Anterograde and Retrograde Traffic between the Rough Endoplasmic Reticulum and the Golgi Complex

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Abstract. The transfer of newly synthesized membrane proteins moving from the rough endoplasmic reticulum (RER) to the Golgi complex has been studied by electron microscopy in HEp-2 cells transfected with cDNAs for chimeric proteins. These proteins consist of a reporter enzyme, horseradish peroxidase (HRP), anchored to the transmembrane domains of two integral membrane proteins, the transferrin receptor and sialyltransferase. The chimeras are distributed throughout the nuclear envelope, RER, vesicular tubular clusters (VTCs) and a network of tubules in the *cis*-Golgi area. At 20°C tubules containing chimera connect the RER to the VTCs and to the *cis*-Golgi network. On transfer to 37°C in the presence of dithiothreitol (DTT), the chimeras are seen to move from the RER and through the Golgi stack. With this temperature shift the direct connections with the RER are lost and free vesicles form; some of these vesicles contain HRP reaction product which is much more concentrated than in the adjacent RER while others lack reaction product entirely.

In cells expressing $SSHRP^{KDEL}$, DAB reaction product remains distributed throughout the RER, the VTCs, and the *cis*-Golgi network for prolonged periods in the presence of DTT and almost all of the vesicles which form at 37°C are DAB-positive.

Together these observations demonstrate that all three chimeras are transported from the RER to the *cis*-Golgi in free, 40–60-nm vesicles at 37°C. They also suggest that the retrograde traffic which carries $SSHRP^{KDEL}$ back to the RER is probably mediated by vesicles with a similar morphology but which, in cells expressing membrane-anchored chimeras, lack detectable reaction product.

One area attracting much current interest is how newly synthesized membrane proteins are transported from their sites of synthesis and membrane-insertion in the rough endoplasmic reticulum (RER)[1] to the Golgi stack. Recently a compartment has been functionally identified *en route* between the RER and the Golgi stack which appears to be involved in the retrieval of RER proteins with a KDEL retrieval signal (Pelham, 1988, 1991; Hauri and Schweizer, 1992; Saraste and Kuismanen, 1992; Lippincott-Schwartz, 1993; Griffiths et al., 1994). The morphology of this intermediate compartment has proved to be enigmatic, and it is unclear whether it constitutes a discrete, separate structure communicating with the RER and the Golgi by vesicular transport, or if it is in direct continuity with either the RER or the *cis*-Golgi. Evidence for the involvement of vesicular intermediates (in at least one step in RER-Golgi transport) has been obtained from yeast genetic studies and experiments employing cell free transport assays. These suggest that RER to Golgi transport has similar biochemical requirements to intra-Golgi transport which is thought to be vesicular (for reviews see Pryer et al., 1992; Schekman, 1992; Rothman, 1994). Furthermore, a variety of vesicles can be synthesized from yeast RER membranes in vitro which are competent to fuse with Golgi stack membranes (Groesch et al., 1990; Salama et al., 1993; Barlowe et al., 1994; Rexach et al., 1994), and distinct vesicular stomatitis virus glycoprotein (VSV-G)–containing vesicles budding from the RER have been observed in morphological studies monitoring VSV-G protein transport in permeabilized cells (Plutner et al., 1992; Balch et al., 1994). Studies showing that a post-RER intermediate structure is maintained under conditions in which the Golgi stack appears to break down (Lippincott-Schwartz et al., 1990; Saraste and Svensson, 1991; Kao and Draper, 1992; Alcade et al., 1994) have supported the idea that the intermediate compartment is indepen-

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1. *Abbreviations used in this paper*: BFA, brefeldin A; HRP, horseradish peroxidase; RER, rough endoplasmic reticulum; ssHRP, soluble secreted form of HRP; ST, sialyltransferase; TfnR, transferrin receptor; T^--TfnR, TfnR lacking the cytoplasmic tail; VSV-G, vesicular stomatitis virus glycoprotein; VTCs, vesicular tubular clusters.

dent from the Golgi complex, whereas electron microscopy has identified direct membrane connections between the RER and tubular-vesicular complexes in the *cis*-Golgi area which suggest instead that the intermediate compartment could be regarded as a functionally specialized region of the RER (Griffiths and Rottier, 1992; Hauri and Schweizer, 1992; Krijnse-Locker et al., 1994). Direct continuities have also been reported between the tubular-vesicular complexes of the *cis*-Golgi area and the cisternae of the Golgi stack (Lindsey and Ellisman, 1985*a*,*b*; Tang et al., 1993; Sesso et al., 1994), while in virally-infected cells connections extending from the RER into the *cis*-most Golgi cisternae have also been described (Krijnse-Locker et al., 1994), suggesting that vesicular transport may not be required until proteins reach the more medial cisternae of the Golgi stack.

The intermediates involved in retrograde traffic from the Golgi to the RER also remain undefined. The observation that tubules extend along microtubules between the Golgi and the RER during initial stages of treatment with the drug brefeldin A (BFA) suggests that tubules can mediate retrograde traffic (Lippincott-Schwartz et al., 1990; Klausner et al., 1992). However, alternative retrograde pathways may also operate since pretreatment of cells with $[AlF_4]^-$ inhibits the BFA-induced appearance of tubules and the relocation of Golgi enzymes, but allows the BFA-induced redistribution of cycling proteins from the *cis*-Golgi to the intermediate compartment to continue (Alcade et al., 1994). Tubular connections have also been proposed to operate in anterograde transport of the KDEL-receptor from the intermediate compartment during recovery from BFA treatment (Tang et al., 1993).

Identifying the intermediates involved in anterograde and retrograde membrane protein traffic between the RER and the Golgi has proved difficult because the number of proteins in transit are usually well below the sensitivity of the techniques available for localizing them. Several studies have relied on the exceptional levels of membrane protein expression induced by viral infection, followed by subsequent detection with immuno-labeling (e.g., Schweizer et al., 1990; Lotti et al., 1992; Plutner et al., 1992; Balch et al., 1994). Others have described the form and distribution of the intermediate compartment itself as defined by "resident" marker proteins (Saraste et al., 1987; Schweizer et al., 1988; Chavrier et al., 1990; Semenza et al., 1990; Saraste and Svensson, 1991; Schweizer et al., 1993; Tang et al., 1993) even though many of these proteins (e.g., p53/58 and the receptor for KDEL) are thought to cycle actively between the intermediate compartment and the RER or *cis*-Golgi. Drug and/or low temperature treatments have also been used extensively in attempts to perturb the pathways between the RER and Golgi (e.g., Saraste and Kuismanen, 1984; Schweizer et al., 1988; Bonatti et al., 1989; Saraste and Svensson, 1991; Lippincott-Schwartz et al., 1990; Jäntti and Kuismanen, 1993 and references therein). Nevertheless, at the present time a consensus view of which elements comprise the intermediate compartment, and how many kinds of vesicular intermediates are involved in transport between the RER and Golgi, has yet to emerge.

We have studied intermediates in the biosynthetic pathway by using the enzyme horseradish peroxidase (HRP) as an amplifying reporter molecule. We previously showed that chimeric molecules between HRP and the signal sequence of secretory proteins could be used to monitor lumenal proteins being transported along the exocytic pathway (Connolly et al., 1994). Here we have extended this approach to follow the movement of membrane proteins from the RER to the Golgi by tagging the signal sequence and transmembrane regions of the human transferrin receptor (TfnR) with HRP. These domains were chosen because they are not thought to contain targeting signals (Trowbridge et al., 1993). We also show that when HRP is anchored to the transmembrane domains and signal sequences of sialyltransferase (ST) (Munro, 1991; Wong et al., 1992) it is directed to, and retained in, specific membranes within the secretory pathway, i.e., the *trans*-most membranes of the Golgi complex. Using these two probes we have been able to identify and characterize the structural intermediates used by these two types of membrane protein during their transport from the RER to the Golgi complex, and to relate them to the structures which HRP tagged with KDEL (Connolly et al., 1994) identifies as belonging to the intermediate compartment.

Materials and Methods

Culture and Transfection of HEp-2 Cells

HEp-2 (CCL 23; ATCC) cells were maintained in culture and transiently transfected using an electroporator (BioRad, Hertfordshire, UK) as described (Connolly et al., 1994), except that cells were electroporated using three pulses at 450 V, 125 microfarads, and infinite ohms. The cells were subsequently plated into 15–24 × 3-cm dishes for each 75% confluent 9-cm starting dish, and maintained as described (Connolly et al., 1994). 17 h after transfection the percentage of the total cell population expressing active enzyme (defined by the presence of DAB reaction product) ranged between 20–80%, and sometimes the level of expression between individual cells varied considerably.

DNA Constructs

DNA manipulations were carried out by standard procedures and reagents were used according to manufacturer's instructions. The construct encoding ssHRP^{KDEL} (pSRα.HRP^{KDEL}) has been described previously (Connolly et al., 1994).

Construction of pSRα.T⁻-TfnRHRP

The transmembrane domain plus a few amino acids either side of the signal-anchor of the TfnR (amino acids 60-91) was obtained by PCR from a cDNA kindly provided by Dr. I. Trowbridge (Salk Institute, San Diego, CA). The primers used (5′ TCGGAATTCGGATCCTCGAGATGAAAAGGTGTAGTGGAAGT 3′; 5′TATGAAGCTTCAAGTCCTCTTCAGAAATGAGCTTTTGCTCCCCTTTAGAATAGCCCAA 3′) generate the TfnR fragment flanked by a new initiating methionine at the amino terminus, and by the c-*myc* epitope recognized by monoclonal antibody 9E10 at the carboxy terminus. In addition, the cysteine at position 89 was changed to serine, since this residue had been implicated in dimerization of the TfnR (Jing and Trowbridge, 1987). The PCR product was digested by XhoI and HindIII using the sites introduced via the primers, and used in a three-way ligation with HRP and the expression vector pSRα. HRP was released from BBG10 (R and D systems, Oxford, UK) with HindIII and BamHI, pSRα (Takebe et al., 1988) was digested with XhoI and BamHI. The ligation gave a construct whose predicted protein product is illustrated in Fig. 1.

Construction of pSRα. STHRP

pSRα.STHRP was made in a similar fashion to pSRα.T⁻-TfnRHRP, using a three way ligation of HRP, pSRα (both made as above) and a fragment of human β-galactoside α-2,6-sialyltransferase. The sialyltransferase frag-

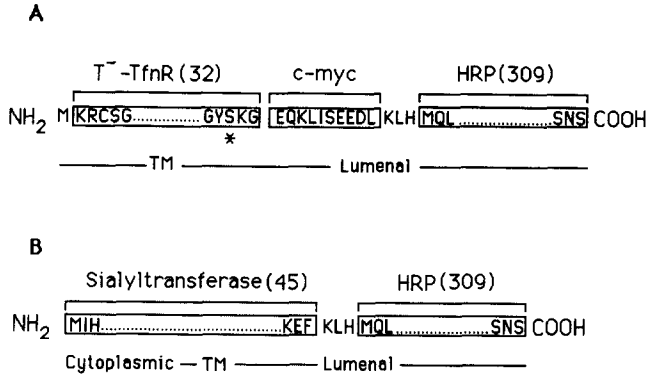


Figure 1. Predicted structure of T⁻-TfnRHRP and STHRP. The main components of T⁻-TfnRHRP (*A*) and STHRP (*B*) are shown in boxed sequences which are linked by the indicated amino acids introduced as a consequence of the cloning strategy. Numbers of residues in each element are shown in brackets. T⁻-TfnR, the transmembrane domain of the transferrin receptor; c-*myc*, epitope of c-*myc* recognized by the antibody 9E10; asterisk shows the serine altered from cysteine as described in Materials and Methods. An approximation of the predicted topology is shown beneath each construct.

ment (amino acids 1-45) was made by PCR from a cDNA (sequence as in Grundmann et al., 1990) kindly provided by Dr. S. Munro. The oligonucleotides used (5′ AGATCTCGAGGATCCACCATGATTCAC 3′; 5′ TTCGAAGCTTGAATTCCTTGGTTTGCAA 3′) allowed us to obtain the sequence flanked with XhoI and HindIII with which the PCR product was digested before ligation to HRP and to the expression vector to give a product which is predicted to express the chimera illustrated in Fig. 1.

20°C and DTT Treatment of Transfected Cells

For untreated samples, transfected cells were fixed directly (see below) 8–24 h after transfection. For long 20°C treatment, ~24 h after transfection the growth medium (DMEM) (Life Technologies Ltd., Paisley, UK) containing 10% FCS) was supplemented with 20 mM Hepes, and the cells were incubated for 16 h in a water bath at 19°C. The medium was supplemented with anti-TfnR antibody ($B_{3/25}$)-gold complexes (prepared as described; Hopkins and Trowbridge, 1983) for the final 1 h of treatment, after which the cells were fixed at 20°C.

For 20°C and DTT treatment, transfected cells were taken 14–17 h after transfection and incubated directly in a water bath at 19°C. After 2 h, the cells were either fixed (20°C) or incubated for an additional 5 min at 20°C in the presence of 20 mM DTT (Sigma Chem. Co. Ltd., Poole, UK). Cells were then rinsed once in DMEM-FCS at 37°C containing 1 mM DTT, and chased at 37°C in DMEM-FCS containing 1 mM DTT, a concentration shown to be sufficient for complete reduction of influenza hemagglutinin disulphide bonds (Braakman et al., 1992*a*). In a few cases cells were incubated for 2 h at 20°C as above, then rinsed and incubated for 5 min at 37°C in the absence of DTT.

Electron Microscopy

Fixation was for 15 min at 37°C or 20°C as appropriate, in 100 mM sodium cacodylate, pH 7.5 containing 2% paraformaldehyde and either 2.5% or 0.5% glutaraldehyde. Samples which had been treated with DTT were fixed in the presence of 1 mM DTT. All samples were then rinsed in 50 mM Tris-HCl, pH 7.5, incubated with H_2O_2 and DAB (Sigma) prepared as described by Graham and Karnovsky (1966). Cells fixed with 2.5% glutaraldehyde were then processed as described previously (Hopkins and Trowbridge, 1983). Cells fixed with 0.5% glutaraldehyde were postfixed in 1% osmium tetroxide/1.5% potassium ferricyanide and treated with tannic acid (Simionescu and Simionescu, 1976) before dehydration and embedding as above. Sections were cut on a Reichert-Jung Ultracut E microtome set at either 50–70-nm (thin), or 200–400-nm (thick), and viewed either unstained, or stained with lead citrate, in a Phillips CM12 transmission electron microscope.

Quantitation

The volume of the total RER occupied by DAB reaction product within a population of transfected cells was quantitated as follows. Electron micrographs at 5,000 times magnification were collected from every transfected cell (defined by the presence of DAB reaction product, regardless of location) in a single section; on average, 20–30 positive cells/section. Each picture was captured using a video camera and Macintosh image grabber, and processed using Photoshop software in conjunction with an Optilab package as follows. The gray levels were thresholded at two (high and low) levels to provide images in which the volume occupied by the total cellular membranes (low threshold) and total DAB reaction product (high threshold) could be quantitated on the basis of the number of pixels occupied. Membranes which did not belong to either the nuclear envelope or the RER cisternae (defined as ribosome-bearing membranes) were deleted manually. To measure the total RER volume, the area contained within RER membranes was filled in manually, and the final number of positive pixels was then calculated as the volume of total RER for that cell. The volume of RER occupied by DAB reaction product was given as the total number of positive pixels on the DAB-positive (high threshold) image after removal of non-RER components.

Preparation of HRP-containing Phosphatidylcholine Liposomes

Unilamellar artificial liposomes containing decreasing concentrations of HRP (type II; Sigma) were prepared at 4°C from L-α-phosphatidylcholine (type XVI-E; Sigma) by the detergent-dialysis method described by Volet et al. (1994). HRP concentrations were chosen to give liposomes containing 2, 0.2, or 0.02 HRP molecules per 50-nm vesicle (i.e., 200, 20, or 2% of the total vesicle population containing one molecule of HRP), and were calculated as follows. For HRP (mol wt = 44 kD), a 1-M solution of 44×10^3 g HRP/l contains 6.02×10^{23} molecules of HRP/l. Therefore one molecule of HRP has a mass of $44 \times 10^3/6.02 \times 10^{23}$ g = 7.3×10^{-17} mg HRP. The volume of a 50-nm spherical vesicle ($4/3\ \pi r^3$) = 6.6×10^4 nm^3 = 6.6×10^{-17} ml. Therefore to obtain one molecule/vesicle a final fluid phase concentration of $7.3 \times 10^{-17}/6.6 \times 10^{-17}$ = 1.1 mg/ml is required. Hence final fluid phase concentrations of 2.2, 0.22, or 0.022 mg/ml were used to obtain 50-nm vesicles containing 2, 0.2, or 0.02 HRP molecules, respectively. Direct measurement of liposome size showed them to be in the 60–80-nm range, and a slightly higher number of molecules/vesicle is, therefore, to be expected. Each preparation was adjusted to a final protein concentration of 2.2 mg/ml with BSA. Liposomes were purified by flotation through a 1.4-M sucrose cushion (50 min, 10,000 *g*, 4°C), and then attached to poly-L-lysine–coated plastic by incubating for 30 min at room temperature. The samples were then fixed (2% paraformaldehyde/2.5% glutaraldehyde), treated with DAB, and processed for EM with tannic acid treatment, as described above.

Results

T⁻-TfnRHRP Is Enzymatically Active Throughout Membrane Boundaries of the Exocytic and Endocytic Pathways

To provide a tracer for intracellular membrane traffic which lacked any known targeting or retention signals, a membrane-associated form of HRP was created using the signal anchor of TfnR (T⁻-TfnRHRP; Fig. 1), and transiently expressed in HEp-2 cells. In all cells expressing the chimeric protein, the overall intracellular morphology was similar to that of untransfected cells (data not shown). Active T⁻-TfnRHRP (monitored by diaminobenzidine [DAB] cytochemistry) was present on the plasma membrane, in cytoplasmic vesicles (50–80-nm diam), vacuoles (<3.0-μm diam) and tubules, and, to a lesser degree, in the Golgi complex (Fig. 2 *a*). In moderately expressing cells incubated at 20°C to inhibit transport from the TGN (see below), DAB reaction product could also be detected throughout the membranes of the nuclear envelope, RER, and all the Golgi cisternae (Figs. 2, *c–g* and 3). The activity

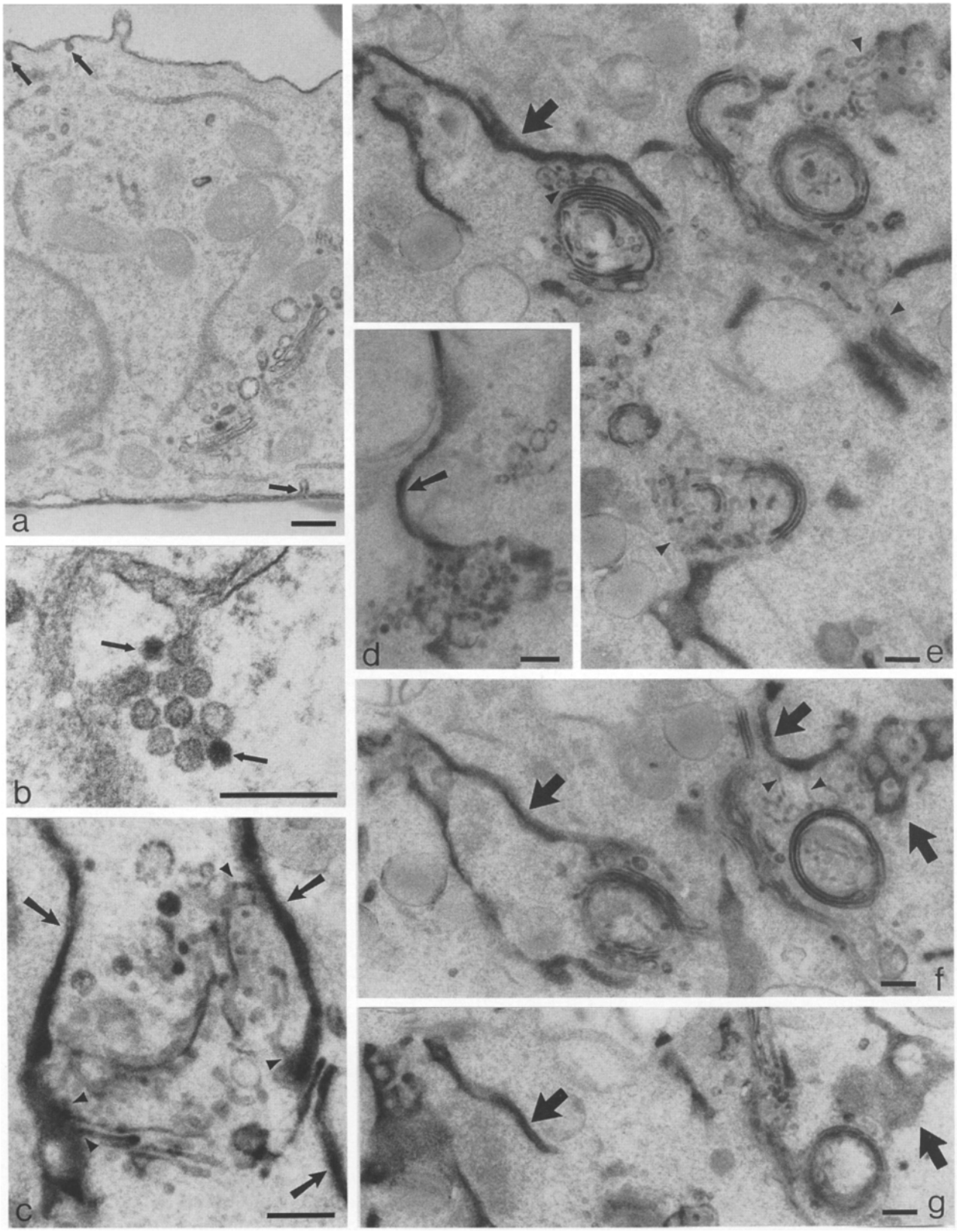


Figure 2. Distribution of T^--TfnRHRP at 37°C and 20°C. HEp-2 cells transfected with T^--TfnRHRP fixed either at 37°C without treatment (*a* and *b*), or after 16 h incubation at 20°C (*c–g*). (*a*) HRP activity is detectable in the RER, Golgi complex, and on the plasma membrane where several invaginations are evident (*arrows*), but is absent from mitochondrial membranes. Note the increase in concentration of DAB reaction product across the Golgi stack. (*b*) VTC associated with the RER in an untreated cell. Some vesicles (*arrows*) contain much higher concentrations of reaction product than the adjacent RER cisternae. (*c*) Thick (~0.5-μm) unstained section showing direct connections between the RER and the vesicular-tubular network of the *cis*-Golgi area at 20°C. Large arrows point to the RER

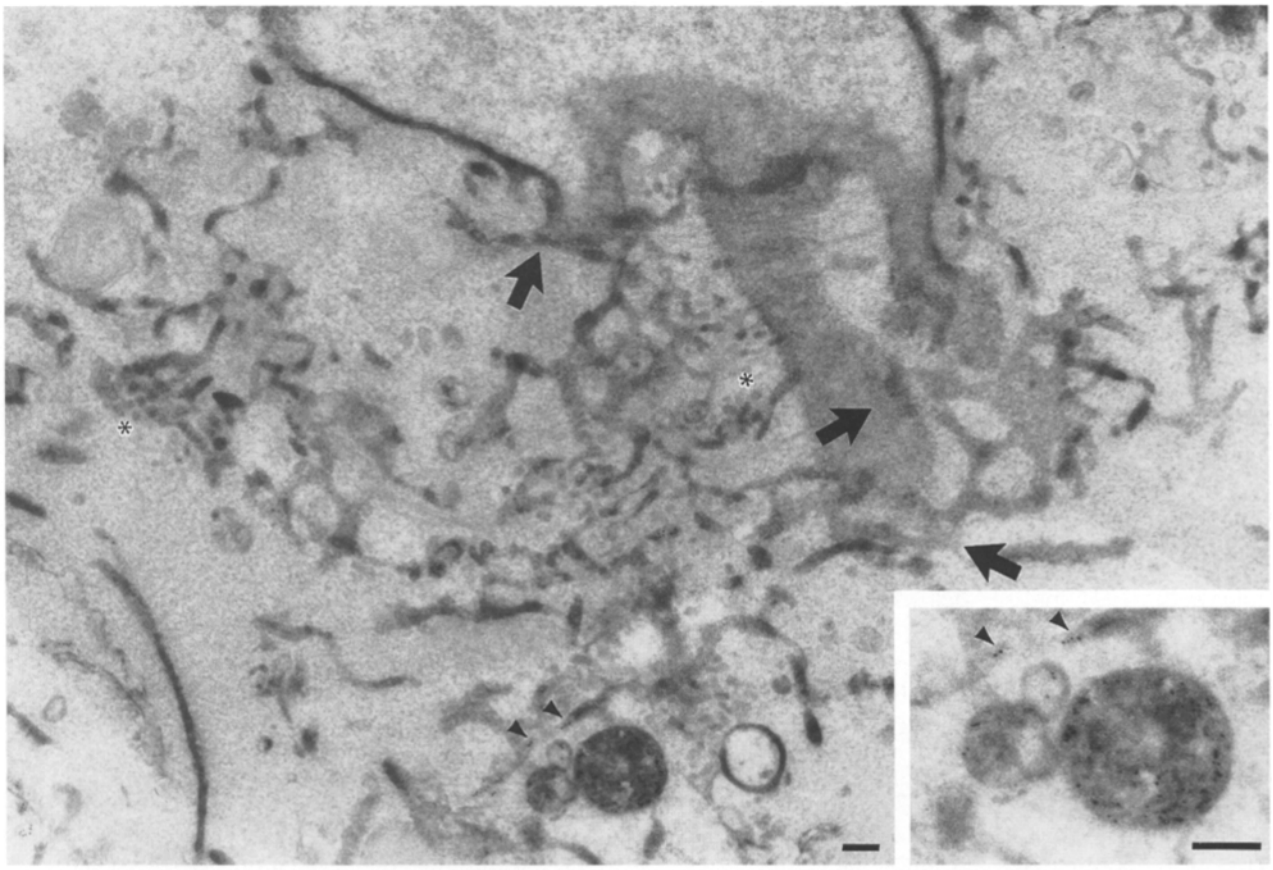


Figure 3. T⁻-TfnRHRP is present in the RER and VTCs at 20°C. Thick (~0.5-μm) section of a HEp-2 cell expressing T⁻-TfnRHRP incubated at 20°C for 16 h, with $B_{3/25}$-gold included during the final hour to label endocytic elements. Note the extensive continuity between the elements containing DAB reaction product. The nuclear envelope is frequently continuous with the highly reticular RER (*arrows*). Asterisks show VTCs associated with either the RER or the nuclear envelope. Arrowheads indicate endocytic elements containing internalized gold particles which are shown enlarged in the inset. Bars, 0.2 μm.

within these early exocytic compartments is newly synthesized protein in transit since, as described in detail below, it can be chased out of the RER and Golgi cisternae. Together these results are consistent with a default route for this chimera through the exocytic pathway.

As described previously for SSHRP (Connolly et al., 1994), variations in the density of the DAB reaction product were seen within vesicular intermediates between the RER and Golgi, and between the cisternae across the Golgi stack. In these confined locations differences in DAB-density probably give a direct indication of the concentration of chimera contained within their membrane boundaries because the cells are fixed before enzyme activation.

To identify endocytic elements containing T⁻-TfnRHRP, transfected cells were incubated for 1 h with $B_{3/25}$-antibody conjugated to gold. These complexes recognize the lumenal domain of endogenous TfnR (Hopkins and Trowbridge, 1983) and should not bind to chimeric T⁻-TfnRHRP. Structures which contained both HRP and gold, and were therefore endocytic, included small 50-nm vesicles and tubules, larger 0.2-μm vacuoles, and multivesicular bodies of 0.2–0.3-μm diam (Fig. 3). DAB-positive structures lacking $B_{3/25}$-gold, and therefore almost certainly intermediates on the earlier stages of the exocytic pathway, predominated in the *cis*-Golgi area.

Distribution of T⁻-TfnRHRP in Cells Incubated at 20°C

The Golgi Area. In HEp-2 cells the morphology of the Golgi area is very complicated at 37°C; the stacks are twisted and convoluted and surrounded by dense populations of small vesicles. To simplify this organization, we incubated cells at 20°C. At this temperature, transport to the TGN continues (Matlin and Simons, 1983; Connolly et al.,

cisternae, arrowheads indicate points of continuity. (*d*) Thick (~0.5-μm) unstained section showing a VTC associated with the RER (*arrow*). Most of the elements are interconnected and appear to contain DAB reaction product, although in these thick unstained sections vesicles without reaction product are not easily detectable. (*e–g*) Consecutive serial thick (~0.5-μm) sections showing the organization of the Golgi area at 20°C. In (*e*) the parallel Golgi cisternae, which probably all belong to a single stack, are seen as concentric whorls. Comparison of *e*, *f*, and *g* shows that each whorl is associated with RER cisternae (*large arrows*) which protrude towards it and are probably transitional elements. The tubular networks of the *cis*-Golgi are clearly displayed (*arrowheads*), lying between these RER cisternae and the concentric whorls of the Golgi stack. Note that at this temperature there are relatively few free vesicles associated with either the RER or the Golgi stack. Bars, 0.3 μm.

1994), but the Golgi stack is less elaborate and quantitation shows the number of 50–80-nm vesicles in the surrounding cytoplasm to be reduced by ~60%. The general organization of the cytoplasm shows no obvious deleterious changes at this temperature even after incubations as long as 16 h when the Golgi stacks become flattened, closely packed, and sometimes roll up to form onion-like profiles (Fig. 2, *e–g*). In cells at 20°C extensive networks of narrow branching 30-nm diam tubules are observed between the *cis*-most of the flattened Golgi cisternae and the RER which protrudes towards them. The RER cisternae probably correspond to the transitional elements described in earlier studies (Jamieson and Palade, 1967; Saraste and Kuismanen, 1984; Merisko et al., 1986; Orci et al., 1994). In unstained thick (~0.5-μm) sections (Fig. 2, *c* and *e–g*) in which DAB-loaded tubules can be readily discriminated from free vesicles, the tubular networks in the *cis*-Golgi area can be seen to be in direct continuity with RER cisternae. Since these networks occupy the same location as the DAB-positive tubules seen in SSHRP^{KDEL}-expressing cells (Connolly et al., 1994; and see below), we presume that they represent the intermediate compartment which has previously been described in this location (Schweizer et al., 1988; Saraste and Svensson, 1991; Hauri and Schweizer, 1992; Krijnse-Locker et al., 1994). At 20°C, all cells actively expressing T$^-$-TfnRHRP displayed DAB reaction product within this *cis*-Golgi tubular network and in all the flattened cisternae of the Golgi stack (Fig. 2).

An additional characteristic of the *cis*-Golgi area at 20°C is rosettes of tubules and vesicles, often containing reaction product, which surround masses of finely fibrous material (Fig. 4 *c*, see also Fig. 8 *a*). These structures, which lie in the cytoplasm adjacent to the *cis*-Golgi cisternae, remain during the first few minutes of warming to 37°C but are rarely seen after more prolonged periods at this temperature.

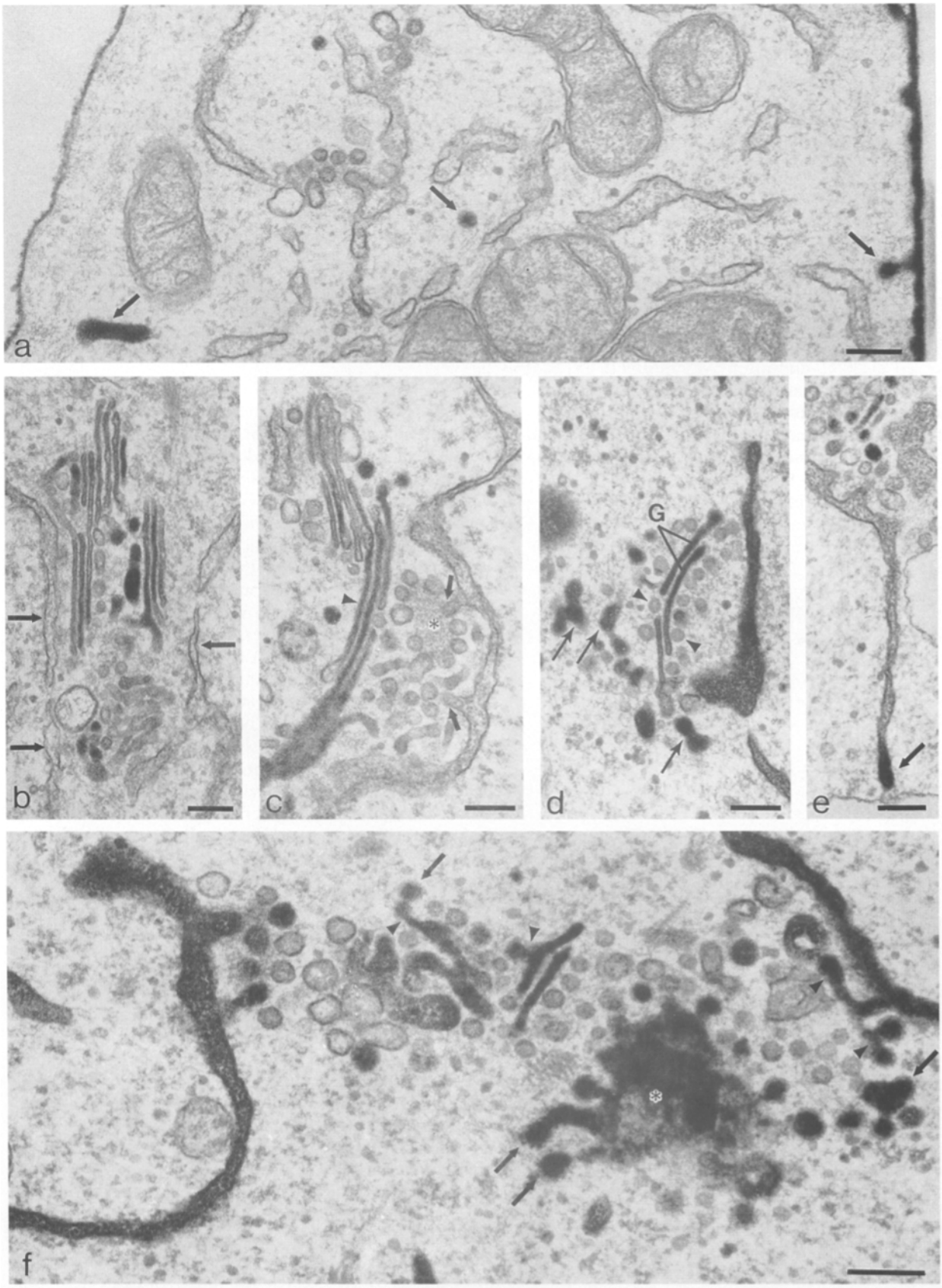
Vesicular-Tubular Clusters Associated with the Nuclear Envelope and Peripheral RER. In addition to the structures in the *cis*-Golgi area, at 20°C T$^-$-TfnRHRP was also detected in compact clusters of 60–80-nm vesicles lying close to the nuclear envelope and peripheral RER cisternae (Figs. 2 *d* and 3). These clusters are also present at 37°C (Fig. 2 *b*) and have many features in common with the vesicular-tubular clusters recently described in cells infected with vesicular stomatitis virus by Balch and his colleagues (1994) and referred to as "VTCs." For convenience we will also use this abbreviation.

At 20°C many of the vesicles within VTCs are directly connected to each other and to the RER by narrow (~30-nm diam) tubules (see also Fig. 8 *c*). In cells where the RER cisternae contain only moderate levels of reaction product, the DAB staining within some vesicles is much stronger than that of the adjacent RER lumen. It is also noteworthy that there are always some vesicles in these clusters which, even in the most strongly expressing cells, lack reaction product entirely (see below). The relationship of the VTCs to the *cis*-tubular networks in the Golgi area is not clear, but since they both contain T$^-$-TfnRHRP it seems likely that, as has been suggested previously (Merisko et al., 1986; Lippincott-Schwartz, 1993), they are both exit sites from the RER.

Enzymatically Active T$^-$-TfnRHRP Is Efficiently Cleared from the Biosynthetic Pathway in the Presence of Dithiothreitol

To determine whether the T$^-$-TfnRHRP detected within the RER and its associated vesicles and tubules at 20°C was newly synthesized protein in transit towards the Golgi, we developed a method for chasing enzymatically active T$^-$-TfnRHRP from these compartments when the temperature was increased to 37°C. Initial experiments indicated that although a soluble secreted form of HRP (SSHRP) drains efficiently from the exocytic pathway in the presence of 100 μg/ml cycloheximide (Connolly et al., 1994), significant levels of T$^-$-TfnRHRP and STHRP (see below) remained within the RER even after prolonged chases of the tracer (data not shown). Therefore, to avoid shutting down protein synthesis entirely, we determined whether the membrane-permeable reducing agent, dithiothreitol (DTT), could efficiently inhibit the production of active peroxidase, since it has been reported that when DTT is added to living cells the oxidizing environment of the RER rapidly becomes highly reducing. Thus, disulphide bond formation is prevented in folding newly synthesized proteins (Braakman et al., 1992*a*,*b*; Tatu et al., 1993; Valetti and Sitia, 1994). Because proteins are retained in the RER if they are incorrectly folded (Hurtley and Helenius, 1989; Pelham, 1989), only proteins which have folded before DTT addition or do not contain disulphide bonds should proceed along the exocytic pathway (Chanat et al., 1993; Lodish and Kong, 1993; Tatu et al., 1993). Since HRP contains disulphide bonds which appear to be required for its activity (HRP run on reducing gels loses its enzymatic activity;

Figure 4. Transport intermediates labeled by T$^-$-TfnRHRP during exit from the RER. (*a*) HEp-2 cell transfected with T$^-$-TfnRHRP incubated for 2 h at 20°C, 5 min with 20 mM DTT at 20°C, and then 1 h with 1 mM DTT at 37°C. Although the HRP activity on the plasma membrane and in endocytic structures (*arrows*) remains strong, T$^-$-TfnRHRP is completely cleared from the RER and its associated vesicles after 1 h at 37°C with DTT. (*b–f*) HEp-2 cells as above incubated at 20°C for 2 h, and fixed either immediately (*b*), or after incubation with 20 mM DTT at 20°C (5 min), and then 1 mM DTT at 37°C for 5 (*c*) or 15 (*d–f*) min. (*b*) At 20°C the transitional cisternae of the RER (*arrows*) lie closely adjacent to the *cis*-Golgi cisternae. Note that relatively few vesicles are present at this temperature. (*c*) Upon transfer to 37°C for 5 min, the number of vesicles and budding profiles (e.g., *arrows*) on the RER has increased. Note the rosette of vesicles surrounding the concentrated mass of finely fibrous material (*asterisk*). In both *b* and *c* the level of T$^-$-TfnRHRP expression within the RER is relatively low which allows the increased concentration of the reaction product within the Golgi stack (*arrowhead* in *c*) to be seen. (*d*) Golgi region showing a strong T$^-$-TfnRHRP signal in the RER, *cis*-Golgi tubules (*arrows*) and flattened cisternae of the Golgi stack (*G*). The adjacent vesicles which have probably arisen upon transfer to 37°C lack reaction product (e.g., *arrowheads*). (*e*) In this cell T$^-$-TfnRHRP has begun to clear from the RER by 15 min at 37°C but there are local concentrations of reaction product in the blunt extremities of the cisternae (*arrow*). (*f*) Note the strong DAB labeling within the RER cisternae and many RER-associated vesicles. In their immediate vicinity there are also vesicles lacking reaction product. Also indicated (*arrowheads*) are 30-nm tubules with connected



vesicles. The DAB reaction product indicated by the asterisk is uneven because this cisternal element is cut in tangential, grazing section. Note the clearly delineated membranes of the RER and the fine cytoplasmic coats on many of the developing buds (*arrows*). In *d*, *e*, and *f* the DAB reaction product within the RER has a granular appearance which is not seen in the budding vesicles and *cis*-Golgi tubules. Bars, 0.2 μm.

data not shown), enzyme synthesized in the presence of DTT should be enzymatically inactive. In addition, because it will have been unable to fold correctly it will probably be retained within the RER.

Preliminary studies showed that during the first 5–15 min of DTT treatment at 20°C there is a significant increase in the level of peroxidase signal in the RER and this effect seemed to increase with higher concentrations of DTT. To both maximize the amount of activity in the RER at the start of the DTT chase and to synchronize the exit of the protein from the RER, transfected cells were therefore incubated for 2 h at 20°C, 5 min with 20 mM DTT at 20°C, and the active T⁻-TfnRHRP was then chased at 37°C in the presence of 1 mM DTT (Figs. 4 and 5) for up to 2 h. At the end of the 20°C incubation and 20 mM DTT treatment, the distribution of T⁻-TfnRHRP was essentially the same as that observed at 20°C, and after 5–15 min with DTT at 37°C (Fig. 4, *c–f*), both the RER and Golgi complex still contained significant levels of DAB reaction product. However, after 1 h the RER was essentially cleared (Fig. 5) and by 2 h the VTCs, the *cis*-Golgi network and most of the cisternae in the Golgi stack were empty. The signal on the plasma membrane and within endocytic vesicles, however, still remained strongly positive at this time (Fig. 4 *a*). As described below, SSHRP^{KDEL} remains enzymatically active within the RER, within VTCs and within the *cis*-Golgi network for over 3 h in the presence of DTT. Therefore, the loss of active T⁻-TfnRHRP from the exocytic pathway which we observe during the DTT chases at 37°C arises because presynthesized T⁻-TfnRHRP drains out of the RER towards the cell surface, and not through degradation or inactivation of already active enzyme.

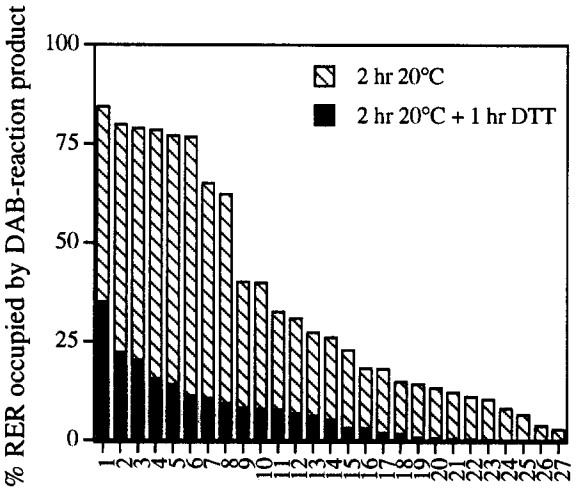


Figure 5. Quantitative analysis of the clearance of T⁻-TfnRHRP from the RER with 1 h DTT treatment. HEp-2 cells transfected with T⁻-TfnRHRP were incubated for 2 h at 20°C and either fixed immediately (*open bars*) or incubated with 20 mM DTT at 20°C for 5 min followed by 1 mM DTT at 37°C for 1 h (*solid bars*). Fig. 4, *b* and *a*, respectively, are examples of preparations of these treatments. The percentage of the total RER within a cell occupied by DAB reaction product was estimated from sections of T⁻-TfnRHRP–positive cells as described in Materials and Methods. Bars on the x-axis represent single cells, arranged in order of decreasing HRP content. The variation in value between cells directly reflects the variation in expression level seen in transiently transfected preparations. Nevertheless, it is evident that the amount of HRP-positive RER within the preparation as a whole is reduced very markedly by the 1 h DTT treatment.

Interestingly, with shorter incubations with DTT (<1 h), it was apparent that the chimera was not being cleared with the same efficiency from all regions of the RER because residual pools of DAB reaction product remained within the blunt ends of the more attenuated RER cisternae (Fig. 4 *e*). These profiles suggest that the drainage of chimera from the reticulum is occurring passively down a concentration gradient.

Characterization of the Transport Intermediates Carrying T⁻-TfnRHRP during RER-Golgi Transport at 37°C

As shown in Fig. 4 (*c–f*), upon initial shift to 37°C for 5–15 min, there was a dramatic increase in the number of vesicles associated with the RER and the Golgi complex, and active budding appeared to be occurring from the membranes of both of these compartments (see also Fig. 7 *d*). The parallel cisternae of the Golgi stack remained flattened during these initial stages (Fig. 4) and it was evident that a very significant increase in the number of vesicles occurred in the *cis*-Golgi area before the stack cisternae themselves become more bulbous. The majority of these new vesicles were 60-nm diam and many were DAB-negative. The tubular network lying between the RER and the *cis*-most cisternae of the Golgi stack also became more extensive; the diameter of the tubules increasing and often extending into ellipsoid (narrowest diameter 80-nm) protuberances, some of which appeared to be free tubulo-vesicles. While these tubules were initially strongly DAB-positive (Fig. 4 *d*), they cleared of reaction product after the first 30 min of chase. Since SSHRP^{KDEL} remained enzymatically active in these structures in the presence of DTT (see below), the disappearance of T⁻-TfnRHRP DAB reaction product indicates that the membrane-anchored protein had moved forward from these elements in the *cis*-Golgi area to the Golgi stack.

Vesicular and tubular elements persisted within the VTCs during the DTT chase at 37°C but within minutes, the vesicles became more widely separated and interconnections between them and the RER were seen much less frequently (Fig. 4 *f*, see also Fig. 8 *d*). Within these first few minutes the number of vesicles within VTCs also increased and although some of them remained strongly DAB-positive, a significant number lacked reaction product entirely (Fig. 4 *f*). During these early changes the lumen of adjacent RER cisternae often remained strongly DAB-positive, and the bud-like extensions which arose from these cisternae always contained DAB reaction product. The origin of the DAB-negative vesicles is thus unclear; they might have arisen directly from membrane of the DAB-positive RER cisternae or they could have arisen from DAB-positive vesicles within the VTCs themselves. In either case the mechanism which creates the chimera-free membrane would need to be very efficient. Alternatively, these DAB-negative vesicles could have been derived from the Golgi area, arriving at these peripheral sites via a long distance retrograde pathway.

DAB-negative Vesicles Do Not Contain HRP

It was important to determine whether the DAB-negative vesicles arising in the *cis*-Golgi area and at the VTCs lacked enzyme entirely. We therefore determined whether single molecules of HRP could be detected within 60–80-nm vesicles by preparing artificial uni-lamellar liposomes containing limiting concentrations of HRP. As shown in Fig. 6, at predicted concentrations of less than one HRP molecule/vesicle DAB-positive liposomes could be detected within the liposome population, and as the concentrations of HRP decreased, the number of positive vesicles decreased proportionally. This indicates that as little as one molecule of HRP can be detected within a 60–80-nm vesicle. We conclude, therefore, that the membranes of the DAB-negative vesicles which appear when RER-Golgi transport increases on transfer to 37°C do not contain any chimera.

HRP Anchored to the Transmembrane Domain of Sialyltransferase Is Retained within the trans-Golgi

As a more stringent test of the HRP-reporter technique and to confirm our observations on T⁻-TfnRHRP, we also followed the trafficking of a chimera carrying a retention signal. For this purpose HRP was anchored to the cytoplasmic tail of sialyltransferase (ST) plus its transmembrane region which is responsible for directing endogenous ST to the *trans*-Golgi (Munro, 1991; Wong et al., 1992) (STHRP; Fig. 1).

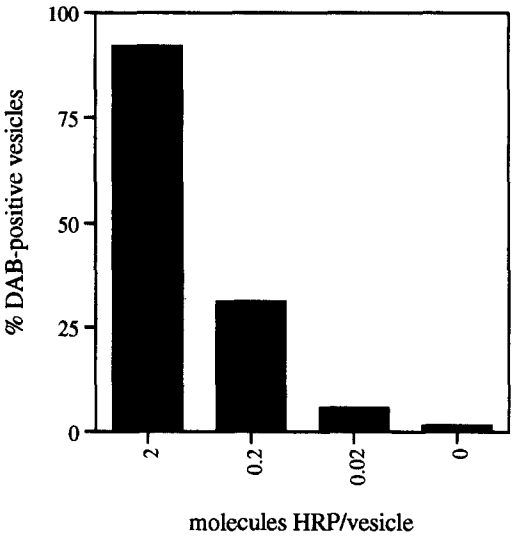


Figure 6. Single molecules of HRP can be detected within 60–80-nm vesicles by DAB-cytochemistry. Artificial 60–80-nm liposomes were made in the presence of HRP at concentrations predicted (see Materials and Methods) to give 2, 0.2, 0.02, or 0 HRP molecules/vesicle (i.e., one molecule (ave) of HRP in 200, 20, 2, or 0% of the resulting vesicle population). The number of DAB-positive vesicles observed is expressed as a percentage of the total number of vesicles in each sampled vesicle population (600 vesicles [average]/sample). Under these processing conditions, at concentrations greater than 1 molecule/vesicle, nearly all the vesicles contain DAB reaction product. Although at concentrations below 1 molecule/vesicle the proportion of positive vesicles is slightly higher than predicted (since the calculations were performed for 50-nm vesicles; see Materials and Methods), it is clear that as the amount of HRP decreases, the number of positive vesicles decreases as predicted if one molecule HRP/vesicle is detectable. The background level of false positives resulting from vesicles appearing electron dense due to tangential section planes is given by preparations made in the absence of HRP.

In cells transfected with STHRP, the DAB reaction product was concentrated within tubules on one side of the Golgi stack. Thick sections (Fig. 7 *a*) show that these tubules and the vacuoles and vesicles associated with them are mostly continuous with the outermost cisternae of the Golgi stack, forming an elaborate tubulo-vesicular structure, which presumably is a component of the *trans*-Golgi network. This indicates that the transmembrane domain of ST successfully targeted active HRP to its predicted intracellular destination. As with T⁻-TfnRHRP, variable levels of expression were observed between individual cells but, apart from rare instances in cells showing exceptionally high levels of expression, no labeling was detected with STHRP chimeras either at the plasma membrane or in early endocytic compartments.

STHRP and T⁻-TfnRHRP Are Transported via the Same Intermediates in the Initial Stages of the Exocytic Pathway

In cells transfected with STHRP and subjected to the 20°C preincubation and the 37°C DTT chase described above for T⁻-TfnRHRP, reaction product, which initially was present throughout the RER and *cis*-Golgi (Fig. 7, *b–d*), was cleared from these elements and their associated tubules and vesicles. At 20°C and initially at 37°C the STHRP DAB signal within some VTC vesicles was much stronger than that within the adjacent RER cisternae while other vesicles within these same VTCs were completely DAB-negative (Fig. 7, *b* and *c*). In general, the distribution of STHRP during the 20°C incubation, and its movement out of the RER, VTC, and *cis*-Golgi elements with subsequent chases with DTT at 37°C, was identical to that seen with T⁻-TfnRHRP. However, unlike the T⁻-TfnRHRP chimera, even after the longest chases the *trans*-Golgi elements remained strongly labeled with the STHRP chimera (data not shown).

The Distribution of SSHRP^{KDEL} in Cells Treated with DTT

We previously showed (Connolly et al., 1994) that HRP tagged with the RER-retrieval signal KDEL (SSHRP^{KDEL}) was present at 37°C throughout the RER, in VTCs, and in vesicles and tubules in the *cis*-Golgi area. This protein is presumed to leave the RER and be recycled back via the KDEL-receptor–mediated retrieval mechanism (Pelham, 1991). To identify transport intermediates on the retrograde pathway, we compared the structures labeled by SSHRP^{KDEL} (which should be present in intermediates on both the anterograde and retrograde RER-Golgi pathways) with those identified on the anterograde pathway in cells expressing T⁻-TfnRHRP and STHRP.

At 20°C SSHRP^{KDEL} was found in all the structures which contained T⁻-TfnRHRP at 20°C (Fig. 8, *a* and *b*). It was, however, excluded from most of the Golgi stack and was never observed in the *trans*-Golgi elements. After 2 h DTT chase (Fig. 8 *e*), the general distribution of SSHRP^{KDEL} was essentially the same as that seen in untreated cells (compare Connolly et al., 1994). Since T⁻-TfnRHRP and STHRP

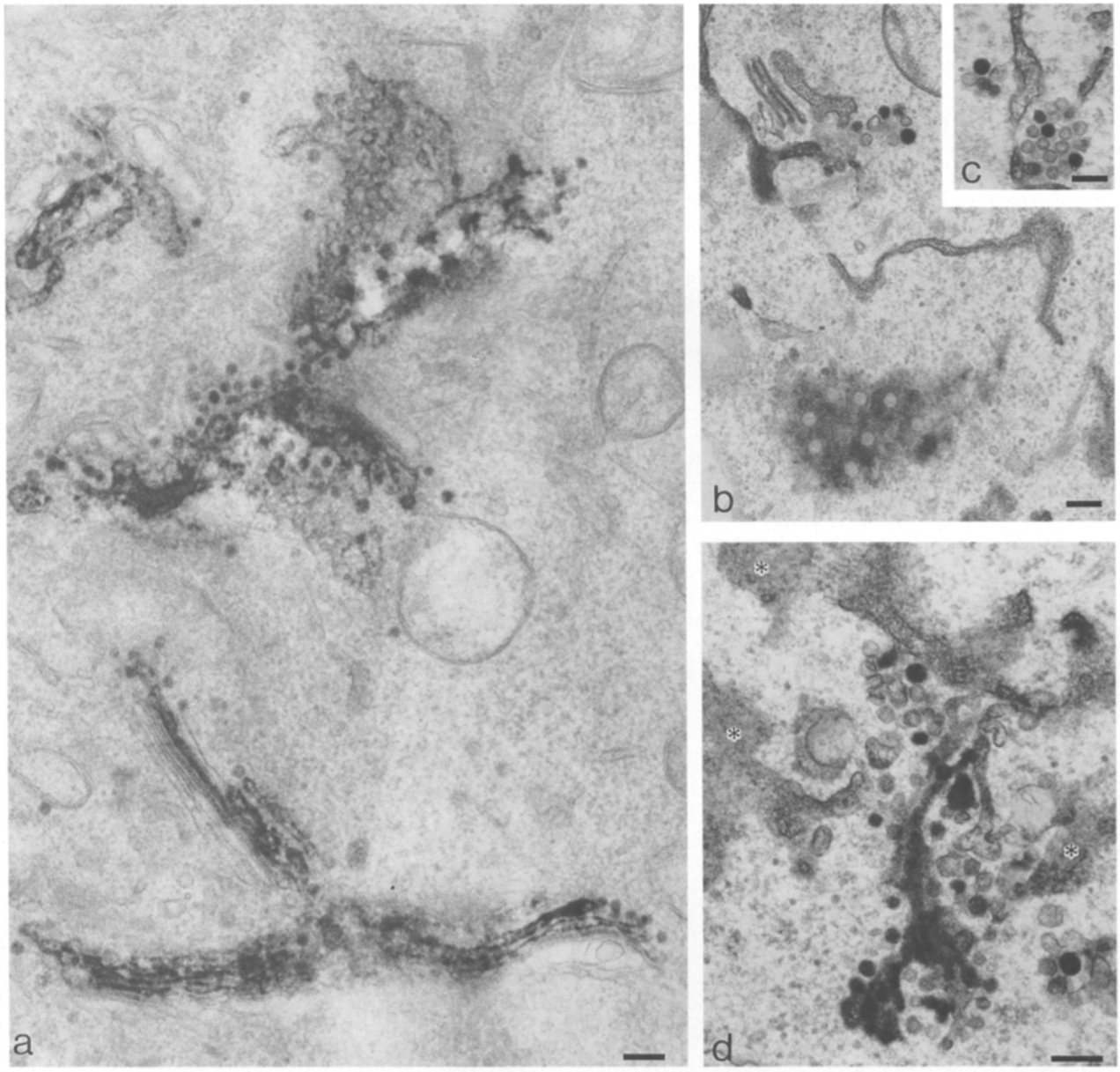


Figure 7. Distribution of STHRP in HEp-2 cells. HEp-2 cells transfected with STHRP were either fixed untreated (*a*), or incubated at 20°C for 2 h and either fixed immediately (*b* and *c*), or after 20 mM DTT at 20°C (5 min) and then 1 mM DTT at 37°C (5 min) (*d*). (*a*) Thick unstained section showing the steady state distribution of STHRP. At this expression level DAB reaction product is detectable predominantly on one side of the Golgi stack. HRP activity is distributed throughout the elaborate reticular network (presumably the TGN) and its associated vesicles, and extends only to 1, and sometimes 2, of the parallel cisternae within the stack. (*b* and *c*) Cells treated at 20°C showing moderate levels of STHRP expression in the RER, nuclear envelope and Golgi cisternae. Note the extreme variation in the concentration of DAB reaction product of the vesicles within the VTCs. Note also that while some VTC elements contain significantly more concentrated reaction product than the adjacent RER and its coated projections, others are completely negative. (*d*) Thick stained section of cells with moderate RER expression showing increased budding profiles on warming to 37°C. Note the granular appearance of the reaction product within the RER lumen and the direct connections between the RER cisternae and vesicles. There appear to be variable levels of reaction product in these RER-associated vesicles, but some of these variations, particularly within the cisternae themselves, are probably due to the tangential plane of the section; for the density of the DAB to be reliably assessed in this kind of structure it needs to be fully included within the thickness of the section. Asterisks indicate RER cisternae. Bars, 0.2 μm.

are cleared from the RER and *cis*-Golgi during this treatment, it is likely that $SSHRP^{KDEL}$ continues to cycle between the RER and Golgi stack.

The grainy distribution of the DAB reaction product within the RER of $SSHRP^{KDEL}$-expressing cells suggests that an ordered lumenal matrix exists in the RER from which DAB is excluded (Fig. 8, *a–b* and *e*). This matrix is also revealed in cells expressing high levels of T^{-}-TfnRHRP and STHRP chimeras (e.g. Figs. 4 *f* and 7 *d*), and is present throughout the RER and nuclear envelope. The DAB reaction product within the VTC vesicles is, however, strikingly different appearing uneven and blotchy (Fig. 8, *b* and *d*). Intriguingly, the content of the evaginated buds of the RER adjacent to VTCs has this same blotchy appearance,

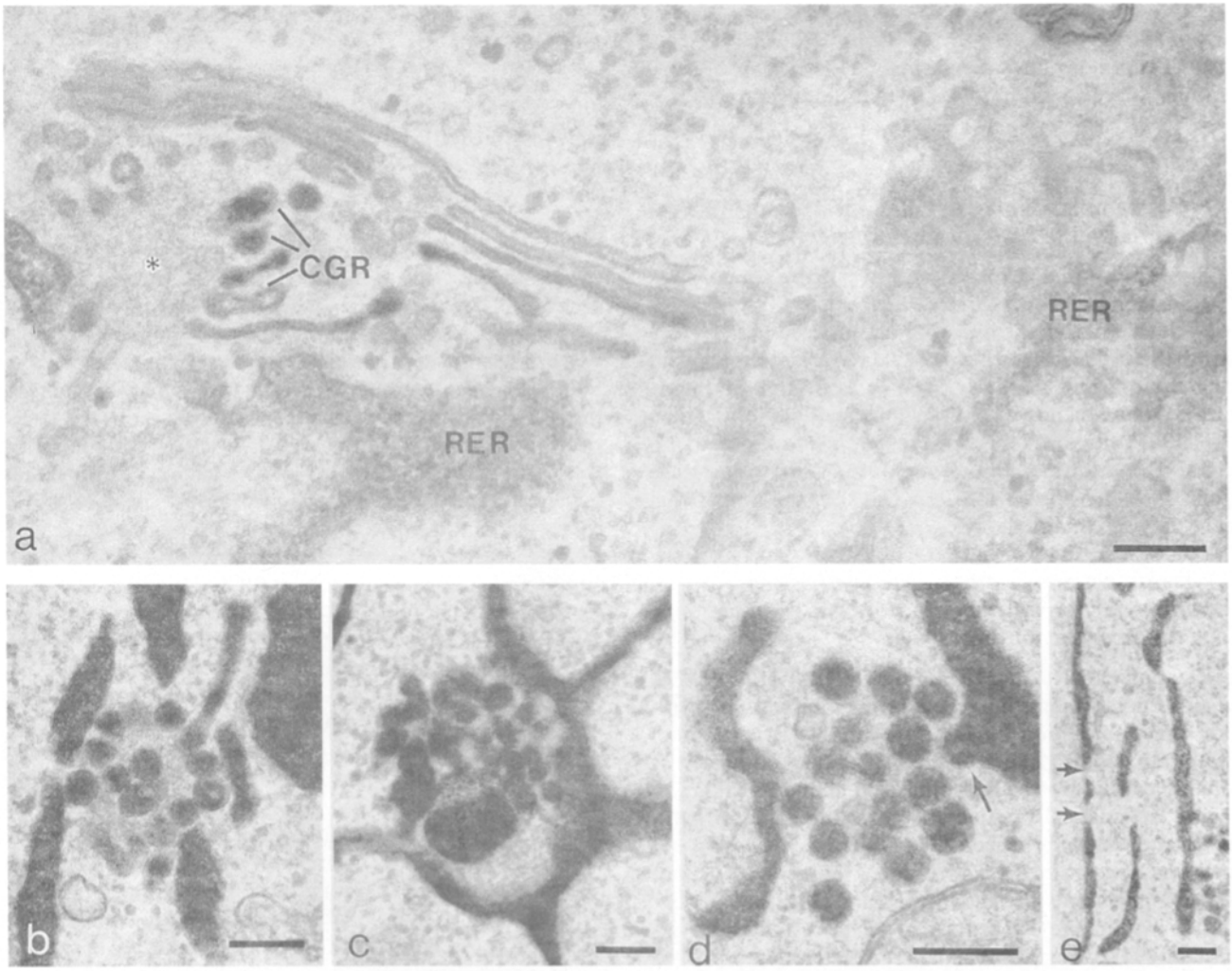


Figure 8. Transport intermediates between the RER and Golgi complex labeled by SSHRP^{KDEL}. HEp-2 cells transfected with SSHRP^{KDEL} were incubated for 2 h at 20°C, and then either fixed directly (*a* and *b*), or after incubation at 20°C with 20 mM DTT (5 min), and then at 37°C for either 5 min with (*c*) or without (*d*) 1 mM DTT, or 2 h with 1 mM DTT (*e*). (*a*) Golgi region in a moderately expressing cell at 20°C. SSHRP^{KDEL} is present in the RER (*RER*), *cis*-Golgi reticulum (*CGR*) and Golgi stack where, typically, it is confined to the *cis*-most cisterna. The asterisk indicates a rosette of concentrated finely fibrous material surrounded by vesicles and tubules, characteristic of 20°C preparations. (*b–d*) VTCs containing SSHRP^{KDEL} reaction product. *b* shows a VTC after 2 h at 20°C. On warming to 37°C (*c* and *d*), some vesicles within VTCs remain interconnected (*c*, *thick stained section*), but others become free and more dispersed (*d*, *thin stained section*). In both *b* and *d*, the content of the vesicular and tubular elements within the VTCs has a distinctive blotchy appearance. In the bud-like evaginations of the RER (shown by the arrow in *d*), the content has a similar blotchy appearance to that of the VTC vesicles and is very different to the even, granular appearance seen in the surrounding matrix of the RER lumen. (*e*) Distribution of SSHRP^{KDEL} after a 2-h DTT treatment. Unlike T$^-$-TfnRHRP, high levels of reaction product remain present within the nuclear envelope (arrows indicate nuclear pores), RER and associated vesicles. Bars, 0.2 μm.

and is distinct from the granular distribution seen in the rest of the RER lumen (Fig. 8 *d*).

The diameters of the *cis*-Golgi tubules (60-nm, average), and the VTC vesicles (80–90-nm) in SSHRP^{KDEL}-expressing cells were greater than the tubules and vesicles in cells expressing membrane-anchored chimeras (30-nm, average, and 50–70-nm, respectively). The great majority, and usually all, of the vesicles within the VTCs contained SSHRP^{KDEL} reaction product (Fig. 8, *b–d*). These vesicles were striking because of their spherical form and the way they appeared full, even distended, with reaction product. Vesicles with the slightly irregular shape of the DAB-negative vesicles observed in the VTCs of T$^-$-TfnRHRP- and STHRP-expressing cells were rarely observed. It thus appears that even though cells are fixed before the HRP is activated, the presence of SSHRP^{KDEL} within small vesicles and narrow tubules fills them to capacity with reaction product and this influences their size. Similar changes are seen in liposomes; those lacking HRP have an irregular outline and appear collapsed while those containing reaction product are spherical and full.

The Distribution of Cytoplasmic Coats

As shown in several figures (especially Fig. 4 *f*), many of the vesicles and membrane domains of cisternae involved in transporting membrane-anchored HRP chimeras bear

cytoplasmic coats. Work currently in progress suggests that it is possible to document the distribution of marker proteins for coats on these membranes. However, the distribution is complex and requires further detailed analysis.

Discussion

HRP-Chimeras As Probes to Follow Membrane Protein Traffic through the Exocytic Pathway

We have shown that HRP reporter molecules anchored to the transmembrane domains of two trafficking proteins can be detected from the time they are inserted in the RER, during their transport through the Golgi complex and along the exocytic pathway. The T^--TfnRHRP chimeras which contain no known targeting information (Trowbridge et al., 1993) travel to the cell surface, while those which include the transmembrane region of sialyltransferase (Munro, 1991; Wong et al., 1992) travel to the *trans*-Golgi where they are retained.

DTT Treatment Shows that Newly Synthesized Membrane-anchored Chimeras Move Anterogradely Along the Exocytic Pathway

The clearance of T^--TfnRHRP and STHRP from the RER and *cis*-Golgi elements observed during inhibition of disulphide bond formation by DTT treatment (Braakman et al., 1992*a*,*b*; Lodish and Kong, 1993; Tatu et al., 1993) is probably caused by newly synthesized chimeras failing to become enzymatically active and enter the exocytic pathway for the following reasons: (*1*) DTT did not inhibit enzyme which was already active and present in the more distal compartments of the pathway, and (*2*) although degradation within the RER may in some instances be increased by DTT (e.g., Wileman et al., 1991; Valetti and Sitia, 1994) $SSHRP^{KDEL}$ remained active within the RER for over 3 h of DTT treatment. The exit from the RER of already active (and therefore presumably folded) HRP in the presence of DTT is consistent with results obtained for other proteins (Braakman et al., 1992*a*; Lodish and Kong, 1993; Tatu et al., 1993).

The stepwise clearance of T^--TfnRHRP and STHRP from the RER and Golgi stack which takes place on transfer to 37°C in the presence of DTT shows that the vesicles derived from the VTCs and the *cis*-Golgi networks are intermediates in the net anterograde movement of these proteins from the RER. Conversely, the persistence of the $SSHRP^{KDEL}$ labeling within these structures during this time indicates that, as with other KDEL-bearing proteins (e.g., heavy chain binding protein; BiP) (Pelham, 1991), both anterograde and retrograde transport of this chimera is continuing. We believe that the structures labeled during the DTT chases are unlikely to be artefactually induced because (*a*) the final destinations of the chimeras were unchanged even after 2 h DTT treatment, (*b*) no major morphological changes occurred during the time required for DTT treatment to clear the RER of HRP activity (there was, for example, no indication that DTT causes a proliferation of RER, as has been suggested by studies on CHO cells [Helenius et al., 1992]), and (*c*) essentially the same DAB-positive structures were seen in the absence of DTT (data not shown).

Structures Involved in RER to Golgi Transport in the Golgi Area

Both SSHRP (Connolly et al., 1994) and T^--TfnRHRP accumulate in the TGN at 20°C indicating that, although the rate of transport may be reduced, anterograde transport from the RER to the Golgi stack continues under these conditions. Analysis of thick sections of cells incubated at 20°C shows that both membrane-anchored chimeras are present throughout the *cis*-Golgi network, the RER cisternae protruding towards it, and within the tubules which directly connect the two compartments. It should be noted that these direct connections were not observed at 16°C when transport between the RER and Golgi stack was inhibited (Connolly et al., 1994). Instead, at 16°C, elements in the Golgi area became extensively vesiculated. It has previously been reported that tubulation of the Golgi stack can occur in vitro and in vivo when ATP levels are depleted (Cluett et al., 1993). Under these conditions β-COP was found to dissociate from the Golgi membranes. Vesicle formation from these tubules was reinstated upon readdition of ATP and subsequent β-COP binding. It is likely that reducing the temperature to 20°C will also reduce energy levels and thus affect the ability of membrane boundaries to vesiculate. This is also consistent with the appearance of rosettes of tubular cisternae surrounding masses of finely fibrous material at 20°C. Similar structures, first observed in anoxic pancreatic cells (Merisko et al., 1986), have also been described in cells in which anterograde transport was inhibited by ATP depletion, BFA treatment or microinjection of β-COP antibodies, and in some instances the fibrous material has been shown to contain coatomer (Hendricks et al., 1992; Oprins et al., 1993; Pepperkok et al., 1993). On warming to 37°C, the dense fibrous masses disappear and direct connections between the RER and *cis*-Golgi network are less frequently seen. Instead, blunt-ended, coated buds protrude from the RER cisternae and large numbers of free vesicles and tubulo-vesicular elements appear in the adjacent cytoplasm. Together, these observations suggest that under 20°C conditions transport from the RER to the *cis*-Golgi network proceeds via direct tubular continuities which have a decreased requirement for bound coatomer. On transfer to 37°C, vesicular transport is reinstated and coatomer is presumably recruited from the adjacent masses of fibrous material which have accumulated at 20°C.

Vesicular Tubular Clusters

In a series of studies designed to follow the transport of VSV-G from the RER to the Golgi complex, Balch and his colleagues (Balch et al., 1994; Pind et al., 1994) described groups of tubular-vesicular elements similar to those identified by the HRP-chimeras in this study, and which they termed VTCs (vesicular tubular clusters). These workers also suggested that VTCs correspond to the fluorescent spots seen distributed throughout the peripheral cytoplasm in cells labeled with markers for the intermediate compartment (Schweizer et al., 1988; Saraste and Svensson, 1991). Our finding that at 20°C VTC-like clusters are often connected directly to the nuclear envelope and peripheral RER clearly demonstrates that these structures are a source of transport intermediates from the RER addi-

tional to those arising from the RER in the *cis*-Golgi area. Because VTCs are seen at similar sites in similar abundance in untreated, untransfected cells, we do not think that the parallel pathway represented by exit at the VTCs is an abnormal phenomenon induced in transfected or virally infected cells. The functional significance of the interconnections between VTCs and RER is not clear but their existence clearly suggests that much of the membrane within VTCs is derived directly from the RER and nuclear envelope cisternal membranes. It is also apparent that these connections, like those between the RER and the Golgi complex, can support transport, which indicates that individual vesicles are not the only means by which these compartments can communicate. The increased number of tubular connections we have observed at 20°C suggests that tubules will readily develop at sites of vesiculation and this could be the explanation for some of the recent difficulties encountered in morphological studies which have tried to determine whether or not vesicular transport steps occur between the RER and the intermediate compartment.

Concentration of Membrane Protein After RER Exit

In HEp-2 cells with moderate levels of expression of membrane-anchored chimeras, some of the components of the VTCs and of the vesicular-tubular structures of the *cis*-Golgi network always contain a much denser content of DAB reaction product than that in the adjacent RER lumen. These increased DAB concentrations suggest that chimeric proteins probably undergo a concentrative process on leaving the cisternal part of the RER. This is consistent with previous reports which proposed that membrane proteins are more concentrated as they exit from the RER (Copeland et al., 1988; Hobman et al., 1992; Balch et al., 1994). However, until we know the extent to which DAB reaction products can diffuse throughout the lumen of the RER compartment, direct comparisons between the density of DAB in the RER cisternae and that developing within the confines of a 60-nm diam vesicle can only be made with caution.

DAB-negative Vesicles Identify Alternative Membrane Trafficking Routes

Some vesicles in VTCs and in the *cis*-Golgi area lacked DAB reaction product in both T^--TfnRHRP– and STHRP-expressing cells. These unstained vesicles were present even when a strong signal was present throughout the adjacent cisternae of the RER and the Golgi stacks. Since the DAB reaction is sensitive enough to detect a single molecule of HRP within a 60-nm diam liposome, it is most unlikely that these vesicles contain active chimera. Recent results suggest that more than one kind of vesicle participates in forward transport from the RER (Barlowe et al., 1994; Oprins et al., 1993; Orci et al., 1994). Thus, as discussed in the Results, these empty vesicles might be a subset of unlabeled anterograde intermediates formed by segregation of membrane proteins upon exit from the RER. However, it seems more likely that they are on a retrograde pathway. This could be a local route responsible for the immediate retrieval of RER resident membrane during the formation of the strongly DAB-positive vesicles of VTCs, and/or it could be returning membrane from the *cis*-Golgi area since light microscope studies on BFA-treated cells clearly suggest that longer retrograde routes also exist (Lippincott-Schwartz et al., 1990).

The Distribution of SSHRP^{KDEL} on Anterograde and Retrograde Pathways

The regular, granular appearance of the DAB reaction product within the RER and the nuclear envelope seen with all three chimeras suggests that a structured matrix exists in the lumen of these locations. Such a structure might, for instance, be involved in retaining soluble glycoproteins and chaperones within the RER. The blotchy appearance of the DAB reaction product within the vesicles of the VTCs is clearly distinct from that in the RER and nuclear envelope, and suggests that differently structured environments exist within the lumena of these two types of organelle. The most intriguing display of this is seen in the content of the buds protruding from the RER membrane adjacent to the VTCs which are often blotchy and nongranular (Fig. 8 *d*). Some of these protrusions are clearly forming anterograde vesicles since this effect is seen with all three chimeras and has also been observed in current studies (unpublished) in which we have been able to inhibit the retrograde pathway for SSHRP^{KDEL}. Thus reordering of the RER matrix appears to occur at the site of budding to allow a free soluble phase to be removed into the forming vesicle. However, in SSHRP^{KDEL}-expressing cells some of these evaginations might also be sites where retrograde vesicles have fused but their content has not yet become integrated into the surrounding matrix of the RER lumen.

In VTCs of cells expressing SSHRP^{KDEL}, the proportion of vesicles without DAB reaction product is greatly reduced. If the DAB-negative vesicles which had failed to incorporate chimera in cells expressing the membrane-anchored proteins are on a retrograde pathway this is to be expected because in cells expressing SSHRP^{KDEL}, these vesicles should now be loaded with chimera being returned to the RER. Thus in the VTCs of SSHRP^{KDEL}-expressing cells both anterograde and retrograde vesicles should be labeled. Interestingly, no morphological differences indicating two types of transport intermediates could be detected between VTC SSHRP^{KDEL}-positive vesicles.

In cells which have not been preincubated at 20°C an additional subset of SSHRP^{KDEL} vesicles in which the DAB reaction product is concentrated beneath the perimeter membrane is also present (Connolly et al., 1994). These vesicles have rarely been observed under the conditions employed in the present study and we do not think, therefore, that they are involved in the mainstream of the membrane recycling which sustains RER-Golgi transport.

Conclusion

Together the observations made in the present study demonstrate that the intermediate compartment includes the *cis*-Golgi network and the vesicular tubular clusters (VTCs) associated with the peripheral RER. They also show that when the temperature is lowered to 20°C, the RER cisternae are directly connected to the *cis*-most cisternae of the Golgi stack. In contrast, at 37°C transport between the RER and the intermediate compartment seems to depend

primarily upon small 60–80-nm diam vesicles. The vesicles which lack membrane-anchored HRP are good candidates for intermediates on the Golgi-RER retrograde pathway but in cells expressing $SSHRP^{KDEL}$ in which both antero- and retrograde pathways should be labeled we have been unable to identify subsets within this population of 60–80-nm vesicles which suggest anterograde vesicles have a distinctive shape, content or other easily discernible morphological characteristic. In the work currently in progress in our laboratory, we are mapping the distribution of marker proteins for the various cytoplasmic coats on these vesicles. We hope that this, together with the indications of their destination given by their cargo of HRP chimeras, will allow us to clearly delineate the various anterograde and retrograde pathways operating between the RER, the intermediate compartments of the *cis*-Golgi and VTCs, and the Golgi stack.

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