Biosynthesis and Intracellular Sorting of Growth Hormone–Viral Envelope Glycoprotein Hybrids

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ABSTRACT Various aspects of the biogenetic mechanisms that are involved in the insertion of nascent plasma membrane proteins into the endoplasmic reticulum (ER) membrane and their subsequent distribution through the cell have been investigated. For these studies chimeric genes that encode hybrid proteins containing carboxy-terminal portions of the influenza virus hemagglutinin (154 amino acids) or the vesicular stomatitis virus envelope glycoprotein (G) (60 amino acids) linked to the carboxy terminus of a nearly complete secretory polypeptide, growth hormone (GH), were used. In in vitro transcription–translation experiments, it was found that the insertion signal in the GH portion of the chimeras led to incorporation of the membrane protein segments into the ER membrane. Effectively, GH became part of the luminal segment of membrane proteins of which only very small segments, corresponding to the cytoplasmic portions of the G or HA proteins, remained exposed on the surface of the microsomes. When the chimeric genes were expressed in transfected cells, the products, as expected, failed to be secreted and remained cell-associated. These results support the assignment of a halt transfer role to segments of the membrane polypeptides that include their transmembrane portions.

The hybrid polypeptide containing the carboxy-terminal portion of HA linked to GH accumulated in a juxtanuclear region of the cytoplasm within modified ER cisternae, closely apposed to the Golgi apparatus. The location and appearance of these cisternae suggested that they represent overdeveloped transitional ER elements and thus may correspond to a natural way station between the ER and the Golgi apparatus, in which further transfer of the artificial molecules is halted. The GH–G hybrid could only be detected in transfected cells treated with chloroquine, a drug that led to its accumulation in the membranes of endosome or lysosome-like cytoplasmic vesicles. Although the possibility that the chimeric protein entered such vesicles directly from the Golgi apparatus cannot be ruled out, it appears more likely that it was first transferred to the cell surface and was then internalized by endocytosis.

Several classes of polypeptides that attain different subcellular destinations or are ultimately exported from the cell are synthesized in ribosomes bound to endoplasmic reticulum (ER)[1] membranes (see references 17, 23, 28, and 39). Precursors of secretory and lysosomal polypeptides, on the one hand, and those of integral membrane proteins on the other, are already sorted into two distinct classes upon completion of their synthesis in the ER. Whereas those in the first group are discharged directly into the ER lumen, those in the second are initially incorporated into the ER membrane and, if destined to other locations, are then transported through the cell in a membrane-associated form.

Considerable evidence shows that a common mechanism, involving the recognition of specific insertion signals in the nascent chains, initiates the incorporation of all these different types of polypeptides into the ER membranes (see references 1 and 39). An additional feature present only in membrane

[1] *Abbreviations used in this paper.* ER, endoplasmic reticulum; G, glycoprotein; GH, growth hormone; HA, hemagglutinin; MDCK, Madin-Darby canine kidney; SV40, simian virus 40; VSV, vesicular stomatitis virus; WGA, wheat germ agglutinin.

polypeptides (halt or stop transfer signal) seems to interrupt translocation of the nascent chain into the ER lumen and to position the mature protein across the membrane (1, 39). Studies on the secretory and membrane forms of immunoglobulins (34, 53), as well as recombinant DNA experiments in which artificial truncated membrane proteins were produced (8, 35), have demonstrated the role of halt transfer signals in leading to the permanent association with the membrane of polypeptides that otherwise would be secreted from the cells.

Once polypeptides are translocated into the ER cisternal lumen or incorporated into the ER membrane, sorting mechanisms, which are yet poorly understood, must operate to determine the ultimate location of each polypeptide within the cell. These mechanisms most likely involve the interaction of sorting signals within the polypeptides with carrier proteins effecting transfer between different compartments, or immobilized receptors that lead to retention in a specific location. Except for some lysosomal enzymes in which modified oligosaccharide chains seem to serve as sorting signals (see reference 46), no other features of the proteins synthesized in the ER have been identified that mediate their posttranslational sorting.

The envelope glycoprotein of influenza virus hemagglutinin (HA) and the envelope glycoprotein (G) of vesicular stomatitis virus (VSV) have provided useful model systems for studying the biosynthesis and sorting of plasma membrane proteins since they have a simple transmembrane disposition and accumulate in large amounts in the plasma membrane of the infected cells from which viral budding takes place (see reference 45). Furthermore, in polarized epithelial cells, which possess distinct apical and basolateral surface domains, intracellular sorting steps also determine that the HA glycoprotein is delivered to the apical surface, whereas the G of VSV is incorporated preferentially in the basolateral aspects of the plasma membrane (32, 33).

In transmembrane proteins, sorting signals could be located in any one of the three domains defined by the phospholipid bilayer, i.e., the luminal, transmembrane, or cytoplasmic portions of the polypeptide. Most previous attempts to identify sorting signals within such proteins have involved deletions or modifications of the cytoplasmic (carboxy-terminal) domain. It was found that deletions in the E2 protein of Semliki Forest virus (7) or the heavy chain of the histocompatibility antigen (H2) (54) did not affect transport of the polypeptides to the cell surface. On the other hand, similar deletions in the G protein of VSV prevented its transport to the plasma membrane and led to the intracellular accumulation of immature glycosylated G polypeptides in pre-Golgi compartments (36). Alterations in the C-terminal region of the HA of influenza (47) or of vaccinia virus (43) have also been obtained which led to intracellular accumulation of the modified proteins. These observations demonstrate that addressing some of those proteins to the plasma membrane does not require intact cytoplasmic segments. However, the failure of other modified proteins to reach the cell surface does not necessarily imply that appropriate sorting signals are contained in these cytoplasmic segments since it is possible that changes in these portions of the polypeptides could alter the quaternary structure of the protein, or even affect the conformation of transmembrane or luminal portions.

Recently we studied the distribution of the VSV G and influenza HA glycoproteins synthesized in cells transfected with the corresponding isolated genes.[2] Both proteins were transported to the cell surface in the absence of other viral components but then showed strikingly different behaviors. Whereas the HA glycoprotein remained in the plasma membrane, and in polarized cells accumulated specifically in the apical domains, the G glycoprotein, after reaching the cell surface, underwent endocytosis and recycling through the cytoplasm. This was easily demonstrated by the accumulation of G in intracellular vesicles when the recycling process was impaired by chloroquine treatment.

In this paper we investigated the role of specific polypeptide segments in membrane insertion and posttranslational sorting and recycling. We constructed chimeric genes and studied, by in vitro transcription–translation and transfection experiments, the incorporation into membranes and the subcellular distribution of the encoded hybrid proteins. These hybrids consisted of carboxy-terminal portions of the HA or G glycoproteins linked to the carboxyl terminus of a nearly complete secretory polypeptide, growth hormone (GH). These experiments demonstrated, in agreement with the findings of Guan and Rose (12) with a similar GH–G hybrid, that linkage of a secretory protein to segments of membrane polypeptides containing putative halt transfer signals results in the cotranslational incorporation of the hybrids into the ER membranes and their retention within the cell. A chimeric polypeptide containing the carboxy-terminal 60-amino-acid residue of G appeared to behave like the intact glycoprotein in that, after chloroquine treatment, it was localized in endocytic vesicles. This suggests that this portion of the G protein contains the information that mediates internalization and recycling of the natural membrane protein. A hybrid polypeptide containing the carboxy terminal portion of HA (154 amino acids) linked to GH accumulated in a juxtanuclear region of the cytoplasm within membrane-bounded modified ER cisternae closely apposed to the Golgi apparatus. Immunoelectron microscopy suggested that the membranous elements in which this chimera accumulated may correspond to a natural way station between the ER and the Golgi apparatus. The striking development of these elements in cells containing large amounts of the chimera may reflect the incapacity of the artificial molecules to undergo further transport along the normal route toward the cell surface.

MATERIALS AND METHODS

Reagents: Radiolabeled compounds were obtained from New England Nuclear (Boston, MA), and restriction enzymes and polymerases from New England Biolabs (Beverly, MA). T4 DNA ligase was kindly provided by Chris Schindler (NYU Medical Center, NY). The DNA linkers and sequencing vectors M13mp8 and M13mp9 (26) were obtained from Bethesda Research Laboratories (Bethesda, MD).

Construction of the Chimeric Genes: The pSV2-gpt expression vector (27), was digested with a *Hind* III-*Bam* HI digestion of pSV2-gpt to remove the gpt gene, the small t intron (9, 15) and the polyadenylation signal. The latter was restored to the vector by first subcloning the *Hinc* II-*Bam* HI 140 base pair fragment of pSV2-gpt that contains the signal into the *Hinc* II-*Bam* HI site of pUC8 (49) to form pUC8PA. The polyadenylation signal was then excised from this plasmid with *Hind* III and *Bam* HI and ligated to the large fragment of *Hind* III-*Bam* HI digested pSV2-gpt to form pSV2PA. A recombinant plasmid suitable for the expression of native rat GH in mammalian cells was then constructed by inserting a GH cDNA flanked by *Hind* III linkers, provided by Dr. J. Baxter (42), into the *Hind* III site of pSV2PA.

To form chimeric genes that encode hybrid proteins containing GH at the N-terminus, the termination codon of GH was replaced with a DNA linker that facilitated the addition of other cDNAs to the 3′ end of the GH gene. To

[2] Gottlieb, T. A. Manuscript submitted for publication.

this effect a partial digestion of the GH cDNA with *Pvu* II was carried out to obtain the 5′ *Hind* III-*Pvu* II fragment that lacks the last three codons of the coding region. This fragment was inserted between the *Hind* III and the *Hinc* II sites within the multiple cloning region of pUC9 (49) yielding plasmid pUC9GHT⁻1 which contained an *Eco* RI restriction site (as well as several others) at the 3′ end of the cloned cDNA.

Two additional plasmids, in which the *Eco* RI site in the multiple cloning region of pUC9 was located in the two other reading frames relative to that of the GH sequence, were derived from pUC9GHT⁻1 by changing the number of base pairs in the linker region. To achieve one frame shift, pUC9GHT⁻1 was linearized by digestion with *Bam* HI and *Xma* I and recircularized after filling in with DNA polymerase the four base pair 5′ extensions at both ends. This was expected to delete one base pair while regenerating both restriction enzyme sites. Probably because of exonuclease contamination in the *Xma* I preparation, this procedure yielded a significant number of plasmids in which either the *Bam* HI site or both the *Bam* HI and *Xma* I sites were destroyed. DNA sequencing of the modified regions of these plasmids showed that two of them, pUC9GHT⁻2 and pUC9GHT⁻3, contained two or four base pair deletions and therefore had the desired frame shifts of the GH sequence with respect to the *Eco* RI site.

Chimeric genes were formed by ligating cDNA fragments of VSV G or influenza HA to the *Eco* RI sites placed at the 3′ end of the GH cDNA. Expression vectors for these genes as well as a simian virus 40 (SV40) early replacement viral vector for expression of the GH–HA chimera were constructed as shown in Fig. 1.

SV40 defective viral particles containing the G–HA recombinant were obtained by transfection of COS cells, which synthesize T antigen constitutively (10). 3 d posttransfection the SVEGHHA virions were harvested after freeze-thawing and sonicating the cells. Viral stocks of high titer were obtained by infecting COS cells with SVEGHHA virions, always harvesting the virus 3 d postinfection to minimize recombination of SVEGHHA DNA with the SV40 DNA integrated in the genome of these cells.

In Vitro Transcription-Translation: The GH cDNA was inserted into the *Hind* III site of the plasmid vector pSP64 (Promega Biotec, Madison, WI), downstream from the bacteriophage SP6 promoter. The GH–G and GH–HA cDNAs were excised from the appropriate pSV2 vectors and subcloned

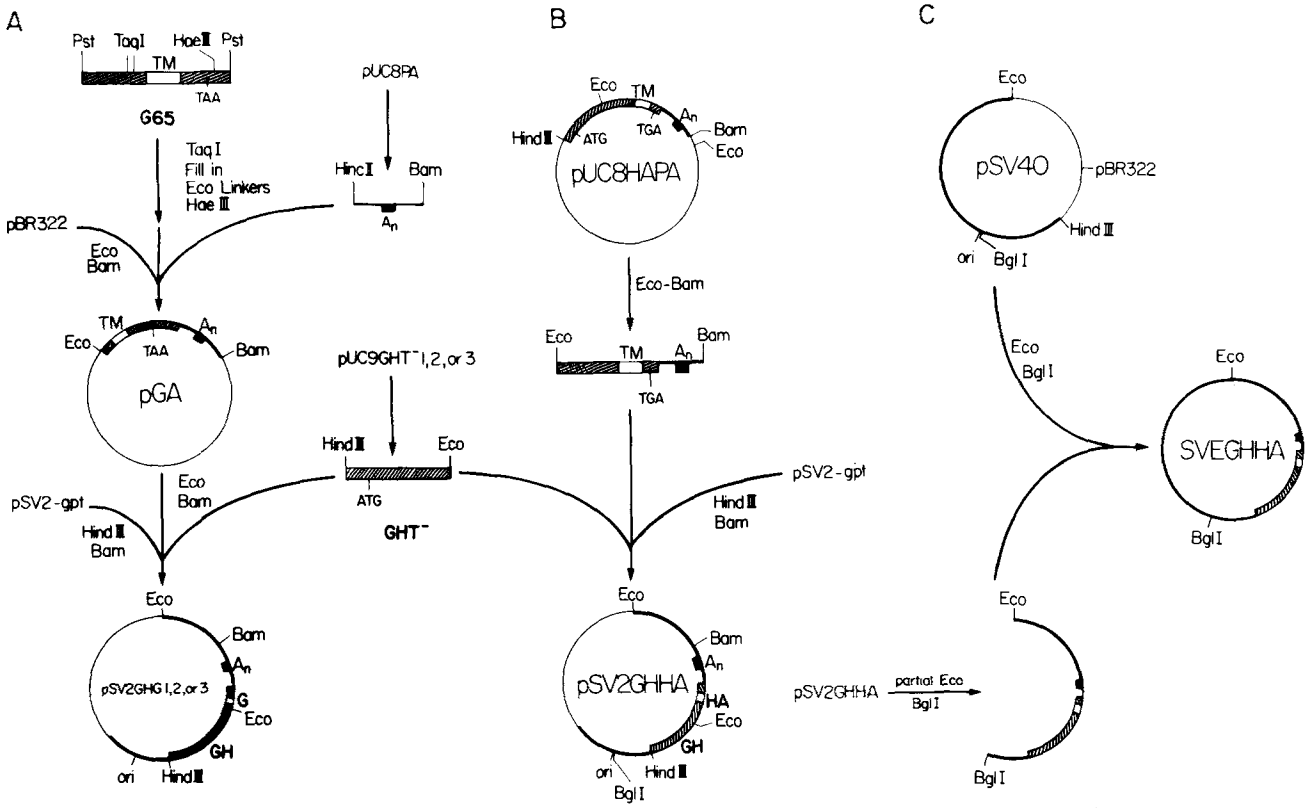


FIGURE 1 Construction of plasmid vectors for the expression of GH and GH–membrane protein hybrids. (*A*) The fragment of DNA encoding the C-terminal 60 amino acids of G (*G65*) was derived from a partial cDNA clone (*pG65*) of the G protein provided by Dr. J. Rose (39). An *Eco* RI linker was added to the *Taq* I site closest to the termination codon, and the fragment was subcloned, together with the SV40 polyadenylation addition signal fragment (excised from pUC8PA), between the *Eco* RI and *Bam* HI sites of pBR322 to form pGA. The *Eco* RI-*Bam* HI fragment of pGA, containing the G cDNA linked to the SV40 polyadenylation signal fragment, was ligated to the inserts of either pUC9GHT⁻1, 2, or 3, and the *Hind* III-*Bam* HI cut pSV2gpt to form the vectors pSV2GHG1, pSV2GHG2, and pSV2GHG3. The GH–G3 hybrid protein included the first 187 residues of GH followed by a sequence of amino acids (RRIGI) encoded by the linker region and the carboxy terminal 60 residues of the G protein. The two out-of-phase GH–G fusion proteins contained the unnatural C terminal peptides, RRIPGNSRACRRIVQ or RRIREFPSC, after the first 187 residues of GH. (*B*) Influenza HA cDNA (a gift from Dr. Peter Palese, Mt. Sinai Medical Center, NY), containing Hind III sites at both ends, was made blunt and inserted into pUC8PA by blunt end ligation between the Pst I (also made flush with Klenow polymerase) and Hinc II sites to form pUC8HAPA. The fragment of HA DNA encoding the C-terminal 154 amino acids was isolated together with the polyadenylation signal, using *Eco* RI and *Bam* HI and combined with the insert from pUC9GHT⁻2 and *Hind* III-*Bam* HI cut pSV2-gpt to form pSV2GHHA. The GH–HA hybrid protein included the first 187 residues of GH followed by the unnatural sequence RRIA and the carboxy terminal 154 residues of the HA protein. (C) An early replacement SV40 viral vector (SVEGHHA) was constructed from pSV2GHHA and from the plasmid PSV40 (a gift of Dr. A. Pellicer, NYU Medical Center). A DNA fragment containing GH–HA DNA sequences and parts of the SV40 early and late regions was obtained from pSV2GHHA by digestion with *Bgl* I and *Eco* RI. A fragment of the plasmid pSV40 excised with *Bgl* I and *Eco* RI was used to provide the rest of the late region and to complete the SV40 origin of replication and early promoter. The two fragments were recombined to form SVEGHHA. *Light lines*, pBR322 sequences; *heavy lines*, SV40 sequences; *hatched lines*, GH, VSV G, or HA sequences; *TM*, transmembrane coding sequence; A_n, polyadenylation signal.

between the Hind III and Hinc II sites of pSP64. After transcription (16), aliquots of the reaction mixture containing 100 ng of RNA were used for translation in the rabbit reticulocyte system (29) with or without the addition of dog pancreatic microsomal membranes (2.0 OD_{280} U/ml) (44). Assessment of accessibility to proteases of the translation products was done as described (38).

Cell Culture and Transfections: CV-1 and COS-7 cells (10) were grown in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum. MDCK cells (originally obtained from Dr. J. Leighton, Medical College of Pennsylvania) were grown in minimal essential medium (Gibco Laboratories) supplemented with 10% horse serum. CV-1 and COS cells were transfected in suspension with 30 μg of CsCl-purified plasmid following the procedure of Chu and Sharp (2). The cells, in suspension, were plated on dishes or coverslips ($5–6 \times 10^4$ cells/cm^2) which were incubated for 5 h at 37°C, followed by a 1-min exposure to 15% (vol/vol) glycerol in growth medium at the same temperature. In some experiments samples were then incubated for 12 h in growth medium containing 10 mM sodium butyrate (11). Cells were labeled with [^{35}S]methionine or prepared for immunofluorescence 36–44 h after transfection.

Nearly confluent cultures of MDCK cells on dishes or glass coverslips were transfected and treated with sodium butyrate (11), as previously described.[2] In some cases butyrate was added again 10–12 h before the completion of the experiment.

Cell Labeling and Immunoprecipitation: Cultures were preincubated for 0.5 h in methionine-free RPMI-medium (Gibco Laboratories) supplemented with 1% dialyzed fetal calf serum and then labeled with [^{35}S]-methionine (specific activity 1,000 Ci/mmol; 125 μCi/ml in the same medium). Cells (1×10^6) were lysed in 0.5 ml of 10 mM Tris-Cl pH 7.4 containing 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% Nonidet P-40 (CV-1 and COS) or 10 mM Tris-Cl pH 7.4 containing 10 mM NaCl, 1.5 mM $MgCl_2$, 0.5% DOC, and 0.5% Nonidet P-40 for 5–10 min at 4°C (MDCK). After removal of the nuclei by centrifugation, immunoprecipitation of the chimeric proteins was done (38) using baboon anti-GH serum (3 μl) (gift of Dr. H. Samuels, NYU Medical Center, NY) and protein A–Sepharose to adsorb immune complexes. The immunoprecipitated protein was eluted from the beads with 250 μl of 10 mM Tris pH 8.0 containing 2% SDS and immunoadsorption repeated before final elution for analysis by electrophoresis on SDS polyacrylamide gels and fluorography (19). When labeled media rather than cell extracts were analyzed, only one cycle of immunoprecipitation was used.

Immunoprecipitated protein released from the protein A–Sepharose beads by boiling for 2 min in 40 μl of 1% SDS was digested with endo H and endo F (3, 38) and the digestion products were analyzed by SDS PAGE.

Indirect Immunofluorescence: Coverslips containing transfected or SVEGHHA-infected cells were fixed for 30 min at room temperature with 2% paraformaldehyde in phosphate-buffered saline (PBS) (33). For internal labeling, coverslips were permeabilized with 0.1% Triton X-100 before application of antibodies at a 1:50 dilution. Baboon anti-GH antibodies were localized with fluorescein isothiocyanate–or rhodamine isothiocyanate–conjugated protein A (Pharmacia Fine Chemicals, Piscataway, NJ), whereas rabbit anti-HA antibodies (strain PR8; a gift of Dr. Peter Palese, Mt. Sinai Medical Center, NY) were detected with rhodamine isothiocyanate–conjugated goat anti-rabbit IgG (Cooper Laboratories, Inc., Malvern, PA). For double labeling with wheat germ agglutinin (WGA) and antibody, pSV2GHHA-transfected cells were first incubated for 1 h at 37°C with unlabeled lectin (200 μg/ml) to cover plasma membrane glycoproteins. Monolayers were then permeabilized with 0.1% Triton X-100 and incubated with a 1:50 dilution of fluorescein isothiocyanate–conjugated WGA (Vector Laboratories, Burlington, CA) for 45 min at 37°C. Unreacted sites of the bivalent WGA were then blocked during a 30-min incubation with 200 μg/ml transferrin (Sigma Chemical Co., St. Louis, MO) before the cells were labeled with anti-GH antibodies and rhodamine isothiocyanate–protein A.

Immunolabeling of Ultrathin Frozen Sections: Infected COS cells were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, infused with 2.3 M sucrose in PBS, and cryosectioned (33, 48). Sections were immunolabeled with anti-GH serum (1:20 to 1:40 diluted in PBS) and protein A–5-nm gold particles conjugates. With minor modifications, embedding in LR White resin (London Resin Co., Ltd., Basingstoke, Hampshire, England) and staining were done as described (14).

RESULTS

Carboxy-Terminal Segments of Viral Envelope Glycoproteins Halt Transfer of GH across the ER Membrane

The hybrid polypeptides GH–G (Fig. 2, *d–g*) and GH–HA (Fig. 2, *h–k*) were synthesized in vitro in the presence and absence of microsomal membranes using artificial messenger RNAs obtained by in vitro transcription of the corresponding cDNAs incorporated into the pSP64 plasmid vector. The products synthesized in the presence of membranes were of slightly lower molecular weight, as expected from removal of the GH signal peptide. The relative change in size due to co-translational processing was smaller for the GH–HA polypeptide (Fig. 2, *h* and *i*) than for the GH–G (Fig. 2, *d* and *f*), presumably as a result of core glycosylation (see below) of the HA component of the GH–HA hybrid protein. Protease treatment indicated that the GH–G hybrid incorporated into microsomes was not completely translocated into the lumen, as was the case with intact GH (Fig. 2, *a–c*) but remained inserted in the membrane with only a small portion exposed on the cytoplasmic face. Proteolysis, indeed, caused an essentially quantitative conversion of the microsome-associated

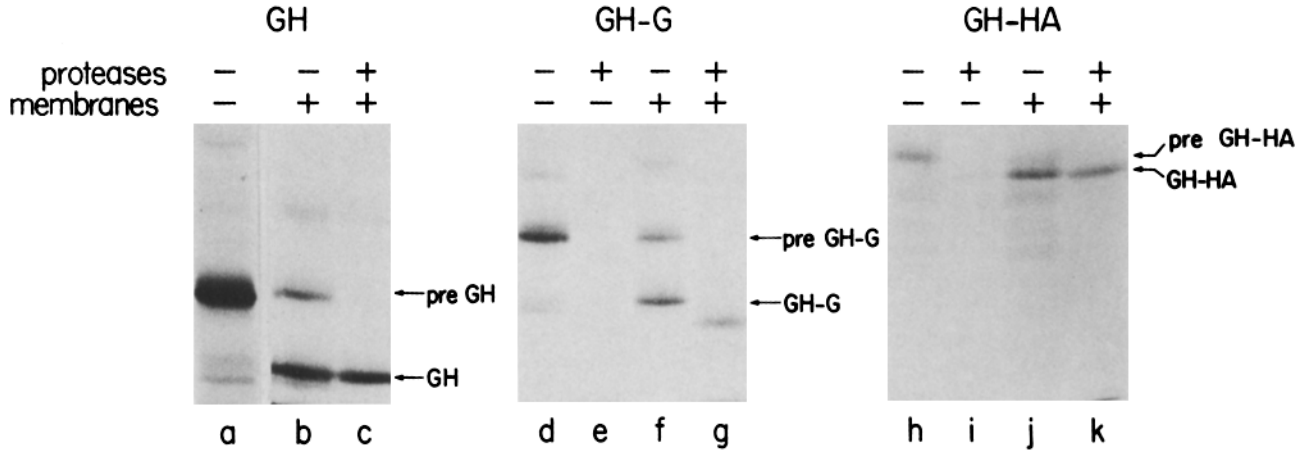


FIGURE 2 Membrane insertion of the GH, GH-G, and GH-HA gene products obtained in an in vitro transcription-translation system. GH and chimeric genes inserted in the plasmid pSP64 were transcribed with SP6 RNA polymerase, and the resulting mRNA translated in the presence or absence of microsomal membranes. The sensitivity of the in vitro synthesized products to a mixture of the proteases trypsin and chymotrypsin was assessed as indicated above each track, to determine the disposition of the polypeptides with respect to the membranes. The [^{35}S]methionine-labeled products were analyzed by electrophoresis on a 10% polyacrylamide SDS gel followed by autoradiography.

hybrid to a slightly shorter polypeptide (Fig. 2, *f* and *g*). These results are essentially equivalent to those obtained with the natural G protein (13) incorporated into microsomes in vitro, which demonstrated that protease cleavage removes only the cytoplasmically exposed carboxy-terminal portion of the polypeptide. That the GH–HA hybrid synthesized and inserted into membranes in vitro attained a similar transmembrane disposition could not be demonstrated in this simple fashion because the cytoplasmically exposed segment of HA represents a much smaller fraction of the total polypeptide chain length. Thus, protease treatment showed extensive protection of the chimera with no noticeable change in electrophoretic mobility (Fig. 2, *j* and *k*). The association of the GH–HA polypeptide with the membrane was in this case inferred from the fact that alkaline treatment, which removes adsorbed and luminal polypeptides from microsomes (6), did not dissociate the GH–HA polypeptide from the sedimentable membranes (not shown). In parallel experiments with transfected cells, when the GH and G cDNAs were fused out of frame, the resulting polypeptides—consisting of the nearly entire GH, followed by unnatural peptides derived from linker oligonucleotides and out of phase translation of G sequences—were translocated across the ER membrane and secreted into the medium. These results clearly demonstrated the presence of a functional halt transfer signal in the carboxy-terminal portion of the envelope glycoproteins included in the hybrids.

Synthesis and Intracellular Distribution of the GH–HA Hybrids in Transfected Cells

In transfected MDCK, CV1, or COS cells the GH–HA hybrid protein was efficiently synthesized and retained within the cells. A polypeptide with the expected molecular weight (M_r 39,000) was recovered from cell extracts, but not from the culture medium, by immunoprecipitation with antibodies directed either against GH (Fig. 3*A*) or denatured viral HA (not shown). Treatment with either endoglycosidase H or F led to a significant increase in the electrophoretic mobility of the hybrid protein (Fig. 3*B*). This demonstrated that the product of the transfected gene was glycosylated, as expected from the presence of a glycosylation site in the HA portion of the hybrid, but that the oligosaccharide chain was not converted from the mannose-rich endo H–sensitive form, to the complex type containing terminal sugars. The hybrid protein did not reach the cell surface, since no immunofluorescence signal was obtained when intact transfected cells were treated with either anti–GH or anti–HA antibodies (not shown). On the other hand, when cells were permeabilized with low concentrations of Triton X-100 before applying the antibodies, a juxtanuclear crescent-shaped region (e.g., Fig. 4*a* for MDCK cells, 4*c* for COS cells) was intensely stained in a small percentage of the cells (2–3%). A similar distribution of label was obtained in a much larger proportion of the cells (>95%) when a defective recombinant SV40 virus bearing the chimeric gene was used to infect COS cells (not shown). In all cases, only a small percentage of the expressing cells showed cytoplasmic labeling patterns expected for proteins localized in the ER, i.e., diffuse cytoplasmic labeling, lattice-like patterns, or intense labeling of the nuclear envelope. Clearly, the juxtanuclear labeling found in the different cell types suggests that the GH–HA hybrid accumulated in a region within or surrounding the Golgi apparatus. This possibility was examined in double-labeling immunofluorescence

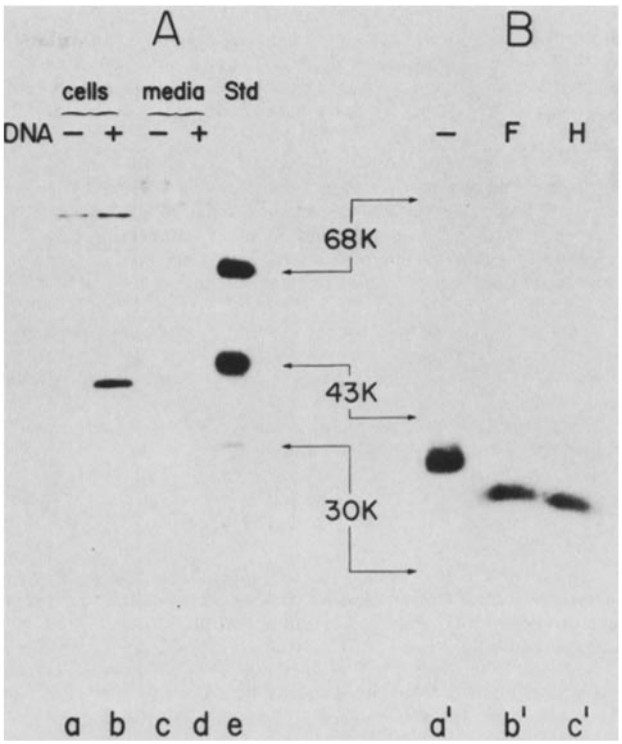


FIGURE 3 The GH–HA hybrid protein synthesized in transfected cells is glycosylated and retained intracellularly. (*A*) CV-1 cells were transfected with pSVGHHA and labeled with [^{35}S]methionine as described in Materials and Methods. Cell extracts and media from control and transfected cells were analyzed by immunoprecipitation, electrophoresis, and fluorography. ^{125}I-labeled bovine serum albumin (68 kD), ovalbumin (43 kD), and carbonic anhydratase (30 kD) were used as protein molecular weight standards (*e*). Similar results were obtained with COS and MDCK cells. (*B*) Immunoprecipitates obtained with anti–GH antibodies from extracts of labeled CV-1 cells transfected with pSV2GHHA were subjected to endoglycosidase digestion and analyzed by electrophoresis. Lane *a'*, undigested; lane *b'*, treated with endo-F; lane *c'*, treated with endo-H.

experiments, in which fluorescein-conjugated WGA (50) was used to identify the *trans* region of the Golgi complex in COS cells (Fig. 4*b*). It was found that the two labels had different distributions (compare Fig. 4, *b* and *c*). Most of the GH–HA positive region was adjacent but did not completely overlap with that stained by WGA. This indicated that the hybrid protein accumulated in a region that might represent either the *cis* portion of the Golgi or possibly transitional elements of the ER located near the Golgi apparatus. The accumulation of secretory and membrane proteins in pre-Golgi compartments has previously been reported in hepatocytes and Semliki Forest virus–infected baby hamster kidney cells (40, 41, 52).

Using the viral vector SVEGHHA, which yields expression of the hybrid gene in a large proportion of COS cells, it was possible to investigate the localization of the hybrid protein by immunoelectron microscopy. When thin frozen sections of infected cells were incubated with antibodies against GH and were localized with protein A complexed with colloidal gold–labeled particles, it was found that the hybrid protein was concentrated in a prominent system of smooth cisternae that surrounded the Golgi apparatus but was continuous with the rough ER (Figs. 5–7). Cisternae of this type were not found in cells not infected with SVEGHHA, or in cells

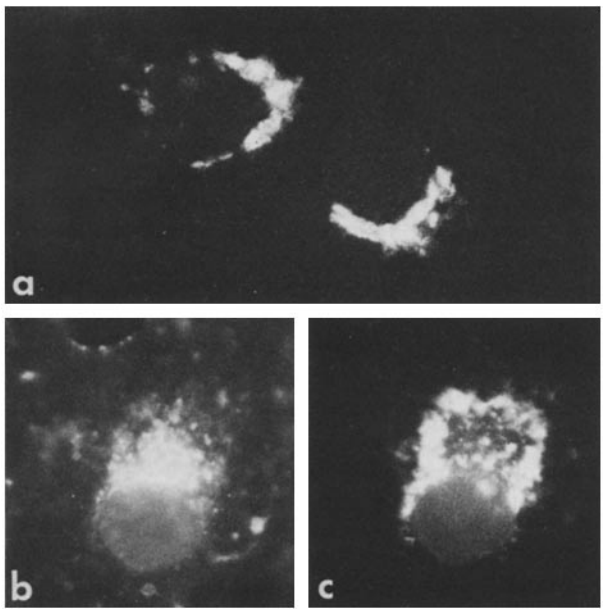


FIGURE 4 Immunofluorescence patterns of cells expressing GH-HA: the GH-HA hybrid protein accumulates in a region of the cytoplasm distinct from that binding WGA. (*a*) MDCK cells were transfected with pSV2GHHA and 36 h later fixed with 2.0% paraformaldehyde and permeabilized with 0.1% Triton X-100. The samples were then labeled with GH antiserum and rhodamine-conjugated protein A as described in Materials and Methods. A crescent-shaped juxtanuclear region containing the GH-HA chimera is intensely stained. (*b* and *c*) COS cells were infected with SVEGHHA, fixed, and permeabilized as described in *a* and double-labeled with anti-GH and fluorescein isothiocyanate-WGA. *b* and *c* correspond to the same cell photographed with two different filters to demonstrate the distribution of fluoresceinated WGA (*b*) or anti-GH antibodies, detected with rhodamine isothiocyanate (*c*). The WGA represents a marker for terminally glycosylated oligosaccharides found in the *trans* Golgi cisternae. × 2,500.

infected with the normal SV40 virus. The cisternae in which the chimera accumulated could be characterized better by standard transmission electron microscopy of Epon-embedded SVEGHHA-infected cells (Fig. 7). Characteristically, the cisternae had a narrow uniform width, and patches of the closely apposed membranes appeared to be joined by a dense material between them. While some of the narrow dense cisternae were continuous with the more dilated ones of the rough ER (Fig. 7, *b* and *c*), others approached very closely the stacks of Golgi cisternae.

Synthesis and Intracellular Distribution of the GH-VSV G Protein Hybrid in Transfected Cells

In contrast to the results just described for GH-HA, initial attempts to detect the expression of the chimeric gene encoding the GH-G hybrid protein by either immunoprecipitation from [^{35}S]methionine-labeled transfected cells or by immunofluorescence were unsuccessful (not shown). A low level of accumulation of GH-G (M_r 26,000) was detected in transfected cells when sodium butyrate (11) was used to enhance transcription from the SV40 early promoter (Fig. 8 *a*). This level appeared even lower than that generally obtained for the GH-HA hybrid in the absence of butyrate enhancement. In parallel experiments cells were transfected with chimeric genes in which GH and G sequences were fused out of frame. In this case (not shown), the resulting hybrid polypeptides consisting of GH followed by peptides derived from translation of linker regions, and out-of-phase G cDNA sequences were produced in substantial amounts and secreted into the medium. We therefore concluded that the failure to accumulate the product of the pSV2GHG3 in-phase chimera did not result from an aberrant splicing of the fused gene transcripts. Moreover, the in vitro transcription-translation experiments previously described eliminated the possibility that as a result of a cloning artifact the linked coding segments contained nonsense mutations or other changes that may have impaired translation. It, therefore, appeared likely that our inability to detect the product of the chimeric GH-G gene after transfection reflected a short intracellular lifetime of the polypeptide. For this reason we assessed the levels of GH-G in transfected cells treated with chloroquine, a lysosomotropic agent that raises lysosomal pH and inhibits the degradative action of lysosomal enzymes (24, 30). A several fold increase in the amount of labeled GH-G hybrid protein detectable by immunoprecipitation with anti-GH antibodies or antibodies that recognize the cytoplasmic domain of the G protein (kindly provided by Dr. T. Kreis, EMBL, Heidelberg) was observed in cells incubated with chloroquine during the 4-h labeling period (Fig. 8, *b* and *c*). Furthermore, immunofluorescence showed that, whereas 3–5% of the transfected cells treated with chloroquine could be labeled with anti-GH or anti-G antibodies, substantially <1% of the transfected cells were labeled in the absence of chloroquine. The immunofluorescence studies indicated that the GH-G hybrid was localized in cytoplasmic endosome- or lysosome-like vesicles which resembled those found in chloroquine-treated transfected cells expressing the intact G protein[2] (Fig. 9). In the larger vesicles the pattern of staining indicated that the protein was membrane-associated, since fluorescence was more intense at the vesicular periphery.

DISCUSSION

In the experiments just described we have analyzed various aspects of the biogenetic mechanisms that are involved in the insertion of nascent plasma membrane proteins into the ER and in their subsequent distribution through the cell. For these studies we used chimeric genes that encode hybrid proteins consisting of nearly the entire GH sequence at their amino termini and portions of the VSV G or influenza HA glycoproteins at their carboxyl termini. In the in vitro transcription-translation experiments we demonstrated that the insertion signal in the GH portion of the chimeras led to incorporation of the membrane protein segments into the ER membrane. Effectively, GH became part of the luminal segment of membrane proteins of which only very small segments, presumably the cytoplasmic portions of the G or HA proteins, remained exposed on the surface of the microsomes. These results support the assignment of a halt transfer role to a segment within the carboxy-terminal portions of the membrane polypeptides. Although this is likely to correspond to the transmembrane portion and downstream charged residues, more precise site-directed mutagenesis studies are required to define the features that compose the signal.

In vitro insertion studies somewhat analogous to those presented here have recently been reported. It was shown that the insertion signal of the bacterial periplasmic protein β-lactamase, equivalent to a secretory protein, can lead the

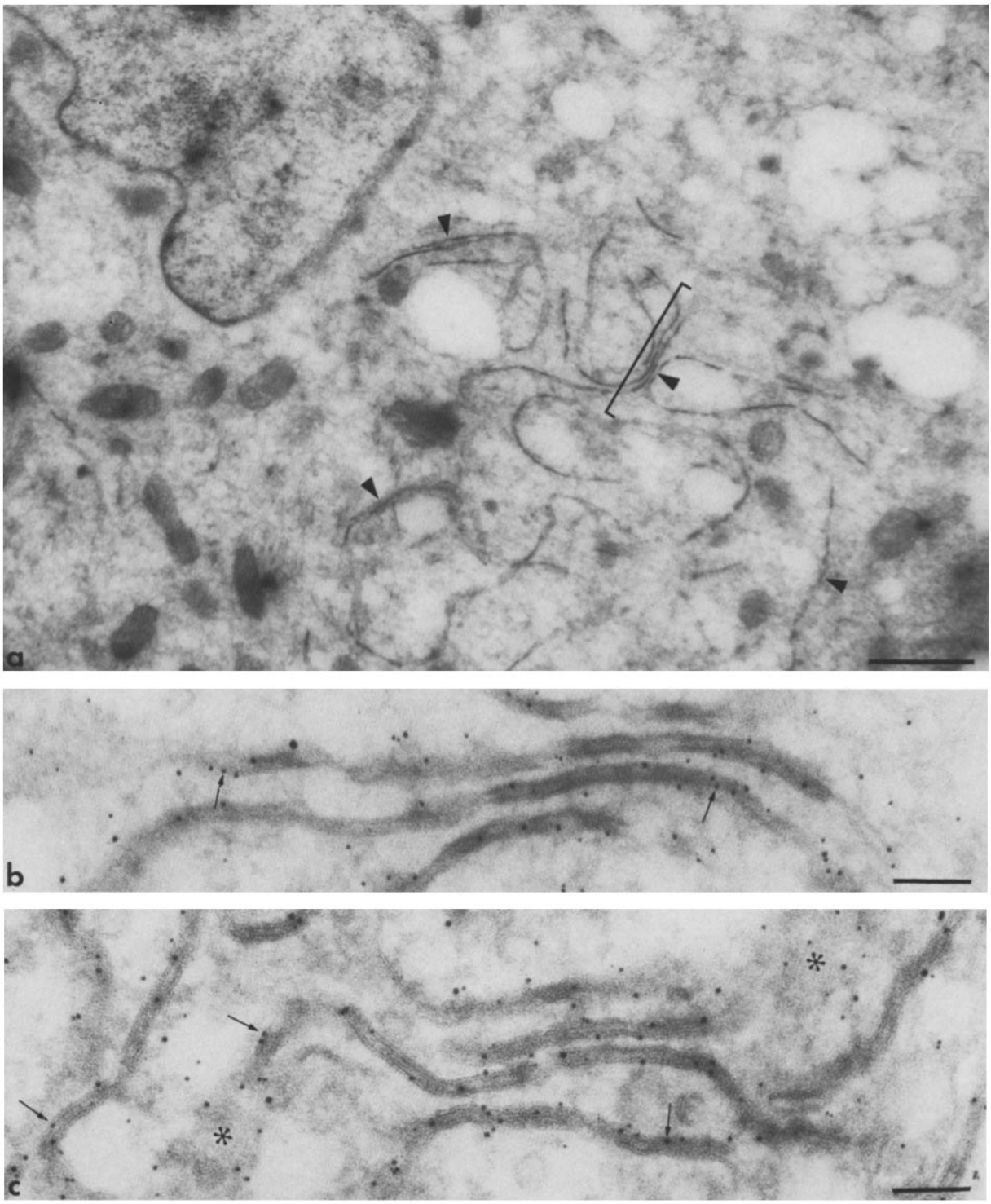


FIGURE 5 The GH-HA chimera accumulates in a system of membrane-bounded cisternae found in the cytoplasm of COS infected with SVEGHHA. Thin frozen sections of COS cells infected with SVEGHHA were immunolabeled with anti-GH antibodies and protein A gold particles (5 nm) as described in Materials and Methods. (a) A system of narrow (~25 nm), darkly stained cisternae (arrowheads) is prominent in the cytoplasm. (*b*) The segment enclosed by the bracket in a is shown at higher magnification. Gold particles (arrows), indicating the presence of the GH-HA chimera, are concentrated in these cisternae. (c) Similar cisternae in another infected cell. The asterisks (*) point to portions of the cisternae sectioned obliquely. (a) × 17,000; bar, 1.0 μm. (*b* and *c*) × 125,000; bar, 0.1 μm.

normally cytoplasmic polypeptide β-globin across the membrane and into the lumen of microsomes (21). However, incorporation of the carboxy terminal segment of the membrane form of the μ heavy chain of immunoglobulin between the lactamase and globin portions of a chimera blocked complete translocation of the hybrid polypeptide, which remained membrane-associated with the lactamase component sequestered within the lumen and the globin portion exposed

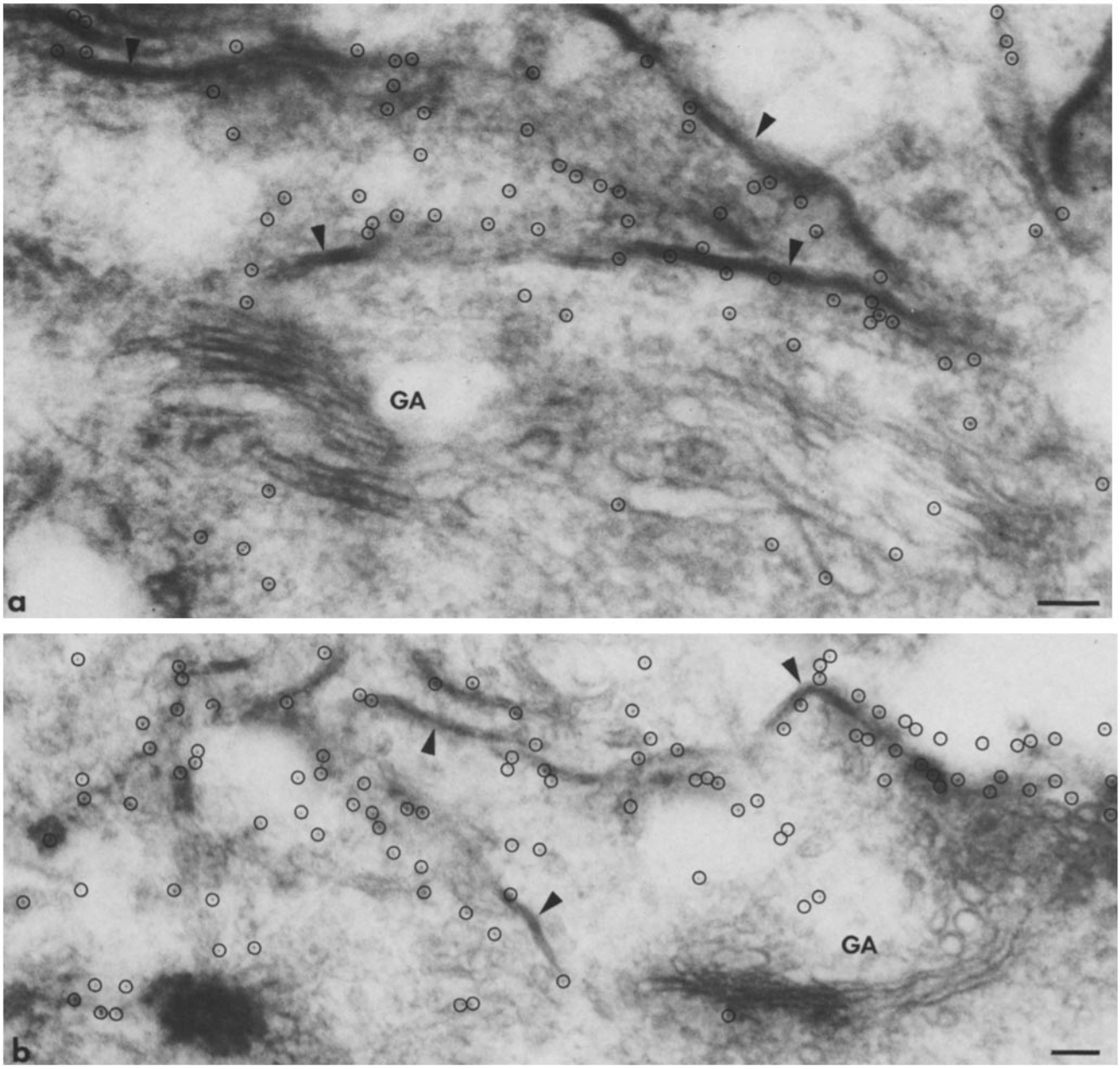
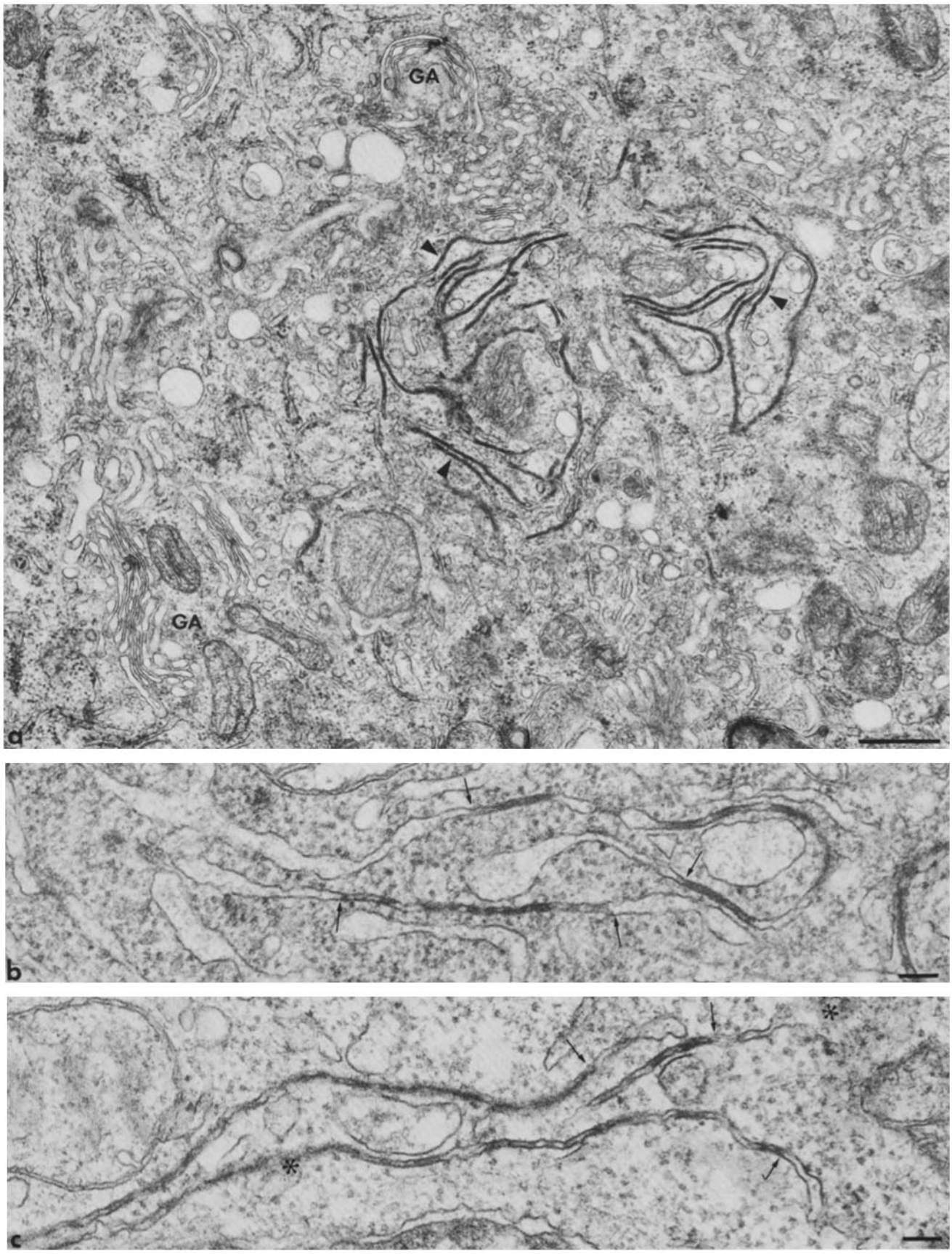


FIGURE 6 Cisternae containing the GH–HA chimera closely approach the Golgi apparatus but remain distinct from this organelle. The specimen was prepared as described in the legend to Fig. 5. Several cisternae (arrowheads), intensely labeled with gold particles (*circles*), are found near the Golgi apparatus (*GA*). Some particles are found in the periphery of the Golgi apparatus, but the Golgi stacks remain unlabeled. (*a*) × 93,000; bar, 0.1 μm. (*b*) × 78,000; bar, 0.1 μm.

on the cytoplasmic surface (53).

When the chimeric genes encoding GH followed by carboxy terminal portions of the VSV G or influenza HA were expressed in intact transfected cells, the products, as expected, failed to be secreted and remained cell associated. Similar findings have been recently reported by Guan and Rose (12) with a slightly different GH–G hybrid, which was shown to be membrane associated and to accumulate in the Golgi

FIGURE 7 The system of cytoplasmic cisternae that develops in SVEGHHA-infected COS cells is continuous with the rough ER. Cisternae similar to those shown by immunolabeling in thin frozen sections to contain the chimera (Fig. 5) were a prominent feature in the cytoplasm of infected cells fixed and embedded for conventional electron microscopy. (*a*) Several cisternae were found in close proximity to the Golgi apparatus (*GA*). (*b* and *c*) In many instances it could be seen that the cisternae, which characteristically had smooth and closely apposed membranes, were in direct continuity with others of the rough ER (arrows), which had a wider lumen. Obliquely sectioned portions are marked by asterisks. (*a*) × 15,000; bar, 1.0 μm. (*b* and *c*) × 68,000; bar, 0.1 μm.


GA
GA
a
b
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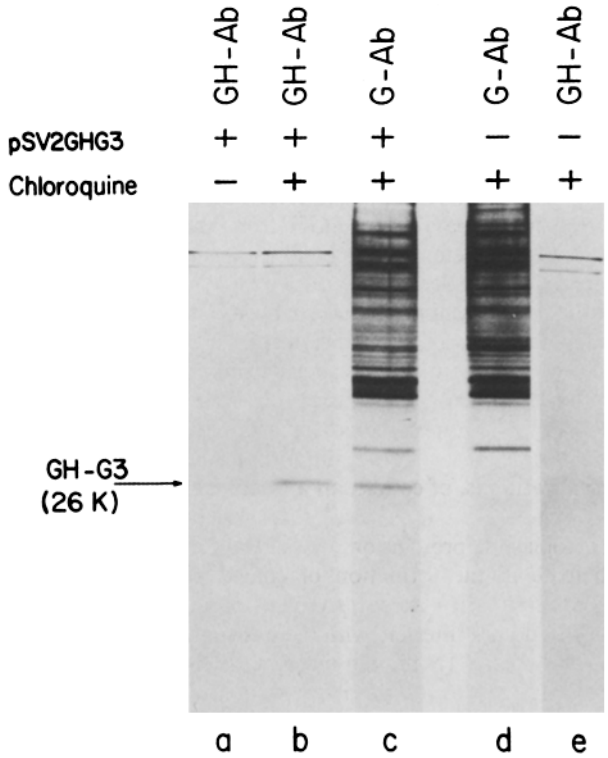

FIGURE 8 Accumulation of the GH–G hybrid in chloroquine-treated transfected cells. CV-1 cells transfected with pSV2GHG3 were incubated in medium containing 10 mM sodium butyrate as described in Materials and Methods. Several dishes (*b*, *c*, *d*, and e) were incubated with chloroquine in labeling medium for 5 h, beginning 1 h before [^{35}S]methionine was added to all the cultures for 4 h. Aliquots of cell extracts were used for immunoprecipitation with anti-GH antibodies (*a*, *b*, and e) or antibodies against the C-terminal protein of G protein (lanes *c* and *d*). The high background in the latter lanes reflects the fact that only a single cycle of immunoprecipitation was done.

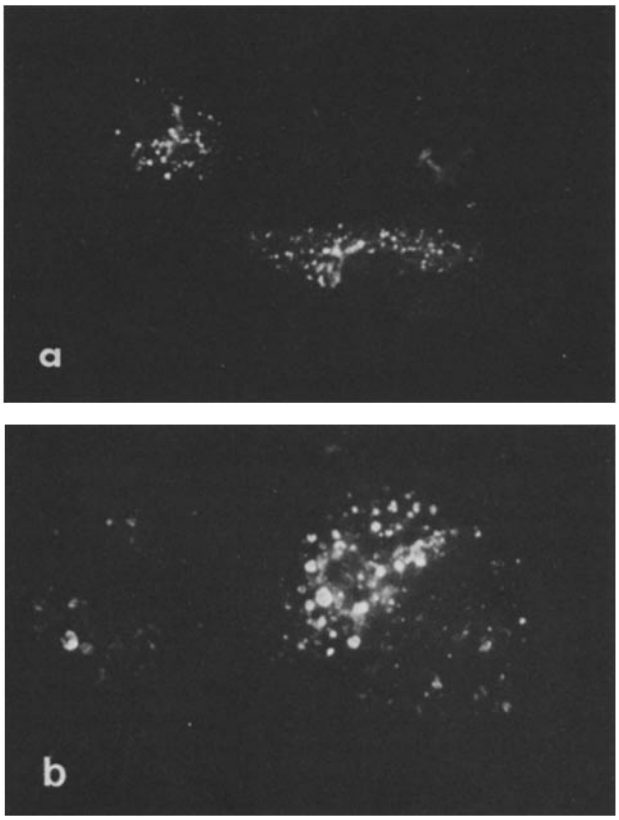

FIGURE 9 Accumulation of the GH–G hybrid protein in cytoplasmic vesicles of chloroquine-treated transfected cells. CV1 (*a*) and MDCK cells (*b*) transfected with pSV2GHG3 and incubated with sodium butyrate received 50 μM chloroquine 36-h posttransfection and were incubated with this drug for a period of 7 h at 37°C. Cells were fixed, permeabilized, and incubated with anti-GH followed by rhodamine isothiocyanate–protein A.

apparatus.

Although considerable experimental evidence indicates that insertion and halt transfer signals are contained in limited regions of polypeptides made on bound ribosomes, it is not at all clear that information for posttranslational sorting is similarly contained in restricted regions of the polypeptide primary sequence. The experiments presented here utilizing hybrid genes expressed in transfected cells can in principle test this hypothesis. However, nonpredictable interactions between unrelated segments of chimeric polypeptides could not only mask existing functional sorting signals but could also block the capacity of the protein to be transported from its site of synthesis to other destinations. Thus, failure of a chimeric polypeptide to reach an expected destination provides no evidence for the absence of the putative corresponding signal. On the other hand, the capacity of an altered polypeptide to reach its expected destination or at least to be transported out of its site of synthesis in the ER, can be taken to indicate the retention of sorting elements. In the construction of chimeras we attempted to minimize interactions between the unrelated linked segments by selecting segments that are defined by the position of the protein with respect to the phospholipid bilayer or correspond to domains that are thought to fold independently of each other. Thus, the entire GH sequence was included, since this could be expected to achieve its native configuration before synthesis of the carboxy-terminal portions of the hybrid emerged from the ribosome. Furthermore, since the C-terminus of most proteins appears to be near the surface of the molecule (31), the addition of extraneous segments at this point may not disturb the native folding configuration.

For the GH–G hybrid, the membrane glycoprotein portion consisted of the entire cytoplasmic and transmembrane domains (37) and only 11 amino acids of the luminal portion. These luminal residues provided a short spacer between the membrane surface and the GH portion. In designing the GH–HA hybrid, we took into account the detailed three-dimensional structure derived from crystallographic data (51) for the H3 variant of HA of influenza which, in its HA2 portion, shows considerable homology with that of the H1 variant used for our experiments (18). The HA segment included, in addition to the cytoplasmic and transmembrane domain, an unusually long alpha helical luminal portion of the protein and a short random coil segment which could serve as a spacer between the GH and the helical domain.

We found that the GH–HA was transported out of the rough ER and accumulated in a pre-Golgi station highly developed in cells that expressed the chimeras, consisting of a cisternal system continuous with the rough ER but devoid of ribosomes and very closely apposed to the Golgi apparatus. It is worth noting that, although the GH–HA chimera did not

proceed through the Golgi apparatus, its behavior was clearly different from that of hybrid proteins described by other investigators (25), which contained segments of both HA and G and appeared to remain in the ER near their site of synthesis. The behavior of the GH–HA hybrid studied here was also different from that of the GH–G hybrid described by Guan and Rose (12), which accumulated in the Golgi apparatus. The failure of the GH–HA to be transported to the Golgi apparatus, despite the presence of the nearly entire GH molecule, may reflect the inability of the GH segment to interact with membrane-associated carriers or receptor molecules whose existence has been invoked to explain differences in the rates of transport of various polypeptides between the ER and the Golgi apparatus (4, 5, 20, 22). Binding to the putative receptors may have been prevented by the tethering of the GH to the long and presumably rigid alpha helical segment of HA contained in the luminal domain of the chimera. In addition, the HA portion of the molecule may either lack a functional sorting signal required for further transport of the natural membrane protein or fail to acquire the appropriate tertiary or quaternary structure necessary for this process. In fact, in the normal HA the alpha helical portion of the polypeptide participates in the formation of an oligomeric complex with two other HA molecules (51).

The finding that transport of the GH–HA chimera was arrested in heretofore unrecognized structures that developed in the transfected cells may be of considerable significance. Although in principle these cisternae represent an unnatural site of accumulation of denatured protein to be degraded, we saw no sign of their incorporation into autophagosome. Based on their continuity with the rough ER and their close approximation to the *cis* face of the Golgi apparatus, we suggest that the cisternae that contain the chimera may represent the amplification of a compartment equivalent to the transitional elements through which proteins are thought to be transferred from the ER to the Golgi apparatus (see reference 28). These structures have not yet been biochemically defined but have been recognized in many ultrastructural studies with different cell types. The overdevelopment of the membranous cisternae in which the hybrid protein accumulated raises the possibility that the chimera remained complexed with carrier molecules which, because of abnormal properties of the chimera, were rendered nonfunctional. If our speculation is correct, future studies with this system may permit a biochemical characterization of the transitional elements and the isolation of the putative carriers, on the basis of their association with the hybrid protein.

The behavior of the GH–G hybrid was in striking contrast to that of the GH–HA just described. The GH–G hybrid did not accumulate to high levels within the transfected cells, apparently because it was efficiently degraded by a lysosome-mediated pathway. This was suggested by the fact that chloroquine treatment of transfected cells led to a substantial increase in the levels of labeled hybrid found in the cells. During such treatment the GH–G protein accumulated in endosome or lysosome-like vesicles. Although the possibility that the chimeric protein entered such vesicles directly from the Golgi apparatus cannot be eliminated, it appears more likely that the chimera first reached the plasma membrane and was then interiorized by endocytosis. This possibility is suggested because the intact G protein expressed in VSV-infected cells, as well as in cells transfected with appropriate expression plasmids, after reaching the plasma membrane, can be quantitatively trapped by chloroquine treatment in vesicles similar in appearance to those containing the chimera.[2]

In contrast to the GH–G polypeptide studied here, a similar hybrid constructed by Guan and Rose accumulated in the Golgi apparatus (12). The two chimeric polypeptides only differed in the presence, within our construct, of a 15-amino-acid segment separating the GH from the transmembrane portion of G protein, and it therefore appears that this allowed progress of the molecule beyond the Golgi apparatus.

If the GH–G chimera studied by us indeed reached the plasma membrane but was efficiently interiorized after chloroquine treatment, information responsible for its endocytosis must be contained in the carboxy-terminal portion of the G protein. The notion that the cytoplasmic part of a transmembrane polypeptide plays an important role in mediating its internalization is, of course, an attractive one since only this portion of the protein is accessible for interaction with peripheral cytoplasmic proteins or cytoskeletal elements which may participate in the formation of coated pits or endosomal vesicles. In fact, it has recently been found (20a) that mutations that do not interfere with the transport of newly synthesized low density lipoprotein receptors to the cell surface, but completely prevent their functional internalization and recycling, are localized in the cytoplasmic segment of the polypeptide. Internalization of the GH–G chimera and its ensuing degradation may be viewed as analogous to the natural behavior of polypeptide hormone–receptor complexes, although in the case of the chimera, degradation of the hormone moiety also entails degradation of the membrane-spanning segment to which the hormone is linked covalently.

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