.0  $\mu$ m.

serve may suggest which of these structures lie before and which lie after interaction of the KDEL-ligand with its receptor. If, as suggested, the binding of ligand to erd2 is associated with an oligomerization into patches of the receptor

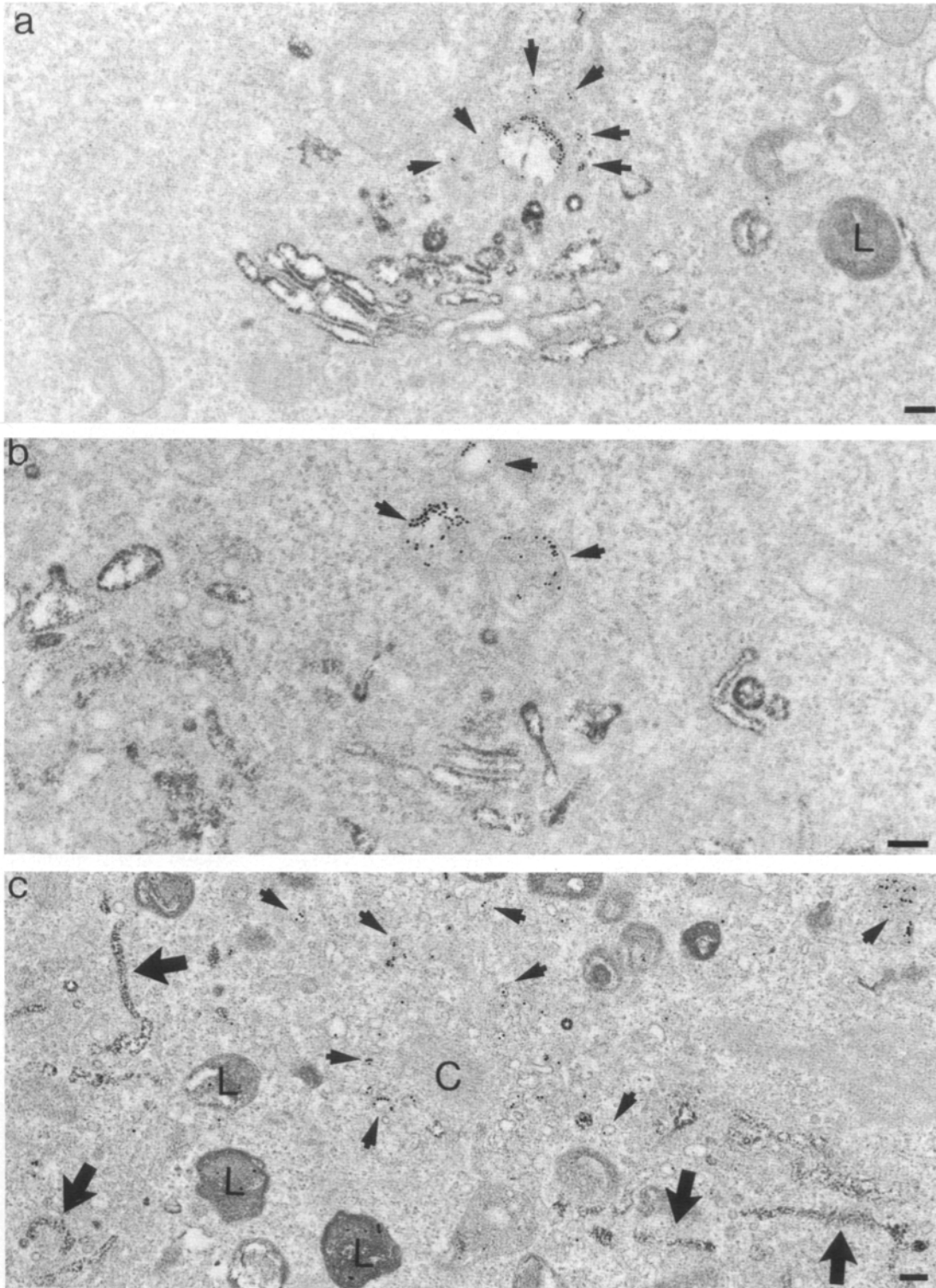
(Lewis and Pelham, 1992; Townsley et al., 1993), thereby enhancing the concentrative effect of a soluble ligand binding to a membrane-bound receptor, then the higher levels of staining observed with ssHRP<sup>KDEL</sup> indicate which elements contain receptor-bound tracer. It should be noted that the regions of high concentration we see within the *cis*-Golgi cisterna, (especially in buds) are similar in size and enhanced staining to the 80-nm vesicles distributed around the Golgi complex, scattered throughout the cytoplasm and in continuity with ER cisternae. Together these elements probably outline the retrograde pathway followed by KDEL-ligand-erd 2 complexes from the *cis*-Golgi to the ER.

The 50-nm-diam vesicles which also stain strongly with DAB could also be on the KDEL retrograde pathway. However, the tubular connections which are often seen to attach them to the RER and nuclear envelope suggest that they are in the process of forming rather than fusing with these cisternal elements. Moreover, the homogeneous distribution of reaction product within these vesicles is very different from the membrane associated staining seen in the 80-nm-diam elements. The increased DAB staining observed in 50-nm vesicles may not, therefore, be due to the HRP ligand bound to the KDEL receptor but may, instead, indicate a less specific, concentrative step taking place on the anterograde pathway. The existence of a concentrative vesicular step on the anterograde pathway between the ER and the *cis*-Golgi has recently been proposed for both a soluble, secretory protein, and a trafficking viral membrane protein (Balch et al., 1994; Mizuno and Singer, 1993).

One of the effects of colchicine that we observed was an increase in the number of 80-nm-diam vesicles. This would be consistent with the view that these vesicles are on the retrograde pathway, since it has been shown that other microtubule-disrupting drugs such as nocodazole and griseofulvin also block transport in this direction (Lippincott-Schwartz et al., 1990). However, the colchicine treatment also leads to an accumulation of ssHRP<sup>KDEL</sup> in the RER suggesting, in agreement with the work of Saraste and Svensson (1991), that microtubule depolymerization also has an influence on transport in the anterograde direction.

The relationship between the compartments containing ssHRP<sup>KDEL</sup> which we have identified, and the elements identified in previous studies of transport between the RER and the Golgi complex will need to be addressed in future work. At the present time it is not possible to obtain a coherent view of how the intermediate compartments identified by marker proteins such as p53 and p63 (Schweitzer et al., 1988, 1990, 1993) relate, for example, to the structures involved in the transfer of viral membrane proteins out of the RER (Hobman et al., 1992). From the effects observed at lowered temperatures and following treatment with microtubule-depolymerizing agents (Saraste and Kuismanen, 1984;

**Figure 8.** HEP2 cells expressing ssHRP<sup>KDEL</sup> during incubation at 15°C. HEP2 cells stably expressing ssHRP<sup>KDEL</sup> were incubated for 20 h at 15°C before fixation and processing for EM peroxidase cytochemistry. The section shows Golgi complexes consisting of whorls of cisternae and a mitochondrial morphology characteristic of the effects of lowered temperature on these cells. Several kinds of vesicles are evident: the small arrowheads indicate 50-nm-diam vesicles lacking DAB, the larger arrowheads indicate coat material which is probably free in the cytoplasm, the arrows indicate  $\sim$ 80-nm-diam vesicles containing DAB reaction product associated with the inner face of their perimeter membranes. In thin sections like this it is difficult to determine if the smaller positively stained profiles are 50-nm-diam vesicles or tangential sections cutting across the periphery of  $\sim$ 80-nm-diam vesicles. RER cisternae are no longer stained with DAB at this lowered temperature. Bar, 2.0  $\mu$ m.



**Figure 10.** HEp2 cells expressing ssHRP that have been allowed to endocytose colloidal gold coupled to antibodies that recognize the transferrin receptor. (a) HEp2 cells stably expressing ssHRP were incubated for 2 h at 20°C in the presence of B<sub>3/25</sub> gold complexes before fixation and processing for EM. This section shows that HRP reaction product is distributed throughout the Golgi stack and within elements in the *trans*-Golgi area. Gold conjugates (arrows) clearly identify the tubules and vacuoles of the endocytic pathway. Lysosomes are not labeled by either tracer. (b) As in a but cells were warmed to 37°C for 10 min in the continued presence of gold conjugate before fixation. Gold-loaded elements are not labeled by ssHRP. (c) As in a but cells were warmed to 37°C in the continued presence of gold conjugate for 30 min before fixation. There are numerous gold-labeled endocytic elements (arrows) surrounding the centriole (C). The *trans*-Golgi elements are largely free of DAB reaction product but some (large arrows) can be identified. Lysosomes (L) remain unlabeled and there are no elements labeled with both gold and HRP. Bars, 0.2 μm.

Saraste and Svensson, 1991; Plutner et al., 1992; Schweizer et al., 1993; Krijnse-Locker et al., 1994; Balch et al., 1994) it is probable that many of the structures described by others as belonging to the intermediate compartment are the same as those described here. However, it should be emphasized that while previous high-resolution studies have described only anterograde traffic between the RER and the Golgi complex, our ssHRP<sup>KDEL</sup> tracer outlines compartments involved in both anterograde and retrograde traffic.

Further progress will probably require comparative analyses in which soluble phase proteins (with and without RER retention signals) and membrane proteins are localized simultaneously.

The staining of ssHRP across the Golgi stack suggests an increasing concentration of this tracer in the more *trans* elements. A concentration of a fluid-phase marker as it traverses the Golgi stack has been reported before (e.g., Bendayan et al., 1980; Pelham, 1989). This increase in concentration would arise if retrograde pathways have a relatively low fluid-phase capacity, but there is as yet no indication that retrograde-directed vesicles are of lower carrying capacity (e.g., smaller) than anterograde vesicles. Alternatively, the increased staining may be due to some other process such as the removal of water from this part of the anterograde pathway.

The distribution of ssHRP reaction product at 20°C clearly identifies the form and extent of the *trans*-Golgi compartment in HEp2 cells. This compartment, originally defined as the TGN in studies using viral proteins (Griffiths and Simons, 1986), is found in the pericentriolar area. Biochemical analyses have revealed in more than one cell type and with several different surface proteins (e.g., Snider and Rogers, 1985; Green and Kelly, 1992) that there is trafficking between the endocytic pathway and the TGN. Experiments with brefeldin A (Wood et al., 1991; Lippincott-Schwartz et al., 1991; Reaves and Banting, 1992) also suggest a close relationship between these two compartments. However, there is little evidence from previous morphological observations that direct continuity exists between them. Our analysis confirms the work of Geuze and others (Geuze and Morré, 1991) that an extensive tubular reticulum exists in the *trans*-Golgi area at 20°C in the absence of viral infection. However, our HRP reporter technique, which should allow detection of even the most narrowly attenuated continuities between cisternae, also fails to identify connections between this compartment and the endosome.

In addition to defining the constitutive exocytic route HRP reporters clearly have wide potential for other pathways. By coupling targeting signals from other proteins to HRP, most of the intracellular pathways which cannot be accessed from the cell surface can be examined. By coupling membrane anchors and transmembrane segments carrying cytoplasmic domains of membrane proteins containing targeting signals to HRP membrane proteins trafficking throughout the cell can be studied. We have already shown that cDNA chimeras between HRP and transferrin receptor and sialyl transferase segments are enzymatically active (unpublished observations): their trafficking is currently being investigated.

Finally, in addition to these morphological studies, it should be possible to exploit the ability of HRP to render colocalized proteins insoluble as in previous studies of the

endocytic pathway (Courtroy et al., 1984; Ajioka and Kaplan, 1987; Stoorvogel et al., 1988; Stoorvogel et al., 1989; Stoorvogel et al., 1991). This would be extremely useful for quantitative studies of protein sorting between exocytic routes.

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