The TGN38 Glycoprotein Contains Two Non-Overlapping Signals That Mediate Localization to the *Trans*-Golgi Network

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Abstract. The membrane-spanning and cytoplasmic domains of CD4 and CD8 were replaced by those of TGN38. After transient expression in HeLa cells, the location of the hybrid proteins was determined using immunofluorescence and quantitative immuno-electron microscopy, FACS analysis and metabolic labeling. The membrane-spanning domain was found to contain a signal that localized hybrid proteins to the TGN. This was in addition to the signal previously identified in the cytoplasmic domain (Bos, K., C. Wraight, and K. Stanley. 1993. *EMBO (Eur. Mol. Biol. Organ) J.* 12:2219–2228. Humphrey, J. S., P. J. Peters, L. C. Yuan, and J. S. Bonifacino. 1993. *J. Cell Biol.* 120:1123–1135. Wong, S. H., and W. Hong. 1993. *J. Biol. Chem.* 268:22853–22862). The different properties of these two signals suggest that each operates by a different mechanism.

PROTEINS synthesized in the ER are funneled through the Golgi apparatus for further processing and sorting (for review see Kornfeld, 1992; Mellman and Simons, 1992; Rothman and Orci, 1992). Proteins first enter the tubulo-vesicular *cis*-Golgi network and progress through the stack comprising *cis*-, *medial*-, and *trans*-cisternae until they reach the tubulo-vesicular TGN (Rambourg and Clermont, 1990). The final stages of N-linked oligosaccharide processing occur in the TGN (Roth et al., 1985; Griffiths and Simons, 1986; Cooper et al., 1990; Griffiths et al., 1989), and proteins are sorted to their correct destination (Griffiths and Simons, 1986; Peters et al., 1991; Kornfeld, 1992). Along the exocytic pathway as far as the TGN, consecutive steps in protein transport appear to be mediated by non-clathrin-coated vesicles (for review see Rothman and Orci, 1992). Transport occurs by default, provided the proteins have been correctly folded in the ER (Gething and Sambrook, 1992). In contrast, resident ER and Golgi proteins are retained in specific compartments, raising questions as to the mechanism by which this occurs.

Resident proteins are localized by retention or retrieval signals (for review see Pelham, 1989; Machamer, 1991; Kornfeld, 1992; Nilsson et al., 1993*b*). Retrieval signals have been found on both soluble and membrane bound proteins of the ER (Munro and Pelham, 1987; Nilsson et al., 1989; Jackson et al., 1990). The carboxy-terminal KDEL motif results in the retrieval of soluble ER proteins lost from this compartment (Munro and Pelham, 1987). This sequence is recognized by a receptor which returns the protein to the ER (Semenza et al., 1990; Lewis and Pelham, 1992). Some membrane proteins contain a double-lysine motif in the cytoplasmic tail which ensures retrieval to the ER (Jackson et al., 1993).

Golgi retention signals were first identified in a viral protein (Swift and Machamer, 1991) and in Golgi glycosylation enzymes (Nilsson et al., 1991; Munro, 1991; Aoki et al., 1992; Burke et al., 1992; Colley et al., 1992; Tang et al., 1992; Teasdale et al., 1992; Wong et al., 1992). In all cases, the retention signal lies in the membrane-spanning domain. More recently, membrane-spanning domains have been implicated in the localization of two proteins to the nuclear envelope (Wozniak and Blobel, 1992; Smith and Blobel, 1993).

So far, none of the proteins studied have been shown to contain both a retention and a retrieval signal though there are indications that more than one signal is present in some of them. When the carboxy-terminal -KDEL signal is removed from the soluble BiP protein, it is secreted very slowly (Munro and Pelham, 1987). When the double-lysine motif is removed from glucuronosyltransferase, an integral membrane protein, little if any escapes from the ER (Jackson et al., 1993). Both these cases suggests that a retention signal is present in addition to the retrieval signal.

To determine whether a protein can contain both signals, we focused on TGN38, a resident of the *trans*-Golgi network (Luzio et al., 1990). TGN38 has a tyrosine-based motif in the cytoplasmic domain that acts as a retrieval signal, return-

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ing the protein from the cell surface to the TGN (Bos et al., 1993; Humphrey et al., 1993; Wong and Hong, 1993). Here we have looked for the second signal and have found it in the membrane-spanning domain.

Materials and Methods

Materials

Chemicals and reagents were obtained from the sources described by Nilsson et al. (1993*a*, 1994). Restriction enzymes, T4 DNA ligase, and polynucleotide kinase for recombinant DNA procedures were purchased from GIBCO BRL (Gaithersburg, MD), New England Biolabs (Bethesda, MD), or Pharmacia Corp. (Uppsala, Sweden). Sequencing reagents and T7 polymerase were purchased from USB Biochemicals. Cell culture media, fetal calf serum, amino acids, and penicillin/streptomycin were from GIBCO BRL. [^{35}S]Methionine/cysteine in vivo labeling mix was from Amersham Corp. (Amersham, UK). Chemicals were purchased from Sigma Chem. Co. (St. Louis, MO), BDH (Poole, UK), or Fisons Inc. (UK).

Recombinant DNA Procedures

The TGN38 cDNA (Luzio et al., 1990) was mutagenized by PCR such that a BamHI site was introduced just after the stop codon. The HindIII–BamHI fragment containing the 5′ untranslated region and the complete coding sequence was cloned between the same restriction sites in the pCMUIV plasmid (Nilsson et al., 1989). The TGN38 cDNA in the mammalian expression vector pUEX (Luzio et al., 1990) was used as a template for PCR amplification under the conditions described by Saiki et al. (1988).

Oligonucleotides used in PCR reactions were generally synthesized with BamHI flanking sequences to facilitate easy cloning into plasmid vectors for sequencing. Oligonucleotides were used at 1 mM in PCR reactions. PCR products were ethanol precipitated, digested with BamHI, and cloned into the pBS plasmid (Stratagene Corp., La Jolla, CA) for sequencing. DNA sequencing was carried out using the dideoxy chain termination method and T7 polymerase (Tabor and Richardson, 1987). Plasmid derivatives were digested with the appropriate enzymes to generate the correct 5′ and 3′ ends for cloning and construction of CD8 and CD4 cDNA chimeras.

CD8 hybrid proteins were constructed such that the first 153 amino acids of the extracellular domain of αCD8 were preserved. This is sufficient for CD8 glycosylation and secretion (Leahy et al., 1992), and should also not affect the ability of CD8 hybrid proteins to form homodimers (Leahy et al., 1992; Boursier et al., 1993). PCR-generated DNA fragments were cloned into the pCMUIV expression vector (Nilsson et al., 1989) using 3 DNA fragments in a ligation. To construct the CD8-MC and CD8-MΔC chimeric cDNAs the ligation mix contained (1) the HindIII–ApaLI DNA fragment that encoded most of the lumenal domain of the CD8 molecule, (2) an ApaLI–BamHI DNA fragment that encoded the membrane-spanning and cytoplasmic domain sequences of TGN38, and (3) the pCMUIV vector as a HindIII–BamHI DNA fragment.

To construct the CD8-M membrane-spanning chimeric cDNA the ligation contained (1) the HindIII–ApaLI DNA fragment that encoded most of the lumenal domain of the CD8 molecule, (2) an ApaLI–SalI DNA fragment that contained the membrane-spanning domain of TGN38, and (3) the pCMUIV plasmid vector as a HindIII–SalI DNA fragment which also contained the cytoplasmic domain of the CD8 molecule.

To construct the CD8-C and CD8-ΔC chimeric cDNAs the ligation contained (1) a SalI–BamHI DNA fragment that contained the cytoplasmic domain of TGN38, and (2) the pCMUIV vector as a BamHI–SalI DNA fragment which also contained the lumenal and membrane-spanning domains of the CD8 molecule.

Cloning of the CD4 cDNA into the pCMUIV plasmid has been described by Nilsson et al. (1989) and we used this CD4 plasmid initially. However, there were no unique restriction sites in the CD4 cDNA at equivalent positions which would have allowed us to insert replacement membrane-spanning and cytoplasmic TGN38 sequences. We thus inserted unique restriction sites within the CD4 cDNA using PCR mutagenesis. Deen et al. (1988) have shown that a CD4 molecule terminating after residue 371 in the extracellular domain is efficiently secreted. As the threonine at 369 is followed by a proline residue, this would make it unlikely that there are any structural features after thr-369 necessary for correct folding and transport of CD4. Codon 367 (Maddon et al., 1985) in the CD4 cDNA (which encodes a serine residue) was mutated from TCC to TCA. This silent mutation created a single HincII site within the CD4 cDNA. Similarly, codons 400 and 401 (encoding arginine residues) were mutated from CGA AGG to CGT CGA. These silent mutations created a unique SalI site in the CD4 cDNA. There were now unique restriction sites on either side of the membrane-spanning region in the cDNA. PCR was carried out on the TGN38 cDNA using oligonucleotides similar to those described above. Instead of the ApaLI site used in CD8 chimera constructions, 5′ oligonucleotides that precede the TGN38 membrane-spanning domain were synthesized with a HincII site. A HincII–SalI or HincII–BamHI DNA fragment containing TGN38 sequences could be fused to CD4 by ligation as described previously. We thus ligated either a HindIII–HincII or HindIII–SalI DNA fragment (encoding the lumenal domain of the CD4 molecule) from pCMUIV-CD4, an appropriate PCR generated DNA fragment that encoded TGN38 sequences, and an appropriate pCMUIV vector. Ligations were transformed into *E. coli* strain JM101 using standard procedures (Sambrook et al., 1989) and clones were analyzed by restriction digestion followed by DNA sequencing. Large-scale preparations of the pCMUIV plasmids (for transfections) were made by alkaline lysis followed by CsCl-ethidium bromide purification (Sambrook et al., 1989).

Cell Culture and Transfections

Monolayer HeLa cells (ATCC CCL185) were grown in DMEM supplemented with 10% FCS, non-essential amino acids, penicillin/streptomycin, and 2 mM glutamine. Cells were grown to 20–30% confluency before transfection by the calcium phosphate method essentially as described by Nilsson et al. (1989). For immunofluorescence, cells were seeded out into 30-mm, 6-well plates (Becton Dickinson, Lincoln Park, NJ) containing sterile coverslips. After one day, cells were transfected with 2 μg of plasmid DNA (CsCl-EtBr purified) and 3 μg of pUC19 plasmid DNA (used as a carrier to increase transfection efficiency). Cells were fixed and processed for immunofluorescence 60–72 h later (Nilsson et al., 1989; Jackson et al., 1990). Transfected cells for electron microscopy and immunoprecipitation were prepared as described by Nilsson et al. (1989) in 100-mm tissue culture dishes and processed 60–72 h after DNA was added.

Indirect Immunofluorescence Microscopy

Cells were processed for immunofluorescence microscopy as described previously (Warren et al., 1984; Nilsson et al., 1991). The rabbit antiserum to TGN38 has been described by Luzio et al. (1990) and was diluted (1:500) in 0.2% fish skin gelatin/PBS before use. GalT was detected using either a rabbit polyclonal antiserum (Roth and Berger, 1982; Nilsson et al., 1993*a*) at a dilution of 1:200 or the monoclonal antibody GT-2 (Berger et al., 1986). Culture supernatants from the QS4120 (Healey et al., 1990) and OKT8 (ATCC CRL 8014) mouse hybridomas were used to detect CD4 and CD8, respectively. The secondary antibodies were horse anti-mouse conjugated to Texas red (Vector Labs, Burlingame, CA) and sheep anti-rabbit conjugated to FITC (Dakopatts, Copenhagen) and both were used at a dilution of 1:100. Rhodamine-conjugated transferrin (Molecular Probes, Eugene, OR) was dissolved in PBS and used at a final concentration of 20 μg/ml; cells were incubated in fresh media containing the ligand for 30 min at 37°C before washing with PBS and fixation. Slides were examined on a Zeiss Axiophot fluorescence microscope using a 63× objective oil immersion lens and a 10× eyepiece.

Electron Microscopy and Stereological Analysis

Transfected cells were fixed in 2% paraformaldehyde/0.2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for 3 h and processed for cryo-immunoelectron microscopy essentially as described by Rabouille et al. (1993). Cell pellets embedded in gelatin were cut into blocks and infused with 2.3 M sucrose overnight. The blocks were frozen in liquid nitrogen, ultrathin cryosections were cut and incubated for 30 min with primary antibodies. Polyclonal rabbit antiserum to TGN38 (Luzio et al., 1990) was used at a dilution of 1:100 and an affinity-purified rabbit antibody to CD4 (Dr. Mark Marsh, MRC Centre, University College London) at a dilution of 1:100. Both were diluted into 0.5% fish skin gelatin/PBS and the bound antibodies were detected using protein A-coupled to 10-nm gold particles used at a dilution of 1:50 (from Cell Biology Department, Ultrecht School of Medicine, Utrecht, Netherlands). The monoclonal antibody 14 to CD8 (Haynes, 1986) was used neat as a culture supernatant and bound antibodies were detected using a secondary goat anti-mouse antibody coupled to 10-nm gold particles at a dilution of 1:20 (Nilsson et al., 1994). Immunolabeling was

followed by staining with uranyl acetate and embedding in methyl cellulose as described by Tokuyasu (1980).

Quantitation of immunolabeling was carried out by first identifying a labeled area of the cell where profiles of the Golgi stack and TGN were readily visible. The TGN was defined morphologically as described in the Results. Labeled cell sections were placed in one of two categories. Cell sections in which no endosomes were labeled consistently contained fewer than 50–60 gold particles whereas cell sections containing labeled endosomes contained more than 50–60 gold particles. In each experiment, 200–1,000 gold particles were counted depending on the type of construct used for transfection (see Fig. 6 legend). The background observed in each experiment was extremely low and therefore ignored.

The relative distribution of immunolabeling was performed by counting the gold particles over the membranes (Golgi stack, TGN, endosomes, and plasma membrane) of selected cells. The linear density of the gold particles at the plasma membrane was estimated by the point hit method. Briefly, pictures were printed at 38,750× or 52,000× final magnification and overlaid with a grid containing a point-to-point spacing of 2 cm (plasma membrane) or 0.5 cm (TGN) (d). The number of intersections (I) of the membrane with this grid were counted. The total number of gold particles over the total membrane was counted and the linear density was then calculated using the formula:- Gold particles/(I X d/magnification).

FACS Analysis

Transfected cells were grown in 30-mm wells and processed for FACS analysis exactly as described by Nilsson et al. (1989). Native and hybrid CD8 proteins were detected using the culture supernatant from the OKT8 hybridoma. Approximately 5×10^5 cells were incubated with the primary antibodies on ice for 20 min followed by gentle washing. The secondary antibody was FITC-conjugated sheep anti-mouse (Vector Labs., Burlingame, CA) used at a dilution of 1:100. After washing, cells were fixed in 3% paraformaldehyde/PBS for 20 min on ice. Immediately before FACS analysis, cells were washed three times with calcium/magnesium-free PBS and resuspended in 0.5 ml of this solution. A Becton-Dickinson FACScan 440 machine was used to analyze the samples. Quantitation of FACS data was performed by calculating two parameters: the amount of positive cells expressing each protein and the arithmetic mean intensity of fluorescence of these positive cells (ΣNi.Ii^2/ΣNili). The mean intensity was multiplied by the number of positive cells which gave the total fluorescence intensity (arbitrary units) detected for the positive cell population. The total fluorescence intensity of the CD8 positive cells was represented as 100% and the total fluorescence intensity of positively gated cells expressing other CD8 hybrid proteins were expressed as a percentage of total CD8.

Immunoprecipitation of Hybrid Proteins

Immunoprecipitation was carried out essentially as described by Jackson et al. (1989). Transfected cells in 100-mm dishes were labeled with 0.2 mCi [^{35}S]methionine/cysteine for 20 min and either lysed in detergent immediately or chased with medium containing excess cold methionine and cysteine. After 15 min on ice the cells were scraped off and lysates centrifuged at medium (12,000 *g*, 10 min at 4°C) and high speed (400,000 *g*, 30 min at 4°C) to remove particulate material. The supernatants were precleared by incubation with mouse IgG bound to protein A-Sepharose (Pharmacia Corp., Uppsala, Sweden) and native and hybrid proteins were immunoprecipitated using QS4120 (anti-CD4) or OKT8 (anti-CD8) antibody bound to protein A-Sepharose. Immunoprecipitates were then washed sequentially twice with lysis buffer, once with lysis buffer containing 0.1% SDS, once with lysis buffer containing 0.5 M NaCl, twice with lysis buffer, and finally once with low ionic strength buffer (20 mM Tris pH 7.5, 0.1% TX-100). After the final wash, immunoprecipitates were divided equally and resuspended in 15 μl of neuraminidase digestion buffer (300 mM NaCl, 100 mM sodium acetate pH 5.5, 14 mM calcium chloride and 1 mM PMSF) containing either 25 mU neuraminidase (Type VIII; Sigma) or buffer (mock treatment). Samples were incubated at 37°C for 4 h before analysis by SDS-PAGE and fluorography.

Results

CD8 and CD4 Hybrid Proteins

We have used both CD8 and CD4 as the reporter molecules. These glycoproteins are members of the Ig superfamily and are normally expressed on the surface of T-lymphocytes (Littman et al., 1985; Maddon et al., 1985). The αCD8 polypeptide consists of 214 amino acids (Littman et al., 1985) and CD4 of 435 residues (Maddon et al., 1985). The amino acid sequences of the membrane-spanning and cytoplasmic domains of CD8 and CD4 are shown in Fig. 1 *A*. Rat TGN38 is a type I glycoprotein with a predicted molecular weight of 38 kD but glycosylation increases this to 85–95 kD (Luzio et al., 1990). Signal peptide cleavage produces a mature protein of 340 residues. The large lumenal domain (residues 1–287) is followed by a single membrane-spanning domain (residues 288–306) and a short cytoplasmic domain (residues 307–340; see Fig. 1 *A*).

Chimeric cDNAs were constructed by replacing domains in CD4 and CD8 with those in TGN38 at identical amino acids in the three proteins (see Fig. 1 *A*). This meant that hybrid proteins containing the membrane-spanning domain of TGN38 also included some of the flanking amino acids. Flanking sequences have been shown to influence substantially the ability of membrane-spanning domains to localize proteins to the Golgi apparatus (Nilsson et al., 1991; Munro, 1991; Colley et al., 1992).

Immunofluorescence Microscopy of CD8 and CD4 Hybrid Proteins

Rat TGN38 cDNA (Luzio et al., 1990) was cloned into the pCMUIV expression vector (see Materials and Methods) and the plasmid introduced into cells by calcium phosphate transfection. At 72 h after transfection, immunofluorescence microscopy showed that TGN38 was localized to a juxtanuclear reticulum (Fig. 2 *A*) very similar to that stained by antibodies to GalT (Fig. 2 *B*), a resident of the *trans*-Golgi cisterna (Roth and Berger, 1982) and the TGN (Lucocq et al., 1989; Taatjes et al., 1992; Nilsson et al., 1993*a*). Higher levels of TGN38 led to an accumulation of the protein in punctate structures in the cytoplasm (Fig. 2 *A*) and at the cell surface (data not shown; see later). These data are consistent with the localization of endogenous TGN38 in NRK cells and expressed protein in transfected CV-1 cells (Luzio et al., 1990).

The membrane-spanning (M) and cytoplasmic (C) domains of TGN38 were grafted on to the lumenal domain of CD8 (see Fig. 1 *B*). CD8 was mostly present on the cell surface though a small amount was present in small punctate structures, especially at high levels of expression (Fig. 2 *C*). The hybrid protein (CD8-MC), however, was restricted to a compact juxtanuclear reticulum (Fig. 2 *D*) that was very similar to that stained by antibodies to GalT (data not shown). The membrane-spanning and cytoplasmic domains of TGN38 were also grafted on to the lumenal domain of CD4. Transiently expressed CD4 was mostly localized to the plasma membrane (Fig. 2 *E*), but the CD4-MC hybrid protein was again localized to a juxtanuclear compartment (Fig. 2 *F*), very similar to that stained by antibodies to GalT (data not shown). There was also faint staining of the nuclear envelope, suggesting some accumulation of both hybrid proteins within the ER (*arrows* in Fig. 2, *D* and *F*).

To show that the membrane-spanning and cytoplasmic domains of TGN38 contained distinct localization signals, each of these domains in both CD4 and CD8 was replaced by that

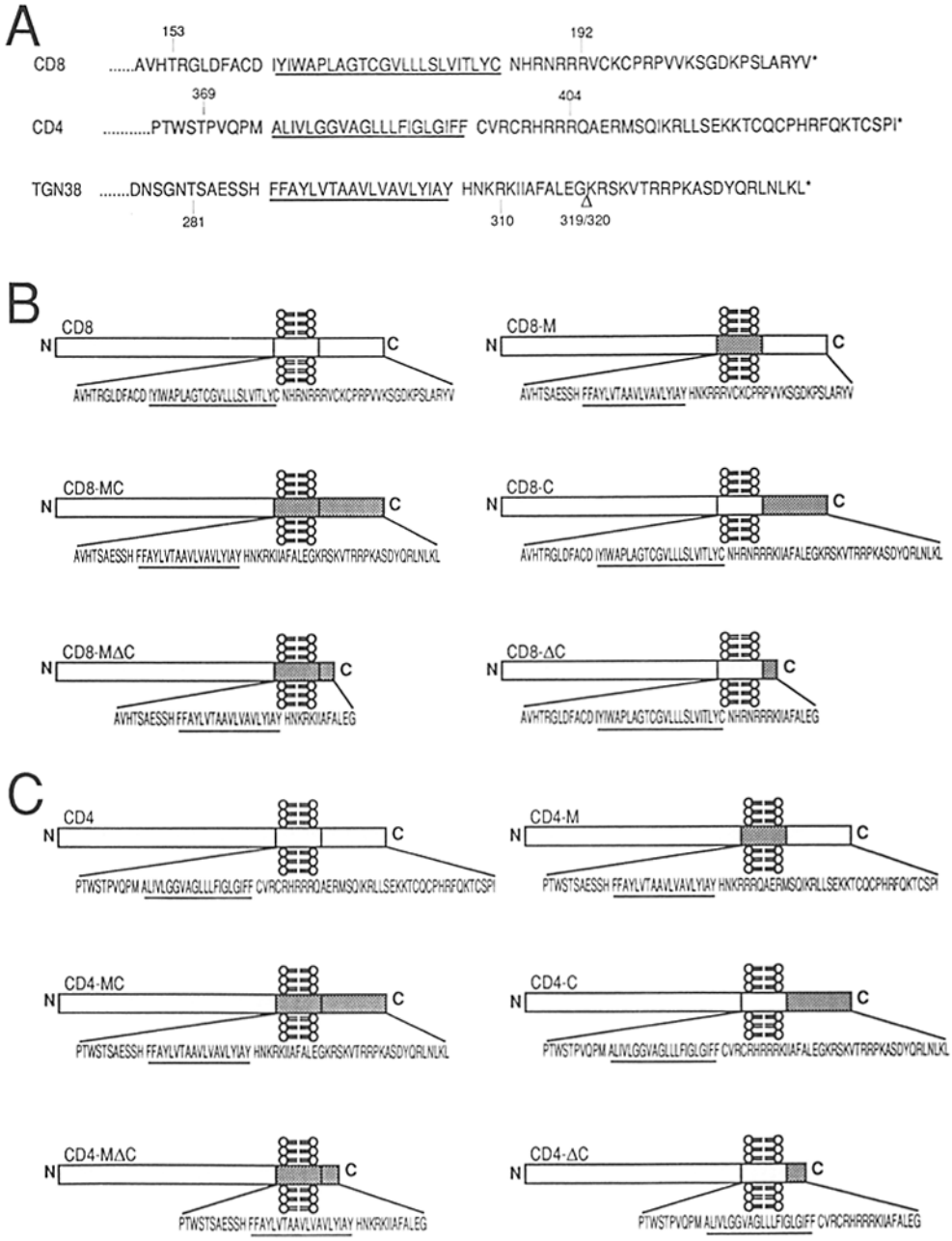

Figure 1. (*A*) The sequences of the membrane-spanning (*underlined*) and cytoplasmic domains of CD8, CD4, and TGN38. The membrane-spanning domain of CD4 is that defined by Maddon et al. (1985) although it could include an additional two amino acids (-CV-) on the cytoplasmic side of the membrane. Amino acid residues at which domains were replaced in the different hybrid proteins are numbered. (*B* and *C*) Line sketches show CD8, CD4, and hybrid proteins containing the membrane-spanning and cytoplasmic domains of TGN38 (*shaded* and *boxed*). Hybrid proteins are defined by suffixes which denote the domains of CD8 or CD4 that were replaced by equivalent domains of TGN38. *M* = membrane-spanning domain and flanking residues; *C* = cytoplasmic domain; and Δ*C* = truncated cytoplasmic domain lacking 21 residues from the carboxy-terminus.

in TGN38 (Fig. 1, *B* and *C*). The TGN38 membrane-spanning domain alone localized the hybrid proteins to the Golgi apparatus. Both CD8-M (Fig. 3 *A*) and CD4-M (Fig. 3 *E*) gave a discrete juxtanuclear staining pattern very similar to that for GalT, especially at low levels of expression (Fig. 3, *B* and *F*, respectively). At higher levels of expression, staining was more diffuse suggesting accumulation in vesicles and at the cell surface (data not shown). This pattern was more frequently observed for CD4-M than for CD8-M.

The cytoplasmic domain of TGN38 contained, as expected, the other Golgi localization signal (Bos et al., 1993; Humphrey et al., 1993; Wong and Hong, 1993). Both CD8-C (Fig. 3 *C*) and CD4-C (Fig. 3 *G*) were detected in a juxtanuclear reticulum very similar to that containing GalT (Fig. 3, *D* and *H*, respectively). At all levels of expression staining of small punctate structures (Fig. 3, *C* and *G*) was also observed and these were shown to be endosomes by immunoelectron microscopy (see below).

Immunofluorescence Microscopy of CD8 Hybrid Proteins Containing a Truncated TGN38 Cytoplasmic Domain

Replacing the membrane-spanning domain of either CD4 or CD8 with that of TGN38 could have activated or corrupted a localization signal in the cytoplasmic domain of the hybrid proteins. CD8 has a tyrosine residue in the cytoplasmic domain but it is the penultimate amino acid that would not normally be expected to operate as a retrieval signal (Fig. 1 *A*). CD4 contains a signal for endocytosis (Shin et al., 1990, 1991) but this does not normally direct the protein to the TGN. Nevertheless, to eliminate the possibility of activating

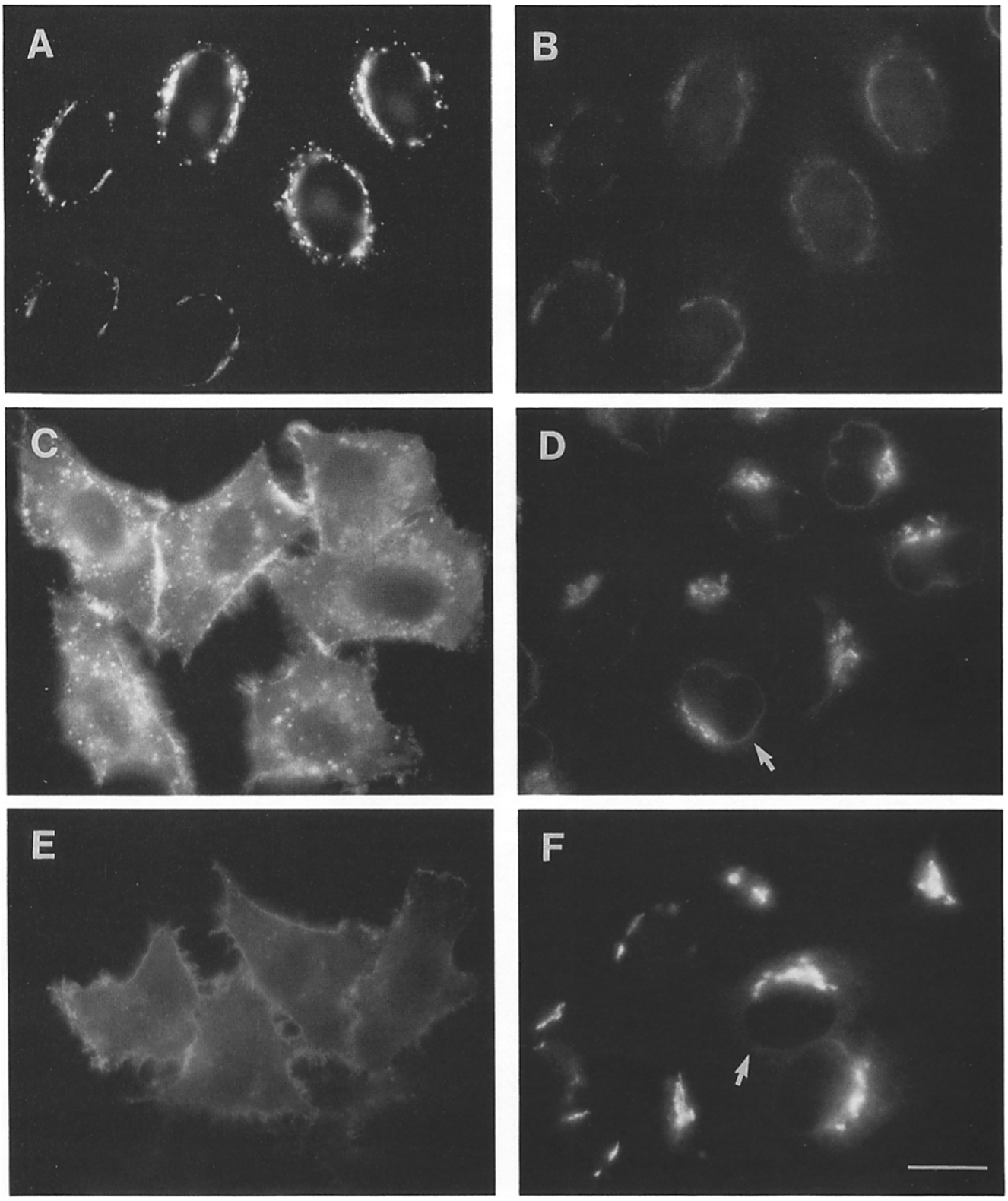

Figure 2. Immunofluorescence microscopy on transfected HeLa cells expressing either TGN38 (*A* and *B*), CD8 (*C*), CD8-MC (*D*), CD4 (*E*), or CD4-MC (*F*). Cells were fixed, permeabilized, and either double-labeled with polyclonal antibodies to TGN38 (*A*) and a monoclonal antibody GT-2 to GalT (*B*), or stained with the monoclonal antibody OKT8 to CD8 (*C* and *D*), or the monoclonal antibody QS4120 to CD4 (*E* and *F*). Arrows in *D* and *F* indicate staining of the nuclear envelope. Bound antibody was visualized using secondary antibody coupled to FITC (*A*) or Texas red (*B–F*). Magnification 800×.

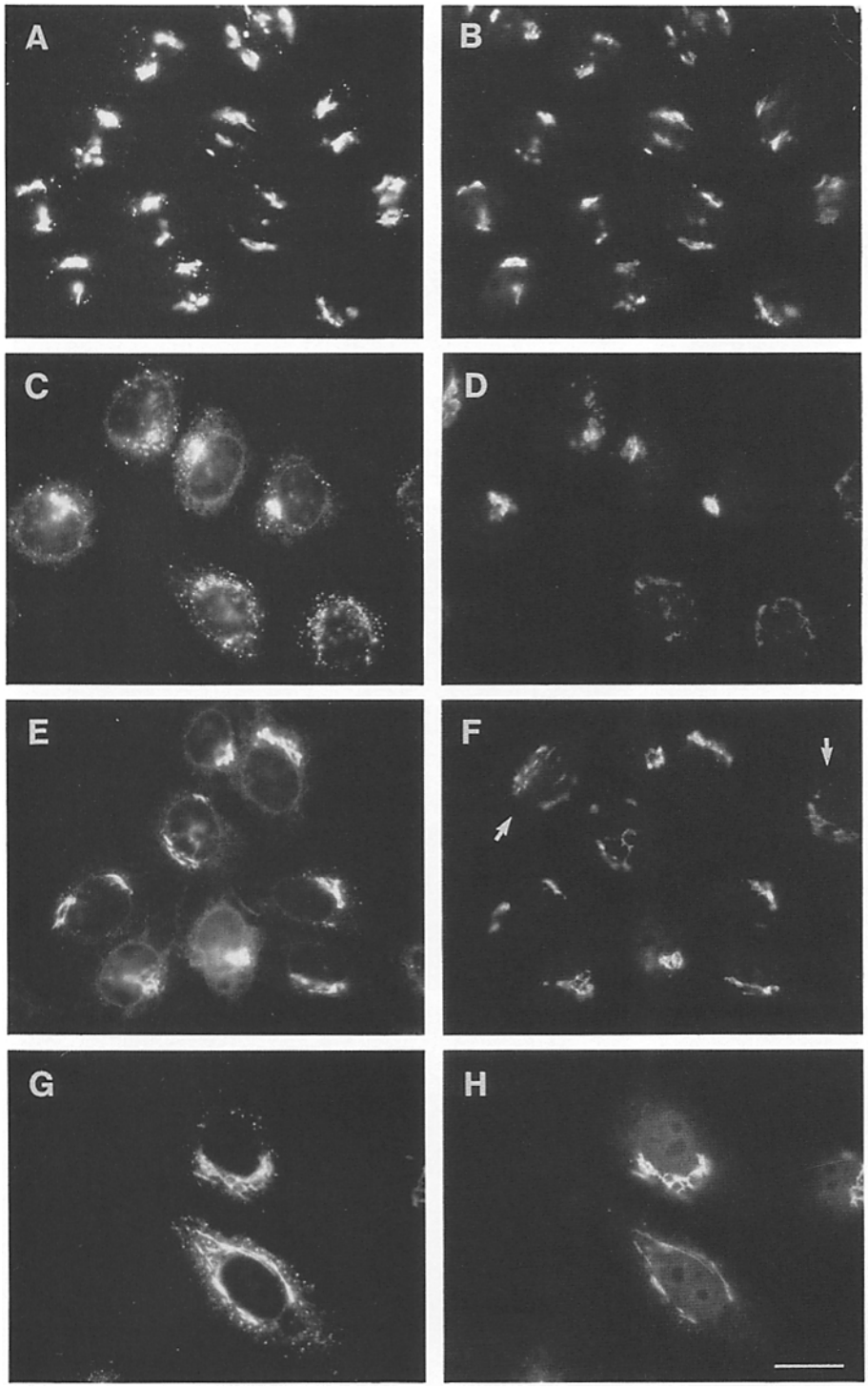

Figure 3. Immunofluorescence microscopy on transfected HeLa cells expressing either CD8-M (*A* and *B*), CD8-C (*C* and *D*), CD4-M (*E* and *F*), or CD4-C (*G* and *H*). Cells were fixed, permeabilized, and double-labeled with either OKT8 to CD8 (*A* and *C*) or QS4120 to CD4 (*E* and *G*) and polyclonal antibodies to GalT (*B*, *D*, *F*, and *H*). Bound antibody was visualized using secondary antibody coupled to Texas red (*A*, *C*, *E*, and *G*) or FITC (*B*, *D*, *F*, and *H*). Arrows in *F* show staining for GalT in nontransfected cells. Magnification 800×.

or corrupting a cytoplasmic signal, we exploited the fact that the localization signal in the cytoplasmic domain of TGN38 has been mapped to the sequence, SDYQRL (Bos et al., 1993; Humphrey et al., 1993; Wong and Hong, 1993). Comparison of the hybrid protein CD8-MC (Fig. 4 *A*) with CD8-MΔC (Fig. 4 *B*), which lacked this sequence, showed that it had no significant effect on the localization of the protein to the Golgi apparatus. Both were localized to a juxtanuclear reticulum very similar to that stained by antibodies to GalT (data not shown). Expression of the CD4-MΔC hybrid protein gave similar results (Fig. 4 *E*).

To show that we had in fact eliminated the localization signal in the cytoplasmic domain of TGN38, we expressed a CD8 or CD4 hybrid protein containing the truncated cytoplasmic domain from TGN38 (CD8-ΔC or CD4-ΔC; Fig. 1 *B*). Unlike CD8-C (see Fig. 3 *C*), the CD8-ΔC hybrid protein was not in the Golgi apparatus but exhibited a cell surface staining pattern and additional accumulation in intracel-

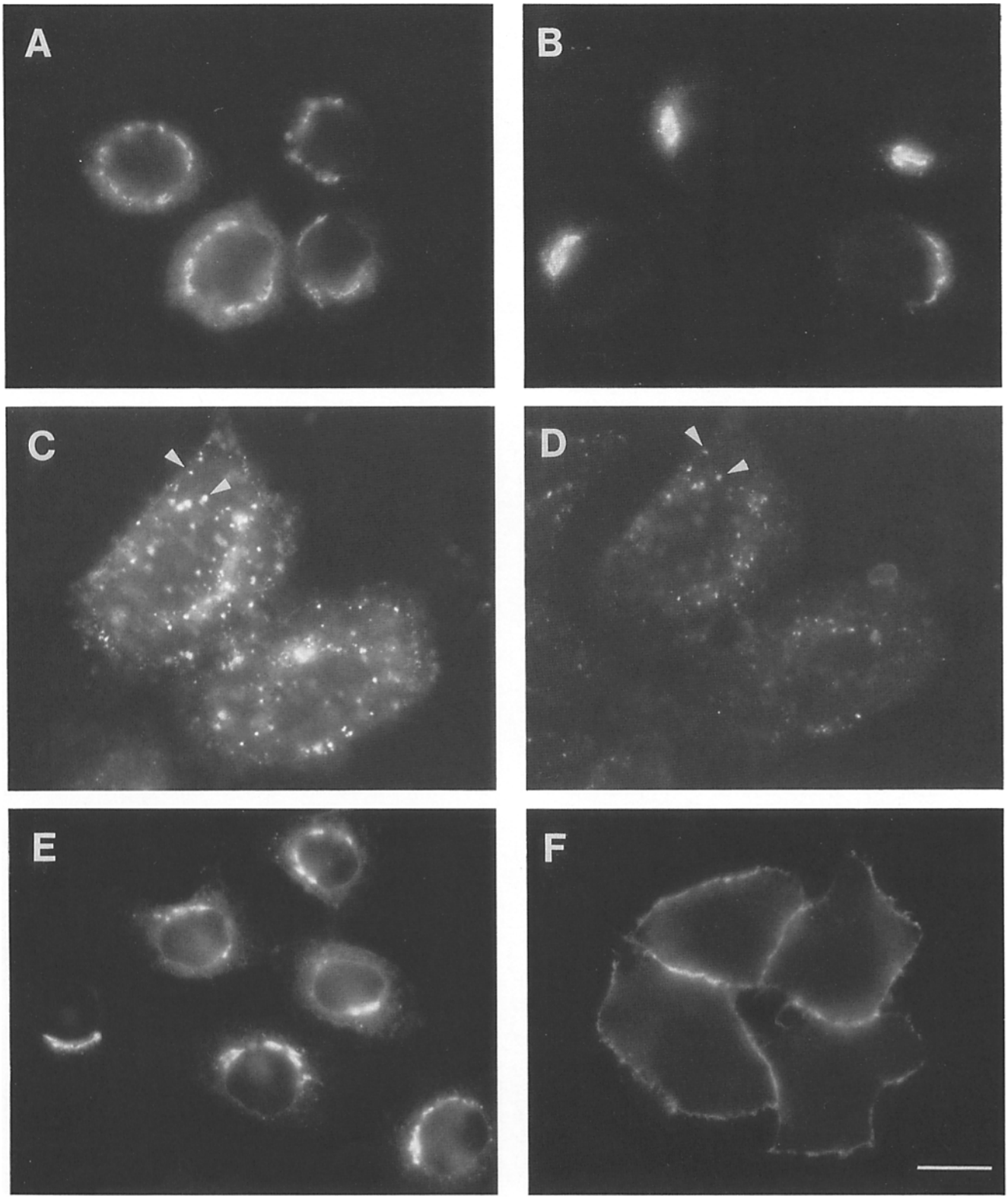

Figure 4. Immunofluorescence microscopy on transfected HeLa cells expressing either CD8-MC (*A*), CD8-MΔC (*B*), CD8-ΔC (*C* and *D*), CD4-MΔC (*E*), or CD4-ΔC (*F*). Cells were fixed, permeabilized, and stained with OKT8 to CD8 (*A–C*) or QS4120 to CD4 (*E* and *F*). Bound antibody was visualized using secondary antibody coupled to Texas red (*A*, *B*, *E*, and *F*) or FITC (*C*). *D* shows the staining pattern for rhodamine-labeled transferrin after incubation with cells for 30 min. Arrowheads in *C* and *D* indicate structures containing both CD8-ΔC and transferrin. Magnification 800×.

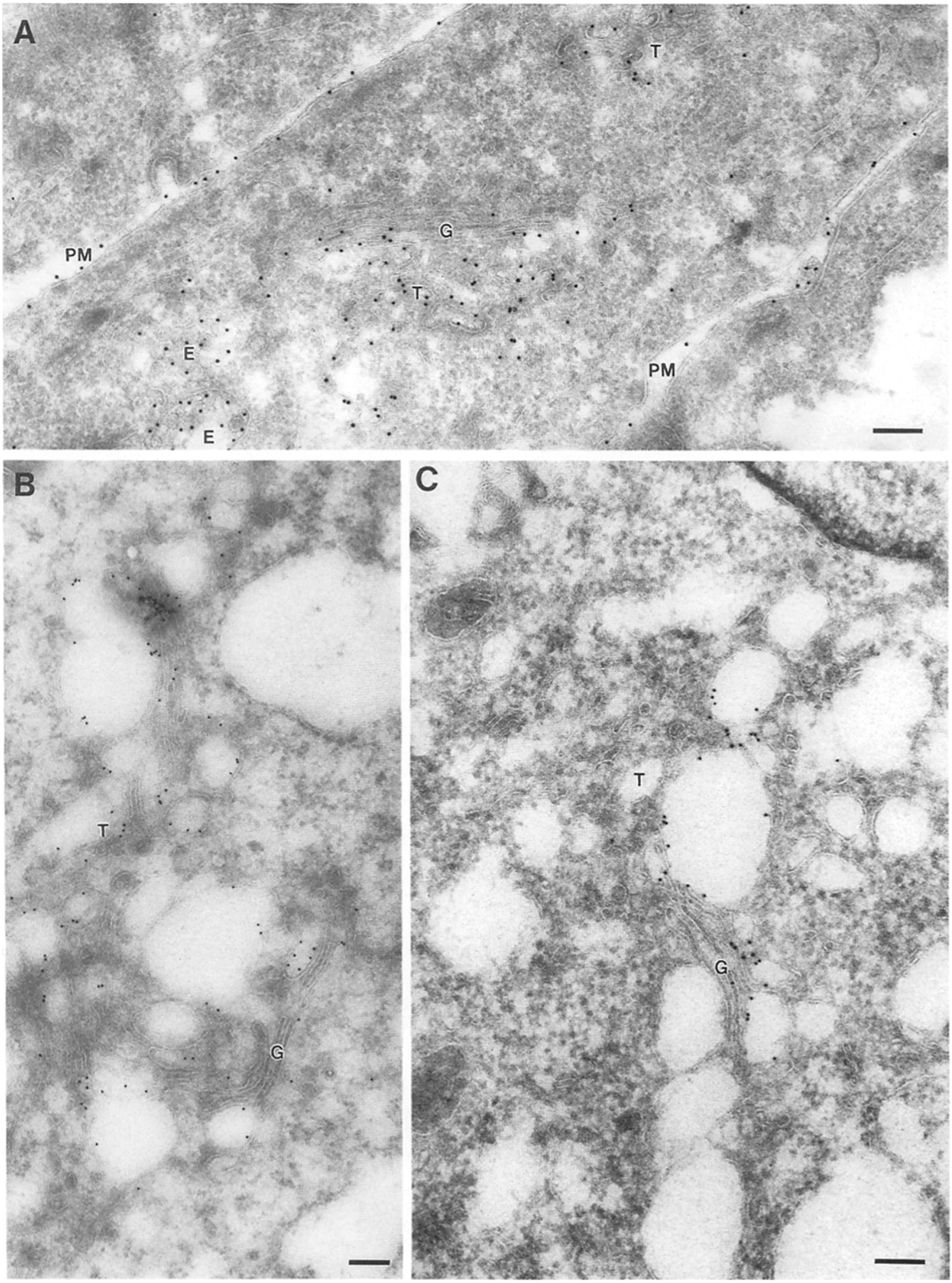

Figure 5. Immunolabeling for TGN38, CD8, and CD4 hybrid proteins in transfected cells. Cryosections of HeLa cells expressing either TGN38 (*A*), CD8-MC (*B*), CD8-M (*C*), CD4-MC (*D*), CD4-C (*E*), or CD4-M (*F*) were immunolabeled with either polyclonal antibodies against TGN38 (*A*); monoclonal antibody 14 to CD8 (*B* and *C*); or polyclonal antibodies to CD4 (*D–F*). Polyclonal antibodies were detected using protein A-conjugated to 10-nm gold (*A*, *D–F*) or secondary antibodies (*B* and *C*) conjugated to 10-nm gold particles. The TGN (*T*) was labeled in all cells and the Golgi stack (*G*) to a much lesser extent. Cells expressing CD4-M (*F*) and CD4-C (*E*) were pretreated for 2 h with 10 μg/ml cycloheximide. Note that higher levels of expression of TGN38 (*A*) and CD4-M (*F*) led to labeling of endosomes (*E*) and the plasma membrane (*PM*). Bar, 0.2 μm.

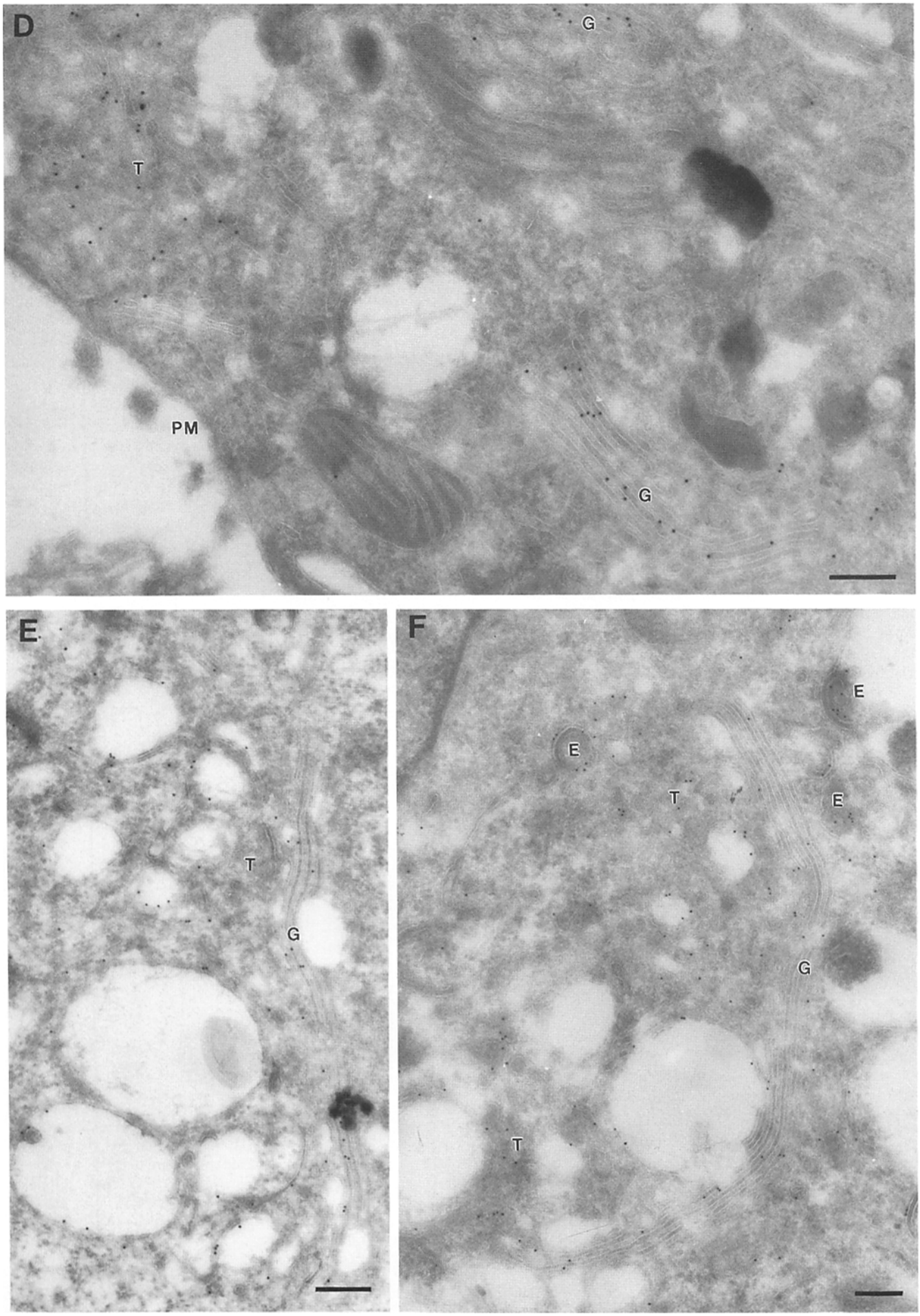

Figure 5.

Table I. Immunolabeling of TGN38, CD4-M, and CD4-C Hybrid Proteins in the TGN, Endosomes, and Plasma Membrane

	Gold particles/ cell section*	Relative distribution over membranes (%)			Linear density (gold particles/μm)‡	
		Golgi apparatus	Endosome	Plasma membrane	TGN	Plasma membrane
TGN38	<60	100	0	0	2.57 ± 1.0	0
TGN38	>60	60 ± 7	29 ± 7	11 ± 7	3.44 ± 0.8	0.81 ± 0.5
CD4-M	<50	100	0	0	0.74 ± 0.4	0
CD4-M	>50	61 ± 8	35 ± 8	4 ± 3	1.78 ± 0.6	0.15 ± 0.12
CD4-C	<55	55.4 ± 17	35 ± 17	9.6 ± 6	0.73 ± 0.2	0.08 ± 0.05
CD4-C	>55	39.3 ± 15	59.5 ± 15	1.2 ± 0.6	1.92 ± 0.8	0.08 ± 0.05

* The two categories of transfected cells, low "expressers" (<) and high "expressers" (>), were defined as described in Materials and Methods. The total number of gold particles counted was 1695 (TGN38), 1036 (CD4-M), and 1302 (CD4-C).
‡ The linear density at the plasma membrane and in the TGN was estimated for the expressed proteins as described in Materials and Methods. The number of gold particles counted as the plasma membrane was 300 (TGN38), 70 (CD4-M), and 60 (CD4-C). Note that for TGN38 and CD4-M, gold labeling at the plasma membrane correlated with labeling of endosomes.

lular punctate structures (Fig. 4 *C*). Some of these were shown to be early endosomes (*arrowheads* in Fig. 4, *C* and *D*) by preincubating the cells with rhodamine-conjugated transferrin for 30 min before fixation and processing. CD4-ΔC was found to be expressed largely on the cell surface of transfected cells (Fig. 4 *F*).

Immunoelectron Microscopy of CD8 and CD4 Hybrid Proteins

To determine the precise location of the hybrid proteins, cryosections of transfected cells were labeled with antibodies which were detected by appropriate gold conjugates. The TGN is defined morphologically as a tubulo-reticular network, part of which is closely apposed to the *trans* side of the Golgi stack. Budding and vesicle profiles coated by clathrin are often observed (Orci et al., 1984, 1985; Griffiths and Simons, 1986). Transiently expressed TGN38 was found in the TGN, the Golgi stack, endosomes, and the plasma membrane (Fig. 5 *A*). The distribution of TGN38 depended on the level of expression. At low levels (<60 gold particles/cell section) TGN38 was found exclusively in the Golgi apparatus (Table I) with most of the protein (85 ± 7%) being found in the TGN (Fig. 6). None was detected in either endosomes or on the plasma membrane (Table I). At higher levels of expression (>60 gold particles/cell section) the percentage of labeling over the Golgi apparatus fell (to 60 ± 7%) and rose over endosomes (to 29 ± 7%) and the plasma membrane (to 11 ± 7%) (Table I). Even at high levels of expression the linear density of labeling over the TGN was four times that over the plasma membrane showing that the protein was still concentrated in the TGN.

Hybrid proteins containing both localization signals gave patterns of labeling almost indistinguishable from TGN38 both morphologically and quantitatively. Both CD8-MC (Fig. 5 *B*) and CD4-MC (Fig. 5 *D*) were present only in the Golgi apparatus at all levels of expression and more than 80% of the label was present in the TGN (Fig. 6).

Hybrid proteins containing only the membrane-spanning domain also gave results very similar to those for TGN38. CD4-M was present only in the Golgi apparatus at low levels of expression (<50 gold particles/cell section; Table I) with 80 ± 8% being present in the TGN (Fig. 6). At higher levels of expression (>50 gold particles/cell section) the percentage of total labeling over the Golgi apparatus fell (to 61 ± 8%) and rose over endosomes (to 35 ± 8%) and plasma membrane (to 4 ± 3%) (Table I). This distribution was not affected by pretreatment of the cells for 2 h with cycloheximide (Fig. 5 *F*). The linear density of labeling over the TGN was 11 times that over the plasma membrane, even higher than that for TGN38 (Table I). The other hybrid protein, CD8-M, was also present exclusively in the Golgi apparatus (Fig. 5 *C*) and most (66 ± 8%) was present in the TGN (Fig. 6). However, more of the protein was present in the Golgi stack than for other hybrid proteins. We also examined cells expressing CD4-M after incubation in cycloheximide for 7 h and found no significant change in the pattern of labeling (data not shown).

The CD4 hybrid protein containing the cytoplasmic domain (CD4-C) was also localized using immunoelectron microscopy. CD8-C could not be detected by immuno-electron microscopy for reasons that are still unclear. More than 84% of the CD4-C in the Golgi apparatus was present in the TGN (Fig. 6) even after 2 h of treatment with cycloheximide (Fig.

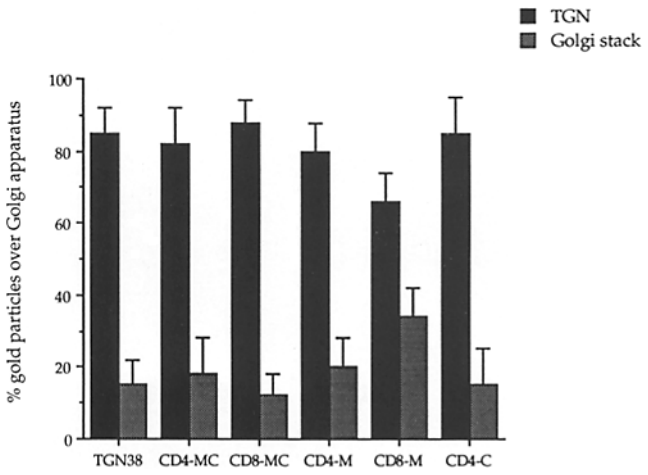

Figure 6. Distribution of TGN38 and hybrid proteins between the TGN and Golgi stack at low levels of expression (see Materials and Methods) in transiently transfected cells. The number of gold particles counted in each experiment were 237 (*TGN38*), 518 (*CD4-MC*), 520 (*CD8-MC*), 150 (*CD4-M*), 250 (*CD8-M*), and 206 (*CD4-C*). The distribution shown was very similar to that observed at higher levels of expression.

5 *E*). However, CD4-C differed from other hybrid proteins and TGN38 in three respects. First, more than 35% of the total protein was present in endosomal structures at low levels of expression and this increased to nearly 60% at high levels. Second, the type of endosomal structure labeled also changed. At low levels, all of the endosomal structures were multivesicular bodies which are late endosomal compartments (Geuze et al., 1988; Hopkins et al., 1990). At high levels of expression, labeling was also present in tubular endosomes. Because of the difficulties of distinguishing between tubular endosomes and the TGN (Geuze et al., 1988; Klumperman et al., 1993), definitive identification of these structures will have to await further analysis using stably transfected cell lines. Third and last, the plasma membrane was labeled at both high and low levels of expression (Table I). The linear density was the same (0.08 ± 0.05 gold particles/μm) suggesting that CD4-C was as efficiently removed from the cell surface and delivered to endosomes. The protein was still concentrated in the TGN since the linear density was 9–24 times higher than that in the plasma membrane (Table I).

As a final control, CD4 was also expressed transiently in HeLa cells. All of the protein was found in endosomes and on the plasma membrane after treatment with cycloheximide and in agreement with studies carried out by Pelchen-Matthews et al. (1991). Little if any was found in the TGN (data not shown). This shows that accumulation of the hybrid proteins in the TGN was not a consequence of the CD4 ectodomain.

Cell Surface Analysis of CD8 Hybrid Proteins

FACS analysis was used to determine the amount of CD8 and CD8 hybrid proteins on the cell surface. Fixed, nonpermeabilized cells transfected with the appropriate cDNA were stained with the OKT8 antibody to CD8 and visualized using a secondary antibody conjugated to FITC (Fig. 7). Mock-transfected cells were used as the negative control and the gate was set such that 1% of these cells scored positive.

The profile of CD8 expression was characteristic of that in a population of transiently transfected cells (Nilsson et al., 1989; Jackson et al., 1990) and 58% scored positive (Fig. 7 *A*, Table II). When both localization signals were present (CD8-MC) only 6% of the cells showed surface staining (Fig. 7 *B*, Table II) and this rose to 19% when the cytoplasmic signal was deleted (CD8-MΔC) (Fig. 7 *D*, Table II) suggesting that the membrane-spanning signal alone could retain the hybrid protein. This was confirmed by expression of CD8-M. Only 13% of the cells scored positive (Fig. 7 *C*, Table II).

The cytoplasmic signal alone could also retain the hybrid protein. Only 11% of cells expressing CD8-C scored positive

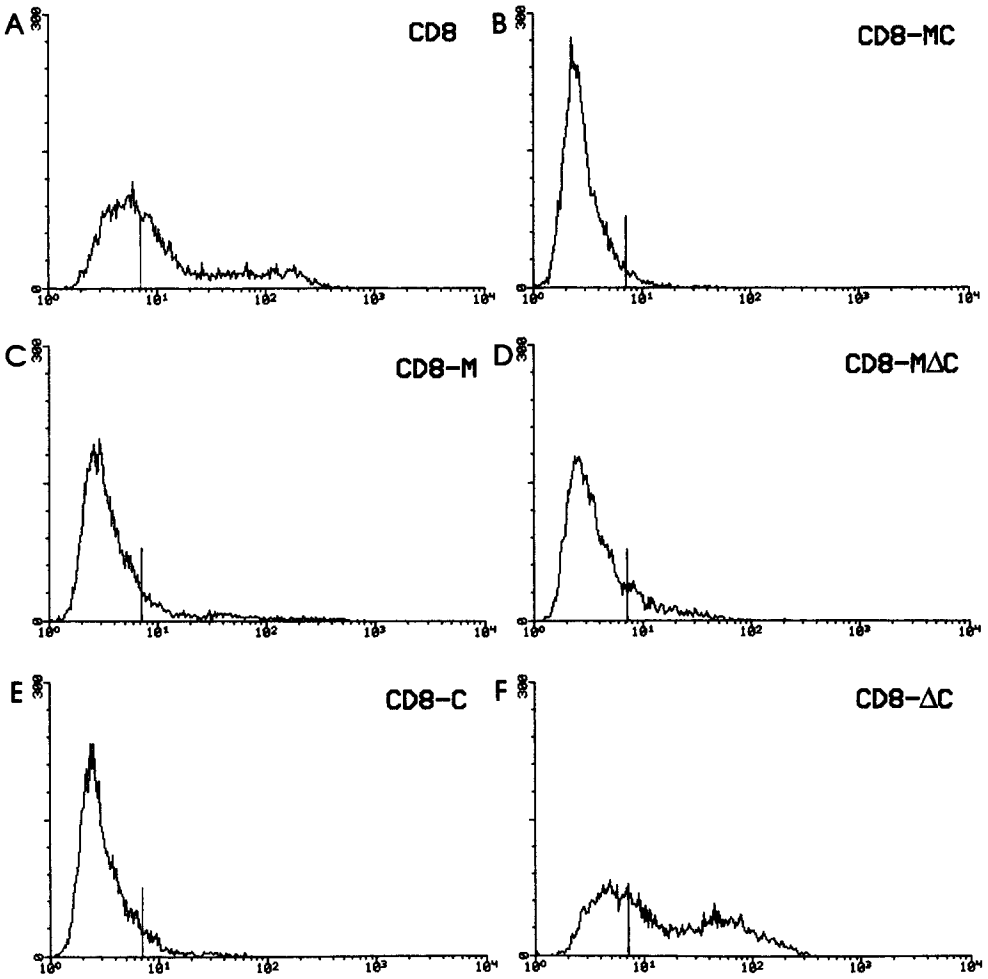

Figure 7. Surface expression of CD8 and CD8 hybrid proteins analyzed using FACS analysis. HeLa cells were fixed and stained with monoclonal antibody to CD8 and visualized using a secondary antibody conjugated to FITC. The gate on the x-axis (fluorescence units) was set using mock-transfected cells as a negative control. Cells scoring to the right of this point were regarded as positive for cell surface fluorescence (see Table II).

Table II. Quantitation of FACS Analysis on Cells Expressing CD8 and CD8 Hybrid Proteins

Expressed protein	% Positive cells*	% Relative intensity‡
CD8	58	100
CD8-MC	6	2
CD8-MΔC	19	11
CD8-M	13	22
CD8-C	11	5
CD8-ΔC	68	123

* The gate was set using mock-transfected cells and 1% were positive. 5,000 cells were analyzed in each experiment.
‡ The total fluorescence intensity was calculated by integrating the area under the positive cells and expressing the results as a percentage of the value for CD8.

(Fig. 7 *E*, Table II). Removal of the tyrosine-based motif in this cytoplasmic signal destroyed its ability to retain hybrid proteins. More than 67% of cells expressing CD8-ΔC scored positive (Fig. 7 *F*, Table II), slightly higher than the number expressing CD8 alone (58%, Fig. 7 *A*, Table II).

The total fluorescence of positive cells for each protein was also calculated by integration of the results presented in Fig. 7. These calculations, presented in Table II, are in close agreement with the percentage of cells scoring positive and provide further evidence that both the membrane-spanning and cytoplasmic signals can, independently, retain hybrid proteins inside the cell.

These data were also supported by immunofluorescence microscopy on non-permeabilized cells (data not shown). There was no surface staining of cells expressing CD8-MC or CD8-C. A few of the transfected cells expressing CD8-M showed low levels of cell surface staining. Cells expressing CD8-ΔC showed high levels of cell surface staining, in agreement with the FACS analysis.

Metabolic Labeling of Hybrid Proteins

CD4 contains two N-linked glycosylation sites (Maddon et al., 1985) one of which is completely processed to the complex bi-antennary sialylated structure. Terminal sialic acid residues are added by α2,6-sialyltransferase which is located in the *trans*-Golgi cisterna and the TGN (Roth et al., 1985). Neuraminidase cleaves off these sialic acid residues producing a small mobility shift on SDS-PAGE (Nilsson et al., 1989; Jackson et al., 1990) that can be used to show that proteins have reached this part of the Golgi apparatus.

Cells expressing CD4 or CD4 hybrid proteins were labeled with [^{35}S]methionine/cysteine for 20 min (see Materials and Methods) and either lysed immediately or chased with excess cold methionine and cysteine for 2 h. Immunoprecipitates were treated with neuraminidase or mock-treated and analyzed by SDS-PAGE (Fig. 8). Newly synthesized CD4 was insensitive to neuraminidase (cf. lanes *1* and *2*) but completely sensitive after a 2-h chase (cf. lanes *3* and *4*). Hybrid CD4 proteins containing either the membrane-spanning (CD4-M) or cytoplasmic signal (CD4-C) were also completely sialylated after a 2-h chase period (lanes *9–16*).

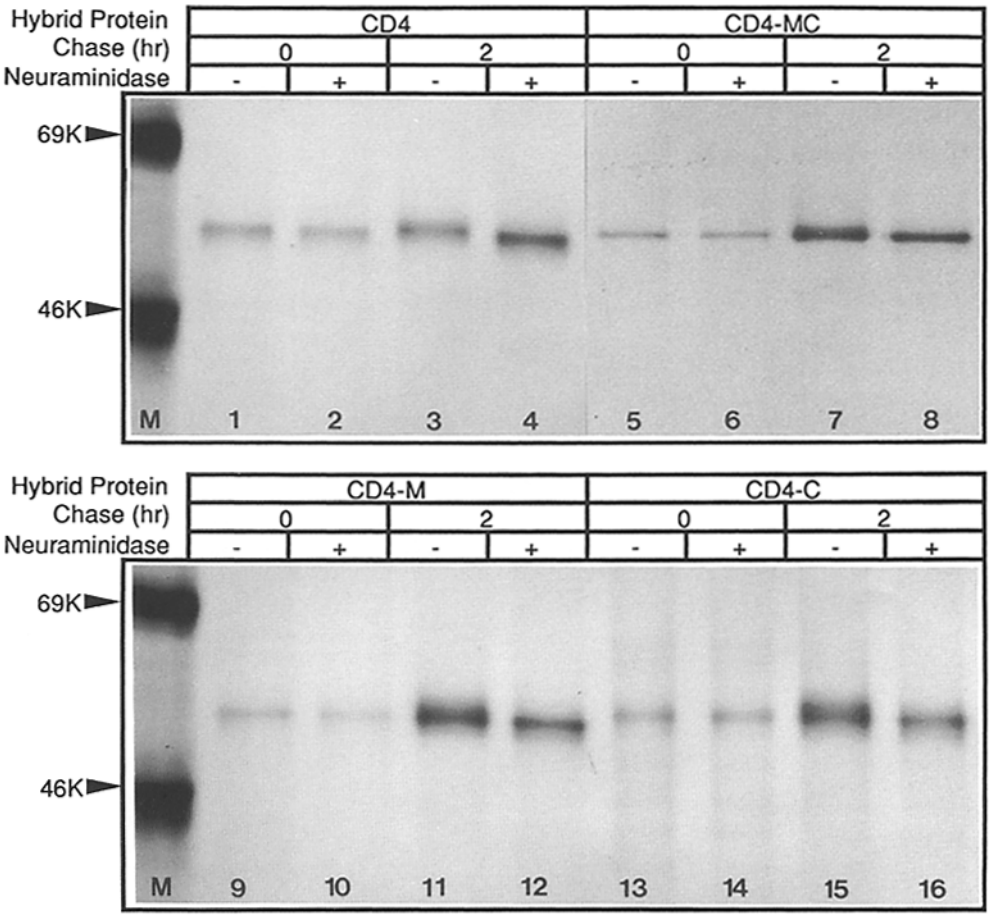

Figure 8. Metabolic labeling of CD4 and CD4 hybrid proteins. Transfected HeLa cells were labeled with [^{35}S]methionine/cysteine for 20 min and chased for 0 or 2 h. Immunoprecipitates were mock-digested (lanes *1, 3, 5, 7, 9, 11, 13,* and *15*) or digested with neuraminidase (lanes *2, 4, 6, 8, 10, 12, 14,* and *16*) and analyzed by SDS-PAGE and fluorography. The molecular size of ^{14}C-radioactive markers are indicated on the left hand side of the panels.

However, CD4-MC, which contains both signals, was incompletely sialylated after the 2-h chase (lanes *7* and *8*) suggesting that transport to the *trans* part of the Golgi apparatus was slower. The reason for this is unclear but was not the consequence of using CD4 as the reporter molecule. CD8-MC was also incompletely sialylated (data not shown) in contrast to hybrid CD8 proteins containing either TGN38 signal (Fig. 9).

Metabolic labeling of CD8 hybrid proteins could not be used to study arrival in the *trans* cisterna and the TGN because the bound oligosaccharides are all O-linked (Littman et al., 1985). Sialylation of O-linked oligosaccharides is thought to occur in the *medial*-Golgi cisterna (Locker et al., 1992). However, pulse-chase analysis combined with neuraminidase digestion showed that CD8-M and CD8-C hybrid proteins had kinetic properties similar to those for native CD8 (Fig. 9). In all cases the half-time for acquisition of terminal sialic acid residues was between 25–30 min. Taken further with the results for the CD4 hybrid proteins, these results show that the presence of either signal had little, if any, effect on synthesis, transport, and processing of the reporter molecules.

Discussion

TGN38 was originally identified as a resident of the TGN (Luzio et al., 1990) but more recent work has shown that it cycles between the TGN and the cell surface (Jones et al., 1993; Reaves et al., 1993). Movement to the cell surface occurs via exocytic vesicles (Jones et al., 1993) and is followed by retrieval which requires a signal present in the cytoplasmic domain that has been localized to the sequence SDYQRL (Bos et al., 1993; Humphrey et al., 1993; Wong and Hong, 1993). Retrieved TGN38 first moves to the endosomes and then to the TGN (Bos et al., 1993; Humphrey et al., 1993). Using plasma membrane glycoproteins as reporter molecules, we have now shown that there is an additional signal for localizing the protein to the TGN. This signal is located in the membrane-spanning domain.

Four independent lines of evidence support this conclusion. First, immunofluorescence microscopy showed that CD4-M and CD8-M hybrid proteins localized to a juxtanuclear reticulum that could also be stained by antibodies to GalT, a marker for the *trans* cisterna and TGN (Roth and Berger, 1982; Taatjes et al., 1992; Nilsson et al., 1993*a*). This staining pattern was not affected by pretreatment with 10 μg/ml cycloheximide for up to 7 h (data not shown) showing that these hybrid proteins were not simply moving slowly along the exocytic pathway. In addition, it is unlikely that a cryptic localization signal in the cytoplasmic domain of either CD4 or CD8 was activated as a result of generating hybrid proteins. Both CD8-MΔC and CD4-MΔC hybrid proteins which lack most of the cytoplasmic domain (including the cytoplasmic signal for TGN38) were also localized to the Golgi apparatus.

Second, quantitative immunoelectron microscopy showed that CD4-M had almost the same distribution as TGN38 at both high and low levels of expression. At low levels of expression, all of the CD4-M hybrid protein and TGN38 was present in the Golgi apparatus, 80% being present within the TGN. At higher levels of expression, CD4-M and TGN38 were also present in endosomes and on the cell surface, again to a similar extent. Even at these higher levels of expression, both proteins were still concentrated in the TGN as compared to the plasma membrane. For TGN38, the linear density in the TGN was four times that in the plasma membrane; for CD4-M it was even higher, being 12 times higher in the TGN than in the plasma membrane. The distribution of CD8-M was also similar to that for TGN38 being exclusively in the Golgi apparatus at low levels of expression. There was, however, more of this hybrid protein in the Golgi stack for reasons that are still unclear.

Third, FACS analysis showed that the membrane-spanning domain reduced the percentage of positive cells from 58%

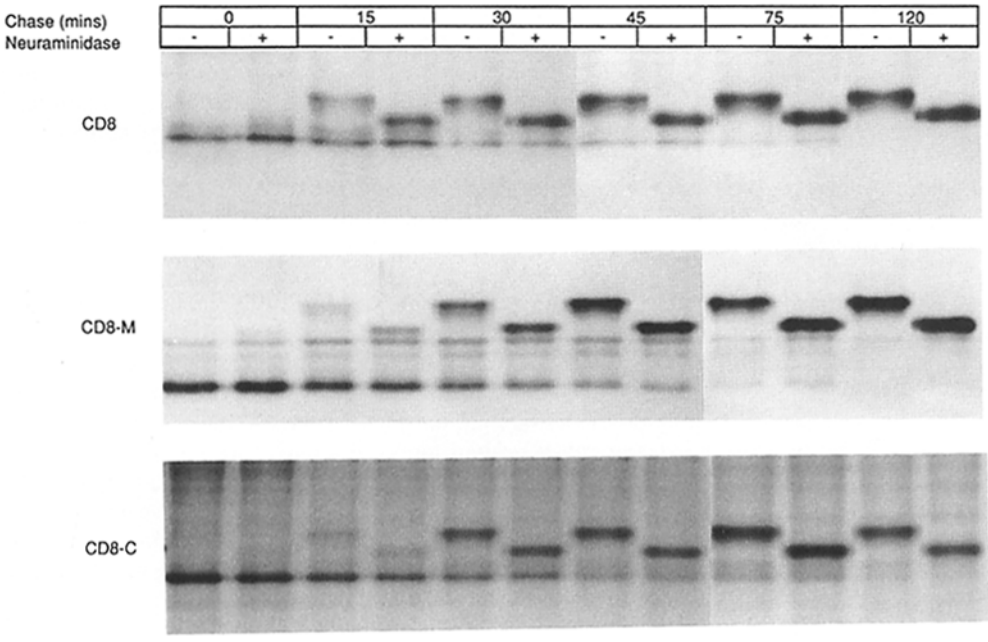

Figure 9. Metabolic labeling of CD8 and CD8 hybrid proteins. Transfected HeLa cells were labeled with [^{35}S]methionine/cysteine for 20 min and chased for 0, 15, 30, 45, 75, or 120 min. Immunoprecipitates were mock-digested (−) or digested (+) with neuraminidase and analyzed by SDS-PAGE and fluorography.

(for CD8) to 13% (for CD8-M), a 4.5-fold decrease. This effect was not the consequence of activating a cryptic signal in the cytoplasmic domain of CD8 since removal of most of this domain (including the TGN38 cytoplasmic signal) still reduced the percentage of positive cells to 19% (for CD8-MΔC), a threefold decrease.

Fourth and finally, metabolic labeling studies showed that all molecules of CD4-M received sialic acid, showing that they had all reached the *trans* cisterna/TGN.

The present studies are at variance with previous studies that identified only the cytoplasmic domain of TGN38 as a localization signal. Removal of most of the domain led to the appearance of the truncated protein on the cell surface (Luzio et al., 1990; Bos et al., 1993). It should be noted that the earlier study was carried out before any evidence existed for retention by membrane-spanning domains, a phenomenon first described for a viral protein (Swift and Machamer, 1991) and Golgi enzymes (Nilsson et al., 1991; Munro, 1991). The expectation was that the signal would be found in the cytoplasmic domain and this appeared to be borne out by the appearance of protein on the cell surface. The likely reason for this, however, was that the levels of expressed protein were so high that they saturated the retention mechanism that operates on the membrane-spanning domain. Even cells expressing the wild-type protein (TGN38), containing both retention and retrieval signals, showed aberrant localization. After 24 h transfection, 50% of the cells had either fallen off the coverslip or showed cell surface staining (Luzio et al., 1990). The more recent study by Bos et al. (1993) overcame this problem by using a similar expression vector (pSVL1) at a low transfection efficiency (~0.15%) to ensure more constant levels of expression in each cell. Unfortunately, their only assay was immunofluorescence microscopy and it was impossible to determine how much of the protein was still present in the Golgi apparatus. These problems did not arise in our system since the expressed levels of hybrid protein were lower and the distributions were quantitated in several independent ways. Two different reporter molecules were also used. However, it is conceivable that other small differences in the systems used are responsible for the apparent discrepancies. Wong and Hong (1993), for example, found that the serine residue in the SDYQRL motif was important for Golgi localization, while Humphrey et al. (1993) found that an identical mutation in their system had no effect. Further studies using stable cell lines will be needed to resolve these differences.

Though the cytoplasmic and membrane-spanning signals can act independently to localize hybrid proteins to the TGN, it is likely that they operate in different ways. The cytoplasmic domain is a clear example of a retrieval signal. This acts after a protein has left the compartment in which it normally functions and returns them from a later compartment. All of the retrieval signals so far characterized have been located in the cytoplasmic domains of proteins and many are based on tyrosine as the critical amino acid set in a particular motif (for review see Collawn et al., 1990; Pearse and Robinson, 1990; Kornfeld, 1992; Matter et al., 1992). The critical motif in the cytoplasmic domain of TGN38 is SDYQRL (Bos et al., 1993; Humphrey et al., 1993; Wong and Hong, 1993) and it operates once the protein reaches the cell surface. TGN38 is then returned to the TGN via endosomes.

The membrane-spanning domain is a typical Golgi retention signal. These act to keep proteins in the Golgi and, so far, all Golgi proteins (Machamer and Swift, 1991) and enzymes (Nilsson et al., 1991; Munro, 1991; Aoki et al., 1992; Burke et al., 1992; Colley et al., 1992; Tang et al., 1992; Teasdale et al., 1992; Wong et al., 1992) have been found to be retained by the membrane-spanning domain. The mechanism of retention is still unclear though two possibilities have been proposed. TGN38 molecules could interact with their neighbors through the spanning domains forming kin oligomers so large that they can no longer enter vesicles budding from the TGN (Nilsson et al., 1994). Alternatively, the length of the membrane-spanning domain might prevent forward movement of the protein past the TGN (Bretscher and Munro, 1993). The latter seems less likely since the length of the membrane-spanning domain (19 amino acids) is longer than the estimated average for Golgi proteins (15 amino acids) but further work will be needed to determine whether either of these mechanisms, or a completely different one, are responsible for retention in the TGN.

Our results suggest that the membrane-spanning domain is an effective retention signal since alone it can confer localization properties on CD4 that make it virtually indistinguishable from TGN38. The predicted effect of its absence would be an increased flux of protein through the TGN to the cell surface. In other words, its absence should put a greatly increased load on to the retrieval system. As fast as the protein is retrieved and returned to the TGN, it would escape to the plasma membrane and need to be retrieved again. This would explain why CD4-C, which has the retrieval but not the retention signal, is present in both endosomes and at the plasma membrane even at low levels of expression. The rate-limiting step in retrieval is not endocytosis because the linear density of CD4-C at the plasma membrane was the same at both low and high levels of expression. The limiting step must be between endosomes and the TGN since CD4-C accumulates in endosomes as the expression level increases.

The only result that is a little puzzling is the presence of CD4-M in endosomes at high expression levels. This hybrid protein would be expected to leak very slowly to the cell surface, but, in the absence of a retrieval signal, it should accumulate there. One possibility is that the endocytic signal in the CD4 cytoplasmic domain (Shin et al., 1991) triggers movement to endosomes; another possibility is that the protein in the endosome is destined for degradation in lysosomes. This latter possibility is more likely for CD8-M which lacks an endocytic signal yet is found in small amounts in what appear to be endosomes by immunofluorescence microscopy.

It is likely that other proteins on the exocytic pathway have both retention and retrieval signals. The sum of the two processes acting on these signals would determine the amount of protein found in a particular compartment. For resident proteins which should spend as much of their time as possible in a particular compartment one would expect retention rather than retrieval to be the dominant process. For recycling proteins, such as receptors, one would expect

the opposite so that substantial amounts of protein would be moving between two compartments. It is not yet clear whether TGN38 is best considered as a resident or a recycling protein (Luzio and Banting, 1993; Stanley and Howell, 1993). That will have to await discovery of its function.

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