**trans-Golgi Retention of a Plasma Membrane Protein: Mutations in the Cytoplasmic Domain of the Asialoglycoprotein Receptor Subunit H1 Result in trans-Golgi Retention**

Johanna M. Wahlberg, Iris Geffen, Françoise Reymond, Thomas Simmen, and Martin Spiess

Department of Biochemistry, Biozentrum, University of Basel, CH-4056 Basel, Switzerland

**Abstract.** Unlike the wild-type asialoglycoprotein receptor subunit H1 which is transported to the cell surface, endocytosed and recycled, a mutant lacking residues 4-33 of the 40-amino acid cytoplasmic domain was found to be retained intracellularly upon expression in different cell lines. The mutant protein accumulated in the trans-Golgi, as judged from the acquisition of trans-Golgi-specific modifications of the protein and from the immunofluorescence staining pattern. It was localized to juxtanuclear, tubular structures that were also stained by antibodies against galactosyltransferase and γ-adaptin. The results of further mutagenesis in the cytoplasmic domain indicated that the size rather than the specific sequence of the cytoplasmic domain determines whether H1 is retained in the trans-Golgi or transported to the cell surface. Truncation to less than 17 residues resulted in retention, and extension of a truncated tail by an unrelated sequence restored surface transport. The transmembrane segment of H1 was not sufficient for retention of a reporter molecule and it could be replaced by an artificial apolar sequence without affecting Golgi localization. The cytoplasmic domain thus appears to inhibit interaction(s) of the extracellular portion of H1 with trans-Golgi component(s) for example by steric hindrance or by changing the positioning of the protein in the membrane. This mechanism may also be functional in other proteins.

Transport of proteins through the secretory pathway appears to occur by default (Pfeffer and Rothman, 1987). In contrast, localization to individual compartments (by retention or retrieval) requires specific signals (Pelham and Munro, 1993), such as the carboxy-terminal sequence KDEL and the cytoplasmic motif K(X)KXX for retrieval to the ER of lumenal and membrane proteins, respectively. The determinants for retention in Golgi compartments have so far been shown to comprise the transmembrane domain with or without flanking sequences. Proteins leaving the Golgi apparatus are sorted in the trans-Golgi network (TGN) largely by signal-dependent mechanisms into specific transport vesicles to secretory granules, apical or basolateral plasma membrane, or endosomes.

In the endoplasmic reticulum (ER), in addition, there are mechanisms to prevent improperly folded or oligomerized proteins from exiting and to degrade them. The ER thus performs a quality control function for secretory and membrane proteins (Hurtley and Helenius, 1989). Many natural and artificial mutations in such proteins have been observed to result in ER retention and degradation, apparently because they affect protein folding or oligomerization. Alterations in the signals for targeted exit from the TGN generally cause missorting rather than TGN localization of the mutant. Only very few mutations have been documented that cause a specific accumulation of the mutant protein in post-ER compartments of the secretory pathway. Such mutations may reflect properties of retention or transport mechanisms in the Golgi. Here we describe mutants of the asialoglycoprotein (ASGP) receptor subunit H1 that specifically accumulate in the trans-Golgi.

The ASGP receptor is an endocytic transport receptor responsible for the rapid clearance of glycoproteins with exposed galactoses from the circulation into hepatocytes (for review see Spiess, 1990). Ligand ASGPs are bound at the cell surface, internalized via clathrin-coated vesicles, and released in the acidic environment of endosomes for subsequent degradation in lysosomes. The ASGP receptor cycles constitutively between the cell surface and early endosomes. The human receptor is at least a trimer composed of two homologous subunits, H1 and H2, which both are required for high affinity ligand binding. The subunits are single-spanning type II membrane proteins with amino-terminal cytoplasmic domains of 40 and 58 residues, respectively. The major subunit H1, which is 2-4 times more abundant than H2, contains all the signals necessary for endocytosis and specific basolateral transport in the plasma membrane.

1. Abbreviations used in this paper: ASGP, asialoglycoprotein; M6P, mannose-6-phosphate.
polarized epithelial cells (Geffen et al., 1989; Wessels et al., 1989). A tyrosine-containing signal necessary for efficient endocytosis and basolateral sorting has been identified in the amino-terminal segment of residues 1-11 (Fuhrer et al., 1991; Geffen et al., 1993). Mutation of the critical tyrosine at position 5 to alanine or deletion of residues 4-11 result in apolar sorting in transfected MDCK cells and in accumulation of H1 at the plasma membrane in transfected fibroblast and MDCK cells.

As part of these studies, an H1 mutant lacking most of the cytoplasmic domain (residues 4-33) was constructed (Geffen et al., 1993). Although this mutant protein exits the ER, it is not detectable at the cell surface. Here we show that it accumulates specifically in a trans-Golgi compartment. The sequence requirement for retention or exit to the plasma membrane was investigated by further mutagenesis. The results indicate that the size of the cytoplasmic domain determines the fate of the protein and modulates an intrinsic ability of the transmembrane and/or exoplasmic portion of H1 to interact with component(s) resident in the trans-Golgi. Such a mechanism may be functional also for other trans-Golgi/plasma membrane proteins.

Materials and Methods

Construction of Mutant Proteins

Mutagenesis of the cDNAs encoding the mutants H1(A4-33A), H1(A4-11), and H1(A12-33) have been described previously (Beltzer et al., 1991; Geffen et al., 1993). H1(A12-33/5A) was constructed using the mutagenesis kit by Amersham Corp. (Arlington Heights, IL), the eDNA of HI(A12-plasmid vector. For Hl(~Pro), a 5' eDNA fragment containing codons 1-24 by PCR from the plasmid pMTR-1 using the mutagenic primer TCC- eDNA with an ATG and an NdeI restriction site for subcloning. The cDNA corresponding to the transmembrane and exoplasmic domains of H1 were amplified similarly with the primer CCGGAC-

The Journal of Cell Biology, Volume 130, 1995 286

To construct the cDNA of AN2 and AN2a a BamHI site was introduced into the aminopeptidase N cDNA at codons 33/34 corresponding to the cytoplasmic end of the transmembrane domain using the mutagenic anti-sense primer TCTCGGGATCCCACTG and the Amersham antisense primer kit. The cDNA fragment 3' of this BamHI site was ligated to the 5' HindIII-BamHI fragment of either H1 or H1(A4-33A) cDNA. From the plasmid 19L, a gift from G. von Heijne (Karolinska Institute, Huddinge, Sweden), a sequence encoding 19 leucines, preceded by a KpnI site and the codons for Met-Gly-Pro-Arg, and followed by a BamHI site was amplified by PCR and ligated in front of the BamHI- EcoRI fragment of H1 cDNA to obtain the construct for H1(A4-33A) Leu19. The construct for H1(A4-33A) was made using the ligating HindIII-NruI fragment of plasmid pSA113 (Spiess and Handschik, 1987) to the H1(A4-33A) cDNA cut with KpnI and blunted.

To generate the cDNA of H1(A4-33A) Leu19 variant of H1(A4-33A) tagged with a tyrosine-sulfation peptide at the carboxy terminus, the 3' (BamHI-EcoRI) fragment of the cDNA of H1(A4-33A) was replaced by that of H1(T5) described by Leitinger et al. (1994). To construct H1(A4-33A) pA, a variant containing a carboxy-terminal e-myc epitope tag, the tyrosine-sulfation tag sequence (KpnI–SacI) of the H1(T5) cDNA was replaced by two complementary oligonucleotides, CGAGAAGTAAGTGCAGCTCTGAGT and CAGGTAAGTCTC- TCTTGAGAACATCCTTGTC, which encode a peptide sequence EOKLISEEDL followed by a stop codon and produce terminal protruding ends corresponding to a KpnI and a SacI site at the 5' and 3' end, respectively. All mutagenized segments were confirmed by DNA sequencing.

Cell Culture and Transfection

MDCK (strain II), COS-7, HepG2, and HeLa cells were grown in minimal essential medium (MEM) with 10% FCS, NIH 3T3 cells in Dulbecco's modified MEM with 10% newborn calf serum, CaCo-2 cells in DMEM with 20% FCS and 1% nonessential amino acids (GIBCO BRL, Gaithersburg, MD), and BHK-21 cells in Glasgow MEM with 5% FCS and 10% tryptose phosphate broth (Gibco). All culture media were supplemented with 2 mM l-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin.

For transient expression, cDNA constructs were subcloned into the SV40-based expression vector pECE (Ellis et al., 1986) and transfected using DEAE-dextran as described by Cullen (1987). The next day transfected cells were trypsinized and seeded onto coverslips and processed for immunofluorescence microscopy 36–48 hours after transfection.

Preparation of the stable MDCK cell lines M1, M1Δ (= M1[A4-33A]), M1T5, M12 (= M[Y+f]), and M12 (M[Y+f]) has been described previously (Wessels et al., 1989; Geffen et al., 1993; Leitinger et al., 1994; Fuhrer et al., 1994). To produce stable cell lines, the cDNA of H1(A4-33A) was cloned into pK21 (with a cytomegalovirus promoter; provided by R. Gentz, Hoffmann-La Roche, Basel), and transfected into MDCK cells together with one tenth the amount of the resistance plasmid pSVneo using polybrene and dimethyl sulfoxide according to Kawai and Nishizawa (1984). Clonal cell lines resistant to 0.5 mg/ml G418-sulfate were isolated and screened for expression by immunofluorescence and immunoblot analysis using a polyclonal antiserum directed against the ASGP receptor. The cDNA of H1(A4-33A) was subcloned into pBamHI (with a murine leukemia virus promoter and the Salmonella his promoter) and transfected into the cell line M2, an MDCK-derived cell line expressing the second ASGP receptor subunit H2 and G418 resistance (Fuhrer et al., 1994). Clonal cell lines were selected in the presence of 2 mg/ml histidinol. The resulting cell lines were named M1ΔT5 and M2ΔT1, respectively.

Biochemical Analyses

The surface/intracellular distribution of H1 variants was assayed by treating intact cells with 1 mg/ml proteinase K in PBS containing 5 mM EDTA for 30 min at 4°C or 37°C. Digestion was stopped by adding 2 mM PMSF. The surface/intraer cellular distribution of H1 variants was assayed by treating intact cells with 1 mg/ml proteinase K in PBS containing 5 mM EDTA for 30 min at 4°C or 37°C. Digestion was stopped by adding 2 mM PMSF. The surface/intraer cellular distribution of H1 variants was assayed by treating intact cells with 1 mg/ml proteinase K in PBS containing 5 mM EDTA for 30 min at 4°C or 37°C. Digestion was stopped by adding 2 mM PMSF.
Antibodies

Antisera used for detection of ASGP receptor subunits were raised in rabbits against the purified human receptor (62-5) or against synthetic peptides corresponding to the carboxy-terminal sequence of either H1 (anti-H1C) or H2 (anti-H2C). For immunofluorescence staining, IgG from anti-H1C and anti-H2C were isolated using protein A-Sepharose. Rabbit polyclonal antibodies anti-human milk galactosyltransferase (N11; from E. Berger, University of Zurich), anti-bovine cation-independent M6P receptor (from B. Hoflack, EMBL), anti-rat TGN-38/41 (from G. Banting, University of Bristol), and anti-human aminopeptidase N (from O. Norén and H. Sjöström, University of Copenhagen) were used as serum preparations. Mouse monoclonal antibodies anti-canine LAMP-1 (AC17; from E. Rodriguez-Boulan, Cornell University), and anti-bovine γ-adaptin (1003; Sigma Immunochemicals, Buchs, Switzerland) were used as ascites preparations. Mouse anti-human ~adaptin (100:3; Sigma Immunochemicals, Buchs, Switzerland) were used as ascites preparations. Mouse anti-human AC17 and N11 was done essentially as described by Wahlberg et al. (1991) and Ktistakis et al. (1991), respectively. Internalization of fluorescein-conjugated dextran (FITC-dextran; Sigma) was done by preincubating the cells on ice for 10 min, adding 10 mg/ml FITC-dextran in medium on ice to the cells, incubating for 10 min on ice and for 5–15 min at 37°C. After several washes in PBS, the cells were fixed in 3% paraformaldehyde and mounted in Mowiol.

Results

A Large Deletion in the Cytoplasmic Domain of H1 Results in Intracellular Accumulation of the Mutant Protein

In the mutant protein H1(Δ4-33A) amino acids 4–33 of the 40-residue cytoplasmic domain have been replaced by an alanine (Fig. 1). In transfected fibroblast and MDCK cells, this mutant protein was efficiently expressed but could not be detected at the plasma membrane. Fig. 2 (A–D) shows immunofluorescence analysis of wild-type H1 and H1(Δ4-33A) expressed by the two stable-transfected MDCK cell lines M1 and M1A, respectively, using an antisera directed against the carboxy-terminal sequence of H1. The wild-type protein was stained on the surface of nonpermeabilized M1 cells (Fig. 2 A). Upon permeabilization the staining reflects the distribution of H1 throughout the secretory pathway, in endosomes and on the cell surface (B). In contrast, H1(Δ4-33A) was not detectable on the surface of nonpermeabilized M1Δ cells (C). Instead, it accumulated intracellularly in defined juxtanuclear structures with mostly tubular appearance (D). A similar stain-
Immunofluorescence localization of wild-type H1 and H1(Δ4-33A). Cells expressing H1 or H1(Δ4-33A) were processed for indirect immunofluorescence microscopy with (B, D, E, and F) or without (A and C) permeabilization and stained using anti-ASGP receptor antiserum. (A and B) M1 cells, a stable MDCK cell line expressing wild-type H1. (C and D) M1Δ cells, a stable MDCK cell line expressing H1(Δ4-33A). (E) Stable NIH/3T3 cells expressing H1(Δ4-33A). (F) BHK-21 cells transiently transfected with H1(Δ4-33A) cDNA. Bar, 5 μm.

The absence of H1(Δ4-33A) from the cell surface was confirmed biochemically by immunoblot analysis of proteinase K digested M1Δ and M1 cells (Fig. 3). In untreated cells, two immunoreactive forms of 40 kD and 45 kD for H1, and 35 kD and 40 kD for H1(Δ4-33A) were detected. The two forms of each protein correspond to the high-mannose glycosylated precursor which is sensitive to endoglycosidase H (endo H) digestion and to the complex glycosylated, endo H-resistant form (Fig. 4, lanes 1–5).

Upon proteinase K digestion at 4°C, 50% of the mature form of H1 was degraded in M1 cells (normalized to the precursor form; Fig. 3, lanes 4 and 5). This corresponded
Figure 3. Surface expression of H1 and H1(Δ4-33A). Intact HepG2, M1, and M1Δ cells were incubated at 4°C or 37°C for 30 min with (+PK) or without (-PK) proteinase K as indicated. After blocking the protease, the cells were solubilized and subjected to gel electrophoresis and immunoblot analysis using anti-ASGP receptor antiserum. Protection of the mature forms was quantified by densitometric scanning, and normalized to the high-mannose precursor form. The complex and high-mannose glycosylated forms of H1 are indicated by C and H, respectively.

Figure 4. Golgi-specific modification of H1 and H1(Δ4-33A). M1 and M1Δ cells were solubilized, digested without (-PK) or with endoglycosidase H (H; lanes 2–5) or neuraminidase (N; lanes 6–9), and analyzed by gel electrophoresis and immunoblot analysis. The stable MDCK cell lines M1TS (lane 10) and M1ΔTS (lanes 11 and 12; two clonal lines with different expression levels) expressing H1TS or H1(Δ4-33A)TS, respectively, were labeled with [35S]sulfate, solubilized, and subjected to immunoprecipitation using anti-ASGP receptor antiserum. The immunoprecipitates were analyzed by gel electrophoresis and fluorography.

H1(Δ4-33A) Undergoes Trans-Golgi Modifications

The fact that H1(Δ4-33A) was found in an endo H-resistant form (Fig. 4, lanes 1–5) indicated that the mutant protein exited the ER and reached at least the medial-Golgi. To test whether H1(Δ4-33A) reached the trans-Golgi, we analyzed the incorporation of sialic acid and sulfation of tyrosine residues, two trans-Golgi-specific modifications (Roth et al., 1985; Baeuerle and Huttner, 1987). Upon neuraminidase digestion of M1 and M1Δ cell lysates, the endo H-resistant forms of wild-type H1 and of H1(Δ4-33A) were both slightly shifted on immunoblots, indicative of desialylation (Fig. 4, lanes 6–9). Subunit H1 is not naturally sulfated. However, we have recently shown that addition of a 9-residue tyrosine sulfation peptide to the exoplasmic carboxy terminus of the protein allows efficient labeling with [35S]sulfate in a stable-transfected MDCK cell line (Leitinger et al., 1994). The tyrosine sulfation tag was introduced into the mutant H1(Δ4-33A) and stable expressing MDCK cell lines, named M1ΔTS, were generated. The tagged protein H1(Δ4-33A)TS accumulated intracellularly like H1(Δ4-33A), as judged by immunofluorescence microscopy and proteinase K digestion (not shown). Upon labeling with [35S]sulfate, radioactivity was incorporated into the endo H-resistant form of H1(Δ4-33A)TS with similar efficiency as into H1TS, the tagged version of H1 (Fig. 4, lanes 10–12). These results indicate that H1(Δ4-33A) reaches the trans-Golgi/TGN.

H1(Δ4-33A) Accumulates in a Late Golgi Compartment

To determine whether H1(Δ4-33A) is localized in the trans-Golgi, endosomes, or lysosomes, the immunofluorescence staining pattern of H1(Δ4-33A) was compared to that of defined markers. As markers for endosomes and lysosomes we used fluorescein-conjugated dextran internalized for 5–15 min at 37°C, and the lysosomal membrane protein LAMP-1, respectively. As illustrated in Fig. 5 (A–C), the staining patterns of these markers differed considerably from the labeling of H1(Δ4-33A). Trans-Golgi was visualized using antibodies directed against human galactosyltransferase transiently expressed in MDCK cells, yielding a staining pattern similar to that of H1(Δ4-33A) (E). Furthermore, endogenous γ-adaptin (a TGN marker; D) and the cation-independent mannose-6-phosphate (M6P) receptor (present in the TGN, endosomes, and the plasma membrane; F) were predominantly immunolocalized to a similar juxtanuclear region as H1(Δ4-33A).

To allow double-labeling experiments, H1(Δ4-33A) was tagged at its carboxy terminus with an antigenic epitope of the c-myc oncogene product (mutant H1[Δ4-33A]myc). This modification did not alter the intracellular distribution. Double labeling of MDCK cells cotransfected with H1(Δ4-33A)myc and human galactosyltransferase cDNAs showed that the tubular structures characteristic for H1(Δ4-33A) were almost indistinguishable from the structures labeled with anti-galactosyltransferase antibody (Fig. 6, A and B). In the stable cell line M1Δ, the structures labeled with the H1-specific antibody also overlapped with the or-
ganelles stained by the anti-γ-adaptin antibody (Fig. 6, C and D). Similar overlap of signals was observed in BHK-21 cells (used because of the species specificity of the antibody) for endogenous TGN-38/41 and transfected H1(Δ4-33A)ex (not shown). These experiments strongly indicate that the mutant H1(Δ4-33A) accumulates in the Golgi, and based on the modification by sialyltransferase and protein sulfotransferase most likely in the trans-Golgi and/or TGN.

This localization is supported by the effect of agents known to perturb specific organelles. Treatment of M1Δ cells with okadaic acid or nocodazole, which are known to induce fragmentation of the Golgi apparatus (Lucocq et al., 1991; Ho et al., 1989b; Turner and Tartakoff, 1989), resulted in redistribution of H1(Δ4-33A) staining throughout the cytoplasm (not shown). The fungal metabolite brefeldin A was recently shown to cause the formation of tubules of the TGN in MDCK cells, while the medial-Golgi stacks were unaffected (Wagner et al., 1994). Under similar conditions, we observed the appearance of thin tubules containing H1(Δ4-33A) as well as galactosyltransferase extending from the juxtanuclear region into the cytoplasm (not shown). In contrast, the distribution of H1(Δ4-33A) was not affected by chloroquine, which causes lysosomal proteins to be depleted from lysosomes (Lippincott-Schwartz and Fambrough, 1987).

Proteins with mutations affecting folding and oligomerization are mostly retained in the ER by chaperones. Mu-

Figure 5. Immunofluorescence localization of H1(Δ4-33A) and different marker proteins. M1Δ cells were processed for immunofluorescence microscopy using antibodies directed against H1(Δ4-33A) (A), LAMP-1 (C; as a marker for lysosomes), γ-adaptin (D; as a marker for TGN), transfected human galactosyltransferase (E; as a marker for trans-Golgi), or cation-independent M6P receptor (F). To stain endosomes, M1Δ cells were loaded for 10 min with FITC-dextran before fixation (B). Bar, 5 μm.
tant H1(Δ4-33A), however, passes these quality control mechanisms and accumulates in a later compartment of the secretory pathway. The kinetics of ER-to-Golgi transport of H1(Δ4-33A), as judged from the conversion of the endo H-sensitive precursor to the endo H-resistant mature form of the protein in pulse-chase experiments, were similar to those of wild-type H1 with 50% conversion after ~3 h (Fig. 7 A). Cross-linking experiments using difluorodinitrobenzene (Shia and Lodish, 1989) in M1 and M1Δ cells showed similar formation of homodimers and -trimers of wild-type and mutant H1 (not shown).

Because conversion to the complex glycosylated form is slow, the half-lives of the mature wild-type and mutant proteins were determined using their sulfatable variants H1Ts and H1(Δ4-33A)Ts which can be specifically labeled in their final forms with [35S]sulfate. The mature mutant protein has a half-life of ~5 h which is somewhat shorter than that of the wild-type H1Ts of ~7 h (Fig. 7 B). In addition, inhibition of protein synthesis for 3.5 h with 100 μg/ml cycloheximide did not change the distribution of H1(Δ4-33A). Together these results argue against rapid missorting of H1(Δ4-33A) to lysosomes with transient accumulation in the Golgi, but rather suggest an efficient mechanism of retention of H1(Δ4-33A) in the Golgi.

**Trans-Golgi Localization Depends on the Size of the Cytoplasmic Domain**

To define the determinants for trans-Golgi accumulation, we prepared a series of H1 cDNA constructs with alterations in the cytoplasmic domain, shown in Fig. 1. These constructs were transiently expressed in MDCK cells and analyzed by indirect immunofluorescence microscopy for accumulation in the typical juxtanuclear, tubular Golgi structures vs transport to the plasma membrane. The results are summarized in Fig. 1. The immunofluorescence patterns of the most relevant constructs are shown in Fig. 8 (A–C).

Two previously prepared mutants, H1(Δ4-11) (lacking the signal for basolateral sorting from the TGN) and H1(Δ12-33), were both transported to the cell surface in stable expressing MDCK cell lines (Geffen et al., 1993). This is in contrast to H1(Δ4-33A) with the combined deletion. Mutation of tyrosine-5 of H1(Δ12-33) to alanine in
the construct H1(Δ12-33/5A) also resulted in transport to the cell surface, arguing against a role of the tyrosine signal in surface transport.

In a series of amino-terminal deletions, H1(Δ2-19) and H1(Δ2-24) were expressed on the cell surface, whereas H1(Δ2-28), H1(Δ2-33), and H1(Δ2-37) accumulated intracellularly in the typical tubular structures. H1(Δ2-24) and H1(Δ2-28) differ only by the four consecutive prolines 25-28. Separate deletion of the proline-rich segment 25-30 in H1(ΔPro), however, did not significantly affect the wild-type distribution. Furthermore, deletion of the juxtamembraneous segment of residues 26-40 did not prevent surface transport. These results show that no single sequence element in the cytoplasmic domain can be made responsible either for retention in the Golgi or for surface transport.

There is, however, a correlation between the size of the cytoplasmic domain and the fate of the mutant proteins (see Fig. 1). Constructs with cytoplasmic domains of 17 or more residues were all transported to the plasma membrane; those with domains of 13 or fewer amino acids were retained in the Golgi. To test this correlation, the cytoplasmic sequence of H1(Δ4-33A) was extended by insertion of an unrelated sequence of 10 residues, the human c-myc epitope, in place of the initial deletion. The cytoplasmic domain of the resulting mutant H1(Δ4-33m) thus consists of 21 amino acids. Upon expression in transfected MDCK cells, the protein did not accumulate intracellularly but was detected on the cell surface (Fig. 8 D). In addition, the cytoplasmic domain of H1 was replaced with that of the transferrin receptor. The resulting fusion protein H1T, with a cytoplasmic tail of 65 residues, was efficiently transported to the plasma membrane (Fig. 8 E). However, upon truncation of this domain to only 11 residues, the fusion protein H1TA was completely retained in the typical Golgi structures (Fig. 8 F). These results confirm that the localization of H1 does not depend on the sequence of the cytoplasmic domain, but rather on its size.

The Exoplasmic Domain in Combination with a Tailless Anchor is Sufficient for Golgi Localization

The transmembrane and/or exoplasmic portions of truncated H1 thus appear to be responsible for Golgi retention, modulated by the cytoplasmic domain. To test whether the transmembrane segment is sufficient for Golgi retention, this domain with the wild-type or the truncated cytoplasmic portion of H1 was fused to the exoplasmic portion of human aminopeptidase N, a type II cell surface protein. Both fusion proteins (constructs AN2 and AN2Δ, illustrated in Fig. 9) were expressed on the cell surface of transiently transfected MDCK cells and did not accumulate in the Golgi (Fig. 9, A and B). Conversely, we tested whether the exoplasmic domain of H1 is sufficient for Golgi localization if anchored in the membrane by an artificial transmembrane segment. In the construct H1ΔLeu19 (Fig. 9), the luminal domain of H1 was fused to a generic hydrophobic sequence of 19 leucine residues preceded by the positively charged sequence MGPR, to warrant efficient membrane insertion. For a control, this short cytoplasmic tail was extended by the cytoplasmic sequence of wild-type H1 in the construct H1Leu19. Whereas H1Leu19 was efficiently expressed on the cell surface of transfected MDCK cells, H1ΔLeu19 accumulated in Golgi structures like H1(Δ4-33A) (Fig. 9, C and D). The particular sequence of the transmembrane segment of H1 is thus not necessary for Golgi localization, whereas the exoplasmic portion of H1 is sufficient for Golgi retention in combination with a membrane anchor that lacks a sizable cytoplasmic domain.

Association with Subunit H2 Partially Restores Surface Expression of H1(Δ4-33A)

In the functional ASGP receptor, H1 is part of a heterooligomeric complex with subunit H2, which has a cytoplasmic domain of 58 amino acids. To test whether association with H2 influences the localization of H1(Δ4-33A), a stable MDCK cell line expressing mutant H1 and wild-type H2 was created. Localization of H1(Δ4-33A) and H2 was assayed separately by immunofluorescence using subunit-specific antisera. In nonpermeabilized cells, both subunits could be visualized on the surface (Fig. 10, A and C). Furthermore, functional receptors could be detected on the cell surface by specific binding of 125I-i iodinated asialoorosomucoid (not shown) which is indicative of correct
hetero-oligomer formation. Thus, association with H2 rescued H1(A4-33A) transport to the cell surface. In permeabilized cells, however, both subunits were also detected in typical Golgi structures (Fig. 10, B and D), indicating that hetero-oligomers were partially retained in the Golgi.

**Discussion**

Most mutations in secretory or membrane proteins either do not prevent surface transport or, if they perturb folding or oligomerization, cause retention and degradation in the ER. Very few mutants have been described that escape ER retention and accumulate in a Golgi compartment on the way to the cell surface. It is conspicuous that in most of these cases the mutation involves the membrane anchor of the protein or the immediate flanking sequences, which have been identified as the segments important for retention of natural Golgi resident proteins. Well characterized cases are growth hormone fused to the transmembrane and cytoplasmic domain of vesicular stomatitis virus G protein (Guan and Rose, 1984), or to an uncleaved transmembrane segment of a GPI-anchored membrane protein (Moran and Caras, 1992); the insertion of an arginine into the signal/anchor domain of influenza virus neuraminidase (Sivasubramanian and Nayak, 1987); two point mutations close to the transmembrane segment of fowl plague virus...
Figure 9. Intracellular localization of H1 fusion proteins. MDCK cells were transiently transfected with the cDNAs of the fusion proteins AN2 (A), AN2Δ (B), H1Leu19 (C), and H1ΔLeu19 (D). The cells were fixed, permeabilized, and stained with anti-aminopeptidase N (A and B) or anti-H1 antibodies (C and D). The structure of the fusion proteins is illustrated schematically (APN, aminopeptidase N). The predominant intracellular localization of the constructs is indicated as in Fig. 1 (PM, plasma membrane; G, trans-Golgi structures). Bars, 5 μm.

Here we describe mutants of a cell surface receptor, the ASGP receptor subunit H1, that pass the quality control mechanisms of the ER, but are retained in a later compartment of the secretory pathway and do not reach the plasma membrane. The site of accumulation was identified as the trans-Golgi/TGN based on biochemical and morphological evidence for the mutant H1(Δ4-33A). The retained protein was modified by the trans-Golgi–specific enzymes sialyltransferase and (when tagged with an appropriate acceptor sequence) tyrosylprotein sulfotransferase. By indirect immunofluorescence microscopy, the mutant was found in juxtanuclear structures that are distinct from endosomes and lysosomes, and that are also stained with antibodies directed against galactosyltransferase and γ-adaptin. Furthermore, the effects of nocodazole, okadaic acid, brefeldin A, and chloroquine on the distribution of H1(Δ4-33A) in MDCK cells were consistent with a trans-Golgi localization.
The nature of the alteration in H1 resulting in Golgi accumulation was analyzed by a series of mutations in the cytoplasmic domain. The results cannot be explained by the accidental generation of a retention motif or the deletion of a hypothetical export signal. However, the localization of H1 mutants correlates with the size of their cytoplasmic domains. Deletion of more than two-thirds of the 40 cytoplasmic residues caused Golgi retention. Extension of the truncated tail of H1(Δ4-33A) by insertion of 10 unrelated residues of c-myc again resulted in surface transport. Similarly, the 65-residue cytoplasmic domain of the transferrin receptor fused to the transmembrane and exoplasmic portion of H1 allowed surface transport, whereas truncation of the fusion protein’s tail to only 11 residues yielded efficient Golgi retention. In contrast, tailless transferrin receptors have been reported to be transported to the cell surface (Gironès et al., 1991; Kundu and Nayak, 1994). Cytoplasmic sequences have been shown by mutational analysis to be necessary for endocytosis, basolateral transport, and sorting to lysosomes (summarized by Sandoval and Bakke, 1994). Mutant proteins lacking cytoplasmic sequences may be missorted to inappropriate post-Golgi compartments, but are generally not blocked in exit from the Golgi. Golgi retention of truncated H1 therefore appears to be due to a specific interaction of the transmembrane and/or exoplasmic portions of H1 with Golgi resident components. This interaction is impeded by large cytoplasmic domains. Coexpression of H1(Δ4-33A) with wild-type H2 resulted in the formation of functional ligand-binding hetero-oligomers detectable on the cell surface. However, since both subunits could also be detected by immunofluorescence microscopy in the typical Golgi pattern, association with H2 only weakened the interaction of H1(Δ4-33A) with the trans-Golgi.

The membrane anchor of H1(Δ4-33A) was found not to be sufficient to retain a reporter protein, the exoplasmic portion of aminopeptidase N, in the Golgi. In addition, the particular sequence of the transmembrane segment is also not necessary for Golgi localization, since it can be replaced by an artificial sequence of 19 consecutive leucines without affecting the fate of the protein. The exoplasmic domain is thus responsible for the interaction of truncated H1 mutants with Golgi components. It remains to be investigated what the potential interaction partners in the trans-Golgi might be.

Mutagenesis of Golgi resident membrane proteins (glycosyltransferases and coronavirus M protein) revealed an involvement of the transmembrane domain and/or the exoplasmic flanking sequence in retention (Nilsson et al., 1991; Munro, 1991; Aoki et al., 1992; Burke et al., 1992;
vent their inclusion into transport vesicles. The retained compartment by interaction via their transmembrane and/or stalk regions. The size of these kin oligomers would prevent their inclusion into transport vesicles. The retained mutants of H1 might participate in trans-Golgi hetero-oligomers by an interaction that is sterically hindered by larger cytoplasmic domains.

An alternative model proposed by Bretscher and Munro (1993) implicates the length of the transmembrane segment in relation to the differing lipid compositions in the compartments of the secretory pathway. Shorter transmembrane segments would be retained in the Golgi, whereas longer ones would be transported further. Hence, it is conceivable that the size of the cytoplasmic domain might have an effect on the positioning of the transmembrane segments of the H1 oligomer within the lipid bilayer and, as a result, on how the cytoplasmic domain is exposed in the lumen.

It has to be pointed out that even the wild-type ASGP receptor is significantly concentrated in the Golgi apparatus. By quantitative immunoelectron microscopy, ~20% of the ASGP receptor has been detected in the trans-Golgi in rat hepatocytes and in HepG2 cells (Geuze et al., 1983, 1984; Zijderhand-Bleekemolen et al., 1987; Stoorvogel et al., 1989). Cycloheximide, which blocks protein synthesis without affecting intracellular transport, caused depletion of poly-immunoglobulin receptor and 5'-nucleotidase from the Golgi, but did not affect the Golgi pools of the ASGP and M6P receptors (Geuze et al., 1984; van den Bosch et al., 1986). Also by immunofluorescence microscopy, the ASGP receptor subunits in HepG2 and transfected MDCK cells can be detected in juxtanuclear Golgi-like structures, although without the obvious tubular appearance of the staining observed for H1(D4-33A) (e.g., Fig. 2 B). This Golgi pool of wild-type ASGP receptor may reflect an intrinsic affinity of the protein to Golgi components that is mediated by the cytoplasmic tail, and that leads to efficient Golgi retention when the cytoplasmic domain is sufficiently truncated.

The situation of wild-type and truncated forms of H1 with respect to intracellular localization is reminiscent of that of β-1,4-galactosyltransferase (Shur, 1993). Two mRNAs are generated by alternative promoters which encode two forms of the enzyme (Lopez et al., 1991). A short form with 11 cytoplasmic residues is localized specifically in the trans-Golgi. A long form with 13 additional amino-terminal residues is less efficiently retained and also found on the cell surface, where it acts as a cell adhesion molecule. Modulation of Golgi retention by the cytoplasmic domain may thus be a general mechanism for fine tuning the intracellular distribution of membrane proteins. Whether in the case of galactosyltransferase this is a specific effect of the additional sequence (e.g., by interaction with the cytoskeleton) or whether, as in the case of H1, simply the additional size of the cytoplasmic domain is responsible for reduced retention remains to be tested.

We thank Katja Huggel for technical assistance, Drs. C. Fuhrer and K. Fiedler for generating the F1(D4-33A) cell line and the AN2 construct, respectively, and Drs. G. Banting, E. Berger, B. Hoflack, L. Kühn, O. Nörén, E. Rodriguez-Boulan, H. Sjöström, and G. von Heijne for cDNAs and/or antibodies. We are grateful to Dr. Gillian Griffiths for initial contributions and for critically reading the manuscript.

This work was supported by grant 31-34008.92 from the Swiss National Science Foundation and by the Incentive Award of the Helmut Horten Foundation.

Received for publication 2 February 1995 and in revised form 6 April 1995.

References


