Truncations of the C-terminal cytoplasmic domain of MG160, a medial Golgi sialoglycoprotein, result in its partial transport to the plasma membrane and filopodia

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SUMMARY

MG160, a type I cysteine-rich membrane sialoglycoprotein residing in the medial cisternae of the rat Golgi apparatus, is highly homologous to CFR, a fibroblast growth factor receptor, and ESL-1, an E-selectin ligand located at the cell surface of mouse myeloid cells and recently detected in the Golgi apparatus as well. The mechanism for the transport of MG160 from the Golgi apparatus to the cell surface is unknown.

In this study we found that differential processing of the carboxy-terminal cytoplasmic domain (CD), consisting of amino acids Arg1159 Ile Thr Lys Arg Val Thr Arg Glu Leu Lys Asp Arg171, resulted in the partial transport of the protein to the plasma membrane and filopodia.

In Chinese hamster ovary cells (CHO), stably transfected with the entire cDNA encoding MG160, the protein was localized in the Golgi apparatus. However, when the terminal Arg171 or up to nine distal amino acids were deleted, the protein was distributed to the plasma membrane and filopodia as well as the Golgi apparatus.

This report shows that the CD of an endogenous type I Golgi protein is important for its efficient retention and identifies a unique residue preference in this process. Cleavage within the CD of MG160 may constitute a regulatory mechanism for the partial export of the protein from the Golgi apparatus to the plasma membrane and filopodia.

Key words: Golgi, Sialoglycoprotein, Plasma membrane, Filopodia, MG160 isoform, E-selectin ligand

INTRODUCTION

Three independent studies identified the same protein from different sources: MG160, which is a 160 kDa medial Golgi sialoglycoprotein isolated from rat brain (Gonatas et al., 1989), CFR, a receptor for acidic fibroblast growth factor isolated from chick embryos (Burrus and Olwin, 1989), and ESL-1, a ligand for E-selectin identified on cell surfaces of murine myeloid cells (Steegmaier et al., 1995, 1997). The amino acid sequence of the rat MG160 is 88% to 95% identical to the human CFR and ESL-1 proteins (Burrus et al., 1992; Gonatas et al., 1995; Steegmaier et al., 1995; Mourelatos et al., 1996). The gene of MG160 has been assigned to human chromosome 16q22-q23 (Mourelatos et al., 1995). In chicken embryos, MG160 appears in the Golgi apparatus of the ectoblast and the primitive endoblast prior to the formation of the primitive streak, and in the notochord, neural tube, somites, cartilage and several other tissues of older embryos (Stieber et al., 1995).

The virtual identity of MG160 to CFR and ESL-1 suggests that the MG160 protein has diverse roles depending on its location. Under steady state conditions MG160 was found exclusively in the Golgi apparatus of several adult rat and chicken embryo cell types (Gonatas et al., 1989; Stieber et al., 1995). However, the recent finding that the E-selectin ligand (ESL-1) is present in both the Golgi apparatus and the plasma membrane and microvilli of 32Dc13 cells and mouse neutrophils strongly suggests that, in certain cells, MG160 is a bitopic protein of the Golgi apparatus and the plasma membrane, with distinct functions in each cellular compartment (Steegmaier et al., 1997; Smalheiser, 1996).

The expression of possible plasma membrane 'isoforms' of MG160 may be regulated by transcriptional, translational or posttranslational mechanisms. To understand the mechanisms involved in the transport of MG160 to the plasma membrane, we examined the contribution of the carboxy (C) terminus (CD) of the protein to its retention within the Golgi apparatus. We found that deletions of one or more amino acids from the CD allowed the partial transport of MG160 to the plasma membrane and filopodia.

The minor changes of the CD of MG160 required for its transport to the plasma membrane support the hypothesis that cleavage within the CD of MG160 constitute a physiological regulatory mechanism.


MATERIALS AND METHODS

Materials

Cell culture media and Geneticin (G418) were purchased from Gibco Laboratories (Grand Island, NY) and Chinese Hamster Ovary Cells (CHO) were obtained from the Cell Center of the University of Pennsylvania. Fetal bovine serum and agarose linked goat anti-mouse antibody were from Hyclone (Logan, Utah). Trans 85S-label (1209 Ci/mmol) was from ICN Biomedicals, Inc. (Irvine, CA). Endoglycosidase H (Endo H) was purchased from Boehringer Mannheim (Indianapolis, Ind.), the enzyme neuraminidase (Vibrio cholerae) from Calbiochem (LaJolla, CA), Protein A Sepharose from Pharmacia (Piscataway, NJ) and NHS-LC-biotin and streptavidin agarose from Pierce Chemical Co (Rockford, IL); the vectors pBK-CMV and p Bluescript SK(−) were obtained from Stratagene (LaJolla, CA). Enzymes used in molecular cloning were from New England Biolabs (Beverly, MA), GIBCO BRL (Gaithersburg, MD) and Boehringer Mannheim. Biotinylated antibodies against mouse IgG, rabbit IgG, the avidin-biotin complexes and the VIP substrate were purchased from Vector (Burlingame CA) and goat anti-mouse colloidal gold from EY laboratories (San Mateo, CA). Oligonucleotides were synthesized by the Nucleic Acid Facility of the University of Pennsylvania.

cDNA constructions

The full-length cDNA of rat MG160 was subcloned from the Bluescript SK(−) vector into the Sal-I-NorI sites of the pBKCMV expression vector. This vector contains the CMV early promoter, an intron with 5′ expression vector. This vector contains the CMV early promoter, an intron with 5′- and 3′- splice sites, SV40 polyadenylation sequences and the selectable neomycin-resistance gene. The upstream lac sequences were excised to maximize eukaryotic expression levels.

This construct is referred to as MG160 (wt), and includes 300 bp from the 5′ untranslated region, the 3513 bp nucleotide coding sequence and 1772 bp from the 3′ untranslated region.

The cDNAs of MG160 with the cytoplasmic domain deleted were created by established PCR techniques. The antisense primer represented sequences encoding a truncated cytoplasmic tail of the desired length by introducing a stop codon at the C terminus followed by a NotI site. The pBKCMV-MG160 as a template and the same 5′-oligonucleotide primer 5′-CGCAACGACACTCTGCAGGA-3′, a PCR product with a unique NotI site was generated. This fragment was digested with Pmel1-NotI and ligated into similarly treated pBCKCMV-MG160. These constructs are referred to as Δ, with the number indicating the terminal amino acid (Fig. 10). The construct designated ΔTMCT (Fig. 10), lacking the cytoplasmic and membrane spanning domains and two contiguous amino acids of the luminal region, was prepared using the 3′-primer 5′-TACAGTCTCAGCGGCCGCCTAGGATGTCA TCACTTGC-3′. To substitute Arg1171 for Lys1171 or Ala1171 the penultimate nucleotide changed to GCC in the latter.

All constructs generated by the PCR were verified by restriction digests and DNA sequencing of double-stranded DNA using the T7 primer and synthetic oligonucleotide primers.

Cell culture and transfections

1 day prior to transfection, 1.0×105 CHO cells were seeded into 10 ml of DMEM supplemented with 10% fetal bovine serum and 1% glutamine. All transfections were carried out using a modified calcium phosphate method (Wigler et al., 1977). The cells were incubated for 48 hours at 37°C before they were used, either for preliminary immunocytochemical studies or for selection of stable transfected colonies in medium containing 2 mg Geneticin/ml. Control transfections were carried out as above, using the pBCKCMV vector without insert DNA.

Antibodies

The methods for the preparation of the rat-specific monoclonal antibody against MG160, mAb 10A8 and the polyclonal antiserum that reacts with MG160 from several species including human, have been reported (Louvard et al., 1982; Gonatas et al., 1989; Croul et al., 1990). The preparation of the synthetic peptide corresponding to the CD of MG160, and of a polyclonal antiserum directed against the CD peptide, were done as follows: the peptide RITKRVTRELKD, comprising the entire carboxy-terminal CD of MG160 (amino acids 1159-1171) was synthesized by the Protein Chemistry Facility of the University of Pennsylvania according to standard methods, using an Applied Biosystems 430A synthesizer described previously (Gonatas et al., 1995). The amino acid sequence of the purified peptide was confirmed by Edman degradation. The isoelectric point of the peptide was estimated using the PCRGene computer program. The peptide was coupled to keyhole limpet hemocyanin using glutaraldehyde and dimethylformamide according to standard procedures (Avrameas and Ternynck, 1971; Green et al., 1982). Antisera were raised by immunizing two female New Zealand rabbits according to previously described protocols (Louvard et al., 1982).

The IgG fraction from mAb 10A8 ascitis was isolated using an AffinityPak™ column of immobilized protein A (Pierce). Monovalent Fab fragments were prepared by papain digestion of the IgG fraction using the ImmunoPure Fab kit 44885 from Pierce, according to the instructions of the vendor.

Purification of anti-CT antibodies using immunoaffinity chromatography with the synthetic peptide

The 13-amino-acid polypeptide corresponding to the C-terminal cytoplasmatic domain of MG160 with an additional amino-terminal cysteine was synthesized by the Protein Chemistry Facility of the University of Pennsylvania. The peptide (5.2 mg) was linked to immobilized iodoacetyl on 1 ml of a crosslinked agarose support (Sulfolink, Pierce) following the instructions of the manufacturer. More than 90% of the peptide was linked to the immobilized matrix, as determined by Ellman’s reagent. 5 ml of an antiserum against CT (the carboxy-terminal cytoplasmatic domain of MG160), raised in a rabbit, was applied to the immunoaffinity column of the immobilized polypeptide. After several washings of the column with PBS, specific antibody was eluted with 0.1 M glycine HCl, pH 2.5, neutralized with 1 M K2HPO4 and concentrated with a 50,000 MW cutoff Centricon filter. By Western blotting of crude rat brain microsomes, the purified anti-CT antibody reacted with a single 160 kDa band corresponding to MG160. By immunocytochemistry with sections of rat brain cerebellum, the antibody stained the Golgi apparatus of Purkinje cells and neurons of the granule cell layer. The immunoaffinity-purified antibody stained the Golgi apparatus strongly and uniformly in subcones of CHO cells stably transfected with Δ1165 at concentrations of 0.5, 1, 2 and 5 μg/ml of antibody. The Golgi apparatus of subcones of CHO cells stably transfected with the entire cDNA of MG160 was stained uniformly and equally well by antisera raised either against the entire protein or its cytoplasmic domain.

Metabolic labelling of cultured cells and immunoprecipitation

Subconfluent monolayers of CHO cells or transfected CHO cells in 35-mm dishes were washed three times with PBS and incubated in methionine/cysteine-free DMEM supplemented with 1% glutamine for 30 minutes at 37°C. Cells were then radiolabelled with 150 μCi/ml of Trans S-label in fresh depleted medium for the indicated time periods. After labelling, the cells were washed five times with PBS and extracted in 500 μl of lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.2% gelatin, 2 mM EDTA, 1 mM PMFS, 2 μg/ml each of aprotinin, leupeptin and pepstatin). The cell extracts were centrifuged at 100,000 g for 15 minutes to ensure removal of debris, and precleared by rotation for 4 hours at 4°C with 25 μl of packed agarose coupled to goat anti-mouse IgG that had previously been incubated with an irrelevant monoclonal antibody. The precleared extract was incubated overnight at 4°C with 300 μl of...
the rat monospecific mAb 10A8 before addition of 15 µl of packed protein A-agarose beads. The beads were then washed once in lysis buffer and five times in lysis buffer containing 0.5 M NaCl. The immunocomplex was boiled in electrophoresis sample buffer and analyzed by SDS-PAGE followed by fluorography (Laemmli, 1970). For pulse-chase experiments, cells were radiolabelled, washed once with complete DMEM and chased for the indicated time with complete DMEM supplemented with 10 mM unlabelled methionine and cysteine. At the end of the chase period, the medium was collected, the cells harvested, lysed and immunoprecipitated as described above.

**Endo H and neuraminidase digestions**

For Endo H digestions the immunocomplex was eluted from the Sepharose beads by heating at 100°C for 3 minutes in 0.5% SDS containing 2% β-mercaptoethanol. The extracted protein was diluted with 1.5 volumes of 0.1 M sodium citrate, pH 5.5, containing 3.5% NP-40 and incubated at 37°C for 16 hours with and without 5 mU Endo H. Neuraminidase treatment was performed by incubating the beads containing the immunoprecipitate at 37°C for 16 hours with 5 mU of neuraminidase from *Vibrio cholerae* in 1% NP-40, 50 mM sodium citrate, pH 5.5, 1 mM CaCl₂ and 1 mM PMSF. Following the incubations, samples were analyzed by SDS and fluorography (Gonatas et al., 1989; Johnston et al., 1994).

**Cell surface biotinylation and immunoprecipitation**

Cells were radiolabelled for 1 hour, chased for 4-4 hours as described previously, washed, and placed on ice. Biotinylation was initiated by the addition of a 1 mg/ml solution of freshly prepared NHS-LC-biotin in PBS. After a 30 minute incubation at 4°C on a slow-moving rocking platform, the reaction was terminated by washing with cold PBS containing 0.1 M lysine. The cells were lysed and the extracted proteins precleared and immunoprecipitated with mAb 10A8. After five washes with high salt lysis buffer, the precipitated protein was recovered by boiling the beads twice in 1% SDS, 0.1 M Tris-HCl, pH 8.0. The combined supernatant was incubated with 25 µl of packed streptavidin-agarose for 1 hour at room temperature and spun down. The supernatant was collected, the cells harvested, lysed and immunoprecipitated as described above.

**Light and electron microscopic immunocytochemistry**

The indirect immunoperoxidase method was described in detail elsewhere (Gonatas et al., 1989, 1995; Brown and Farquhar, 1989). In brief, for the detection of cell-surface MG160, cells grown on coverslips were washed at 4°C with Dulbecco’s modified Eagle medium (MEM) and incubated for 2 hours on ice with undiluted supernatant of mAb 10A8, or with a polyclonal antisera against MG160. The cells were washed in cold DMEM and fixed at room temperature with 1% paraformaldehyde in 0.1 M phosphate buffer. Following fixation the cells were incubated with a biotinylated anti-mouse antibody and peroxidase was detected with VIP as substrate with the ABC method according to instructions by the vendor (Vector, Burlingame, CA).

For the ultrastructural detection of MG160, cells were fixed with periodate-lysine-2% paraformaldehyde and permeabilized with 0.005% saponin, prior to the incubations with appropriate antibodies and the detection of horseradish peroxidase with VIP or diaminobenzidine tetrahydrochloride (DAB), using the method of Graham and Karnovsky (1966).

For the ultrastructural demonstration of cell surface MG160 with colloidal gold, cell monolayers were incubated on ice with mAb 10A8 followed by goat anti-mouse IgG adsorbed with 10 nm colloidal gold.

**RESULTS**

A diagram of the amino acid sequence of MG160, derived from cDNA cloning and sequencing, summarizes the principal features of the protein (Fig. 1). The amino acids 1-26 at the amino terminus represent the cleavable signal peptide (SP) sequence of the protein; the lumenal domain is encoded by amino acids 27-1136, which includes two glutamine (CAG) repeats and 16 cysteine-rich repeats; amino acids 1137-1158 correspond to the transmembrane domain (TM); the 13 amino acids of the cytoplasmic C-terminal domain (CD), 1159-1171, which are conserved in the chicken, mouse, rat and human, are identified by their letter code (Gonatas et al., 1995; Mourrelatos et al., 1996).

**Light microscopic immunocytochemistry**

The endogenous MG160 is localized in the GA

The endogenous MG160 of wild-type (wt) CHO cells was localized by staining fixed and detergent-permeabilized cells with a polyclonal antisera against rat MG160, which reacts specifically with the GA of several species, including human (Croul et al., 1990). All cells contained perinuclear profiles consistent with the Golgi apparatus (Fig. 2). By ultrastructural cytochemistry, one to two cisternae of the Golgi stack were stained (not shown). The cell surface of these cells was unstained by either light or electron microscopic immunolabeling.

**Light microscopic immunocytochemistry**

The full-length cDNA from rat MG160 is expressed exclusively in the GA of CHO cells

To selectively label rat MG160 expressed in transfected cells, we used the mouse monoclonal antibody 10A8, mAb 10A8, which is specific for the rat homologue and fails to react with...
the endogenous MG160 protein of CHO cells (Gonatas et al., 1989). Virtually all fixed and detergent-permeabilized CHO cells, stably transfected with the cDNA of rat MG160, contained the MG160 protein in focal or perinuclear structures typical of the Golgi apparatus (Fig. 3A). The localization of the mAb 10A8 into two to three medial cisternae of the Golgi stack was confirmed by immunoelectron microscopy (Fig. 6A). The MG160 protein was not detected on the cell surface of unfixed cells stably transfected with the cDNA of MG160 and incubated with mAb 10A8 (Fig. 3B).

The entire transmembrane and cytoplasmic domains of MG160 are required for its retention in the GA

Fully processed protein was found in the culture medium (see below) of cells transfected with a cDNA lacking two amino acids contiguous to the transmembrane domain and the entire transmembrane and cytoplasmic domains (ΔTMCT). The requirement for both the transmembrane domain (TM) and the cytoplasmic domain (CT) of MG160 for Golgi retention was confirmed by light microscopic immunocytochemistry. In CHO cells transiently transfected with ΔTMCT, MG160 was found diffusely within small cytoplasmic granules in a small number of cells; the compartment containing MG160 was not further investigated (Fig. 3C). The surface of the ΔTMCT transfectants did not contain MG160 (Fig. 3D).

The deletion of one or more amino acids from the CD of the MG160 protein result in its transport to the cell surface and filopodia

When cells were transfected with the following deletion mutants of MG160: Δ1170, Δ1169, Δ1167, Δ1165, Δ1163 and Δ1162, the protein was found on the plasma membrane and filopodia of unfixed cells incubated with mAb 10A8. However, in contrast to the expression of the MG160 protein in virtually all cells stably transfected with the entire cDNA of the protein (Fig. 3A,B), only approximately 5-10% of cells transfected with Δ1170, R1171A and Δ1165 showed cell surface and/or Golgi localization of the protein; furthermore, the positive cells formed small clusters. An example of surface labeling in cells stably transfected with Δ1170 is shown in Fig. 3E,F. Furthermore, the replacement of the last arginine (R1171) with the uncharged amino acid alanine (R1171A) did not prevent the transport of the protein to the cell surface of stable transfectants (Fig. 4A,B).

The last arginine (R1171) is crucial for the efficient retention of MG160 in the Golgi apparatus

As shown in Fig. 3E,F, the deletion of the last arginine, R1171, resulted in the localization of the MG160 protein at the cell surface. To further investigate the contribution of charge in Golgi retention, the positively charged arginine was replaced with lysine, which bears a similar charge to that of arginine (R1171K; also see Discussion). CHO cells stably transfected with R1171K were immunostained with mAb 10A8. In fixed and detergent-permeabilized cells the protein was not found in either the Golgi apparatus or at the cell surface (Fig. 4C,D); instead, immunoreactive MG160 was found in numerous cytoplasmic granules, the nature of which was not investigated.

An antiserum against the CD identifies the MG160 protein within the GA but fails to detect the truncated CD of the protein at the plasma membrane

In order to distinguish between full-length MG160 and its ‘isoforms’ lacking segments of the CD domain, a polyclonal antiserum was raised against the 13-amino-acid CD peptide. The specificity of the antiserum was confirmed by ELISA and by Western blots of the expressed CD fusion protein with glutathione-S-transferase, and of rat brain microsomes (data not shown). The anti-CD antiserum did not stain the surface of cells stably transfected with Δ1165, Δ1170, Δ1169, Δ1167 and Δ1165, which nevertheless displayed cell-surface MG160 when incubated with mAb 10A8. As shown in Fig. 4E,F, cells stably transfected with the deletion mutant Δ1165 showed prominent intracytoplasmic and cell surface staining after incubations with the mAb 10A8. In all deletion mutants of the CD the intracellular localization of the mAb 10A8 was obscured by the intense staining of the cell surface (Fig. 4E). In these cells, the Golgi localization of the mAb 10A8 was confirmed by immunoelectron microscopy (Fig. 6B). Similarly, the polyclonal antiserum against MG160 reacted with the protein in both the Golgi apparatus and the plasma membrane of fixed and permeabilized Δ1165 cell transfectants (Fig. 5A). In contrast, the anti CD antiserum failed to detect the truncated CD of the protein at the cell surface, although it detected the protein in the Golgi apparatus (Fig. 5B).

In order to exclude the possibility that the absence of the cell-surface staining by the anti-CD antibody was due to weak labeling, we used immunoaffinity-purified antibody at a concentration of 5 μg/ml. Under these conditions, the antibody reacted with the MG160 protein in the Golgi apparatus of fixed and permeabilized Δ1165 cells, but failed to stain the plasma membrane (Fig. 5B); furthermore, the immunostaining of the Golgi apparatus obtained by the anti-CD antibody was as strong as that obtained by the antiserum against the entire MG160 protein (compare Fig. 5A with B).

In summary, the immunocytochemical studies showed that an intact CD of the MG160 protein was required for its efficient retention in the GA, and that several deletion mutants of the
CD, including the deletion of R^{1171}, resulted in the transport of the protein to the plasma membrane and filopodia.

**Immunoelectron microscopic localizations of the MG160 protein in the GA and at the plasma membrane**

The localization of the mAb 10A8 in the Golgi apparatus of CHO cells stably transfected with the cDNA of the MG160 protein was confirmed by immunoelectron microscopy, which revealed the exclusive localization of the immunoperoxidase reaction product into two to three medial cisternae of the organelle (Fig. 6A). The presence of MG160 in both the Golgi apparatus and on the plasma membrane of CHO cells stably transfected with the Δ1165 deletion mutant was confirmed by immunoelectron microscopy (Fig. 6B). Lastly, using double immunogold and immunoperoxidase labeling, we excluded the possibility of a diffusion artefact causing the localization of MG160 on the filopodia (Fig. 6C).

**Immunoprecipitations**

Fully processed MG160 is secreted by cells transiently transfected with ΔTMCT, a deletion mutant lacking the transmembrane and cytoplasmic domains.

CHO cells transiently transfected with the ΔTMCT mutant, lacking the transmembrane and cytoplasmic tail of MG160 and two lumenal amino acids, were pulse-labeled, chased, immunoprecipitated with mAb 10A8 and analyzed by SDS-
PAGE, as described in Materials and methods. At the end of a 1-hour pulse virtually all radioactivity was associated with the cell lysate (C) (Fig. 7A). 2 hours after labeling, most of the MG160 protein was immunoprecipitated from the culture medium (M) and very little from the cell lysate (C) (Fig. 7A). The MG160 protein recovered from the cell lysate was sensitive to neuraminidase and resistant to Endo-H digestions, indicating that the protein was fully processed during its transport through the GA (not shown; Gonatas et al., 1989).

In cells stably transfected with $\Delta$1165, a mutant lacking the last six amino acids of the CD, the MG160 protein is found within cells, on the cell surface and in the culture media.

Stably transfected cells with $\Delta$1165, a deletion mutant lacking the last six amino acids of the CD, were labeled for 1 hour with Trans S-label and chased for 1, 2 and 4 hours (Fig. 7B). The lysed cells and media were immunoprecipitated using the ratspecific mAb 10A8 and analysed by SDS-PAGE. In addition, the cell surface ‘isoform’ of MG160 was also studied by biotinylation (see below). As stated above, virtually all MG160 protein was found in the culture medium after a 2-hour chase of cells transfected with $\Delta$TMCT (Fig. 7A). In contrast, most of the MG160 protein was found in the cell lysates of cells transfected with $\Delta$1165 (Fig. 7B). In the $\Delta$1165 transfectants the amounts of the total cellular MG160 protein (TC), of the cell surface ‘isoform’ (S) and of the protein in the culture media (M), were estimated after biotinylation followed by SDS/PAGE and densitometry (Fig. 7B). The amount of MG160 from the total cell lysate (TC) was given a value of 100, and the amounts of MG160 at the cell surface (S) and in the culture media (M) were expressed as percentages of the total cellular.

Fig. 4. (A,C,E) Localization of the rat MG160 protein in fixed and detergent-permeabilized CHO cells stably transfected with two point mutants and one deletion mutant as indicated, and immunostained with mAb 10A8. (B,D,F) Localization of the rat MG160 in cells incubated with mAb 10A8 prior to fixation. In B and F, the prominent staining of the MG160 protein on the cell surface obscures the intracellular Golgi stain, which was confirmed by immunoelectron microscopy (Fig. 6B). In D there is no MG160 protein on the cell surface, while the intracellular protein is distributed diffusely in a compartment probably corresponding to the rough endoplasmic reticulum (C).
MG160. During this experiment the cell surface MG160 represented a small fraction of the total cell-associated protein, while the cell-surface protein progressively decreased with time and a larger percentage of the total cell-associated protein was found in the culture medium (Fig. 7B). These results are consistent with the interpretation that in the Δ1165 transfectant a fraction of the MG160 protein is continuously transported from the Golgi apparatus to the plasma membrane and filopodia, which are progressively shed into the medium as cells adhere to the plastic and move. This interpretation is consistent with the finding that after a 4-hour chase the amount of MG160 in the culture medium is almost as high as that of the cell-associated protein, while the surface ‘isoform’ of the protein is virtually absent. In contrast in the ΔTMCT mutant, after a 2-hour chase, virtually all labeled protein was found in the culture medium and very little in the cell fraction (compare Fig. 7A, 2 hrs, columns C for the cell fraction and M for the medium, with 7B, 2 hrs and 4 hrs chase, columns TC for total cellular fraction and M for the culture medium).

Processing of MG160

In order to determine the extent of processing of the MG160 protein, cells stably transfected with Δ1165 were labeled for 1 hour with [35S]methionine/cysteine and chased for 3 hours before lysis; the cell lysates and the culture media were immunoprecipitated with mAb 10A8 and the immunoprecipitates digested with neuraminidase and Endo H. The MG160 protein from both the cell lysates and the culture media was sensitive to neuraminidase and resistant to Endo H digestion, indicating that it had acquired sialic acid (not shown; Gonatas et al., 1989).

To rule out the possibility of a lysis artifact, CHO cells were pulsed with [35S]methionine/cysteine for 1 hour, washed and lysed together with unlabeled CHO cells transfected with the cDNA of MG160. The combined cell lysate was immunoprecipitated with mAb 10A8 and analyzed by SDS-PAGE and fluorography, which did not disclose any labeling (not shown).

The cell surface ‘isoforms’ of MG160 are confirmed by labeling with NHS-LC-biotin

In order to further examine the cell surface ‘isoform’ of the MG160 protein, CHO cells stably transfected with Δ1165, Δ1170 and R1171A, were studied by surface biotinylation and immunoprecipitation (Figs 8 and 9A). The cells were incubated at 4°C with the non-permeant probe NHS-LC-biotin. Cells stably expressing MG160(wt) or the two deletion- and one point-mutants were pulsed for 1 hour with [35S] methionine/cysteine, chased for 2 hours, placed on ice and incubated with the biotinylating agent to label surface proteins. The cells were then lysed and the MG160 protein was immunoprecipitated with mAb 10A8. This fraction represented the total amount of cellular MG160. Another sample of the immunoprecipitate was dissociated and incubated with streptavidin-agarose beads to bind the biotinylated surface molecules, while the supernatant containing the non-biotinylated intracellular MG160 was collected. These fractions were subjected to SDS-PAGE and fluorography. As shown in Fig. 8, cells transfected with MG160 (wt) expressed the protein only in the cell lystate. In contrast, cells transfected with the three mutant forms of the protein expressed MG160 in the cell lystate, at the cell surface and in the medium (Fig. 8B, 9A). It must be pointed out that this result was semiquantitative, since all the radioactivity could not be eluted from the agarose beads, as shown by scintillation counting.

In contrast to the expressed Δ1165, Δ1170 and R1171A mutant forms of the protein recognized by mAb 10A8, cells stably transfected with the R1171K point mutant did not express surface MG160 protein (Fig. 4D and Fig. 9A). To further examine the synthesis of MG160 in R1171K transfectants, cell lysates were immunoprecipitated with the polyclonal antiserum against MG160, which reacts with both the endogenous and transfected proteins, but the immunoprecipitate was probed with mAb 10A8, which reacts only with the transfected rat MG160. The results of this experiment showed a single band of rat MG160 with an apparent molecular mass significantly smaller than that of the 160 kDa of the control (wt) MG160 protein (Fig. 9B). The results indicate that in the R1171K transfectants the MG160 protein either failed to mature and exit from the rough endoplasmic reticulum, or exited from the rough endoplasmic reticulum and was subsequently degraded (Fig. 4C).
Quantitation of cell surface and 'secreted' MG160

The radioactivities from the cell lysates, the cell surface 'isoforms' of MG160 and the culture media of cells stably transfected with Δ1165, Δ1170 and R1171A were then calculated by a phosphoimager analysis. Cells grown to confluence were labeled for 1 hour with [35S]methionine/cysteine and chased for 2 hours; at the end of the chase the culture media were immunoprecipitated with mAb 10A8, and cells were biotinylated at 4°C for 1 hour before cell lysis and the recovery of surface MG160 by streptavidin (see Materials and methods). In Δ1165 and Δ1170, the cell-surface radioactive MG160 was 8% and 11% of the total cellular radioactivity, respectively, while in R1171A, the cell surface MG160 was 3% of the total radioactive protein. The amounts of the radioactive MG160 in the culture media, expressed as percentages of the radioactivities from the entire cell lysate, were 10 in Δ1165, 6 in Δ1170, and 18 in R1171A. These results are consistent with the densitometric analysis performed on the fluorogram from immunoprecipitates obtained from the Δ1165 mutant (Fig. 7B).

Fig. 6. Immunoelectron microscopic localizations. (A) Cells stably transfected with the entire cDNA of the rat MG160 were stained with mAb 10A8. Note the prominent staining of mostly medial cisternae of the Golgi apparatus and the absence of staining on the plasma membrane. (B) Δ1165 mutant; the arrows show the surface staining of two adjacent cells, while stained cisternae of the Golgi apparatus are marked by g. (C) Δ1165; cell surface localization of mAb 10A8 visualized by immunoperoxidase and immunogold. Arrowhead, several gold particles on a filopodium.

Fig. 7. Immunoprecipitations with mAb 10A8. (A) In ΔTMCT, cells transiently transfected with this mutant form of the cDNA lacking the transmembrane and cytoplasmic domains, were pulsed for 1 hour with Trans S-label and chased for 1 or 2 hours. At zero time (0, the end of the pulse), most of the protein was found in the cell lysate (C) and very little in the culture medium (M). After a 2-hour chase, most of the protein was secreted in the medium, M and very little left in the cell lysate, C. (B) Δ1165 cells stably transfected with a mutant lacking the six last C-terminal amino acids were pulsed for 1 hour and chased for 1, 2 and 4 hours. At the end of the chase periods, the media and cell lysates were immunoprecipitated with mAb 10A8; in another sample, the surface MG160 protein was labeled with NHS-LC-biotin and recovered, as described in Materials and methods. The immunoprecipitates were subjected to SDS-PAGE and fluorography, and band intensities were measured by densitometry. The autoradiograms show a faint signal from the surface MG160 (S), a progressive decrease of the intensity of the total cellular protein (TC) and a progressive increase of the protein in the culture medium (M). The figures below the lanes correspond to the densitometric measurements. In order to compare the results, the counts derived from the total cell immunoprecipitate at a given time point were assigned a 100 value and the counts of the cell surface (S) and of the MG160 protein recovered in the culture medium (M) were expressed as percentages of the total cell (TC) densitometric counts. Thus after a 1-hour chase, the MG160 protein in the culture medium was approximately 9% of the total cell (TC), while after a 4-hour chase the MG160 protein in the cell lysate (TC) and the culture medium (M) were 100 and 81, respectively.
showed MG160 in the culture medium (Media), at the cell surface immunoprecipitate from a single dish was used to determine cellular and surface protein, while in Fig. 8, the immunoprecipitate from two entire these experiments half of the biotin as previously; however, in surface-labeled with NHS-LC-biotin (Pierce). The control cells were transiently transfected with the pBKCMV vector alone. The culture media (Media) and the cell lysates were immunoprecipitated with rat-specific mAb 10A8 (Total Cellular). Biotinylated cell surface proteins were recovered after incubation with Streptavidin-Agarose (Cell Surface). The supernatant from the incubation with Streptavidin-Agarose was also recovered and comprised the intracellular MG160 (Intracellular). Samples were analysed by SDS-PAGE and fluorography. Cells transfected with the entire cDNA of MG160 [MG160 (wt)] showed a 160 kDa protein only in the total cell lysate (Total Cellular), whereas cells transfected with Δ1165 showed MG160 in the culture medium (Media), at the cell surface (Cell Surface) and within cells (Intracellular).

The plasma membrane ‘isoform’ of MG160 does not recycle through the Golgi apparatus

The endocytosis of the mAb 10A8, the monovalent Fab fragments of the mAb 10A8, and the polyclonal antiserum against MG160 by cells stably transfected with Δ 1165, was then examined. These experiments, performed with a variety of conditions of time, temperature and antibody concentration, showed that the antibodies were internalized in multiple scattered granules, consistent with an endosomal-lysosomal compartment, but not in the GA (not shown). Therefore, we concluded that the truncated form of MG160, which is transported to the plasma membrane, does not recycle into the Golgi apparatus. It is noteworthy that the CD of MG160 does not contain the SDYQRL motif of TGN38, which is required for the return of the TGN38 protein from the cell surface into the TGN (Banting and Ponnambalam, 1997; Ponnambalam et al., 1994; Bos et al., 1993; Humphrey et al., 1993; Wong and Hong, 1993).

The results of the experiments conducted with point or deletion mutants of the CD are summarized in Fig. 10.

DISCUSSION

With the exception of cells transiently transfected with the ΔTMCT mutant, all experiments were performed with subclones of stably transfected CHO cells. Cell surface MG160 was never observed in subclones of cells transfected with the entire cDNA of the MG160 protein. (Fig. 3B). It should be emphasized that virtually all cells stably transfected with the entire cDNA of the protein (wt), expressed it only in the Golgi apparatus, in contrast to the expression of the cell surface ‘isoform’ of MG160 in a small percentage of cells transfected with the deletion and point mutants. Therefore, it is highly unlikely that the difference between the exclusive Golgi localization of the abundantly expressed entire (wt) protein, and the cell surface and/or Golgi localization of the various mutants which displayed limited and variable levels of expression, is due to variable levels of protein (Figs 3, 4 and 8A,B). If the overexpression of the MG160 protein accounted for its cell surface phenotype, then cells stably transfected with the entire protein should have displayed most of the cell surface ‘isoform’; they had none (Fig. 3A,B).

The half-life of MG160 is approximately 12 hours, but the time of transit of the protein from the rough endoplasmic reticulum to the Golgi apparatus, and from the Golgi to the cell surface, has not been determined (Johnston et al., 1994). The localization of the MG160 protein in the Golgi apparatus of certain cells may reflect a long transit time of the protein from the Golgi to the cell surface (Fig. 3E,F). Therefore, the apparent lack of morphological phenotypic homogeneity of cells transfected with deletion or point mutants is probably due to the properties of the protein.

As expected, in cells transfected with the ΔTMCT mutation, a construct lacking the entire transmembrane and cytoplasmic domains, the MG160 protein was not retained in the GA but secreted in the culture medium (Fig. 7A). However, it is of interest that in this mutant the MG160 protein recovered from the culture medium was fully processed, indicating that the
### Point Mutants of the cytoplasmic tail (CT) of MG160

<table>
<thead>
<tr>
<th>aa 1159-1171</th>
<th>RITKRVTRELKDR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mutation</strong></td>
<td><strong>Location</strong></td>
</tr>
<tr>
<td>R1171A</td>
<td>Golgi (G), Plasma membrane (PM), Medium (M)</td>
</tr>
<tr>
<td>R1171K</td>
<td>Protein fails to mature and exit from the RER</td>
</tr>
</tbody>
</table>

#### Deletion mutants of the CT

<table>
<thead>
<tr>
<th>aa 1159-1171</th>
<th>RITKRVTRELKDR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mutation</strong></td>
<td><strong>Location</strong></td>
</tr>
<tr>
<td>aa 1159-1170 (Δ1170)</td>
<td>G, PM, M</td>
</tr>
<tr>
<td>aa 1159-1169 (Δ1169)</td>
<td>G, PM *</td>
</tr>
<tr>
<td>aa 1159-1167 (Δ1167)</td>
<td>G, PM *</td>
</tr>
<tr>
<td>aa 1159-1165 (Δ1165)</td>
<td>G, PM, M</td>
</tr>
<tr>
<td>aa 1159-1163 (Δ1163)</td>
<td>G, PM *</td>
</tr>
<tr>
<td>aa 1159-1162 (Δ1162)</td>
<td>G, PM *</td>
</tr>
<tr>
<td>aa 1159-1161 (Δ1161)</td>
<td>Probably in RER</td>
</tr>
<tr>
<td>aa 1159-1160 (Δ1160)</td>
<td>Probably in RER</td>
</tr>
<tr>
<td>aa 1158 (Δ1158)</td>
<td>Entire CD Deleted</td>
</tr>
<tr>
<td>ΔTMCT (Δ1133)</td>
<td>Two aa of the luminal domain and the entire transmembrane and CT domains deleted</td>
</tr>
<tr>
<td>Mature protein in M</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 10. Summary of results obtained with CHO cells transfected with point or deletion mutants of MG160. Amino acids (aa) 1159-1171 of the entire carboxy-terminal cytoplasmic tail of MG160 are shown. Point mutations are designated by the wild-type amino acid preceding the codon number and the mutant residue follows, i.e. R1171A. Stable CHO transfectants were used in experiments with R1171A, R1171K, L1168K, Δ1170 and Δ1165. Asterisks indicate experiments in which the culture media (M) were not immunoprecipitated with mAb 10A8.

The experiments have also shown that the CD of MG160 is crucial for the efficient retention of the protein in the Golgi apparatus. When one to nine of the 13 amino acids of the CD of MG160 were deleted, a part of the protein was transported to the plasma membrane and filopodia of CHO transfectants (Figs 3, 4, 7B, 8, 9A). The MG160 protein was also found in the culture media of cells transfected with the Δ1170, R1171A and Δ1165 deletion mutants. In these mutants the ‘secreted’ form of MG160 was probably due to the shedding off into the medium of low density filopodia containing MG160 rather than to an authentic secretion of the mutant proteins, which possess an intact transmembrane domain and 12 (Δ1170) or 7 (Δ1165) of the 13 C-terminal amino acids of the CD (Fig. 10). The differences of cell surface and intracellular MG160 among the mutant forms of the protein used may have been caused by different levels of expression of the mutant protein (Figs 8A,B, 9A). However, cells stably transfected with MG160 (wt) expressed the protein only in the Golgi apparatus and never on the cell surface (Figs 3A,B, 8A).

The positive charge of the terminal arginine (R1171), isoelectric point 10.76, is not sufficient to explain the retention of MG160 in the Golgi apparatus, since its replacement with lysine, isoelectric point 9.54, resulted in the formation of immature protein which was probably arrested in the endoplasmic reticulum (Figs 4C,D, 9A,B), (Fieser and Fieser, 1956). The amino group at the ε position of lysine probably reacted more avidly with adjacent molecules than the positively charged guanidino group of arginine (Lehninger at al., 1993). The substituted terminal lysine probably formed covalent linkages with adjacent cytosolic polypeptides, which inhibited the transport of MG160. This interpretation is supported by experiments showing that lysine is a much more effective inhibitor than arginine in blocking the insolubilization of proteins by glutaraldehyde (Avrameas and Ternynck, 1969).

A great deal of information has been obtained concerning the signals that specify the retention of glycosylation enzymes in the GA (Machamer, 1993; Munro, 1995; Nilsson and Warren, 1994). These enzymes of the GA have the topography of type II membrane proteins, displaying a single transmembrane domain, a luminal C terminus and a cytoplasmic N terminus. The MG160 protein has a type I topography and, hence, a comprehensive review of the information derived from the enzymes of the GA is beyond the scope of this paper. However, the concept that a cytoplasmic molecular ‘scaffold’ contributes to the retention of a protein in the GA should be discussed.

Two type II medial Golgi proteins, the N-acetylglucosaminyltransferase I and the mannosidase II, interacted reversibly with an intercisternal extracellular matrix, which presumably contributed to their specific retention in the medial Golgi (Shusarewicz et al., 1994). An analogous cytoplasmic molecular ‘scaffold’ of peripheral membrane proteins, such as GM130, may contribute to the retention of intrinsic membrane proteins in the cis-cisternae of the Golgi apparatus (Nakamura et al., 1995). The deletion of R1171 resulted in protein localization at the cell surface (Fig. 3F). Apparently, the last arginine (R1171) of the t13-amino-acid cytoplasmic domain (CD) of the medial Golgi protein MG160 is crucial in bestowing conformational or other properties for its interaction with putative extracellular or peripheral membrane protein matrices, which constitute an important retention mechanism of the protein in the medial Golgi. Furthermore, the substitution of the R1171 with K1171 (R1171A) drastically altered the properties of the CD of MG160 so that the mutated protein either failed to exit from the RER or was not retained in the Golgi apparatus. Future studies may resolve this issue and may contribute to the characterization of peripheral membrane or extracellular proteins interacting with MG160 and mediating the retention of the protein in the medial Golgi.

One hypothesis proposed is that the oligomerization of a membrane protein contributed to its Golgi localization (Machamer, 1993; Weisz et al., 1993). Pursuing this hypothesis we performed several experiments under a variety of conditions of protein solubilization and electrophoresis, which did not disclose MG160 oligomers (not shown). Therefore, we concluded that the oligomerization of the MG160 protein does not constitute a retention mechanism.

The MG160 protein is the only type I intrinsic membrane protein of the Golgi stack of mammalian cells so far sequenced and expressed; hence, comparisons between MG160 and the expressed type I exogenous viral proteins or the endogenous type II enzymes of the Golgi apparatus are not easy to interpret (Machamer, 1993; Nilsson and Warren, 1994; Munro, 1991,
The membrane-spanning domain of the TGN38 protein contains a signal for the TGN localization of hybrids of the TGN38 protein with the CD4 and CD8 proteins, which are normally localized in the plasma membrane; the cytoplasmic domain of the TGN38 protein contains the motif SDYQRL (S, Ser; D, Asp; Y, Tyr; Q, Gln; R, Arg; L, Lys), which is required for the internalization of the protein from the cell surface into the TGN (Ponnambalam et al., 1994; Banting and Ponnambalam, 1997; Bos et al., 1993; Humphrey et al., 1993; Ponnambalam et al., 1994; Rajagopalan, 1994).

When the six residues RKPRE (E, Gly; K, Lys; P, Pro; R, Arg) of the cytoplasmic domain of calnexin were deleted, the protein exited from the RER and localized in the Golgi apparatus, while the deletion of the entire cytoplasmic domain of calnexin resulted in localizations in the Golgi apparatus and the plasma membrane (Rajagopalan et al., 1994).

The examples of TGN38 and calnexin suggest that the cytoplasmic domains of type I proteins contain signals for their efficient retention in the appropriate compartments, and in the case of TGN38, for the recycling of the protein from the plasma membrane to the TGN. The cytoplasmic domain of MG160 is considerably shorter than that of TGN38 and calnexin, and probably unique, since the deletion of a single amino acid, the terminal arginine (R1171), allowed the partial transport of the MG160 protein from the Golgi stack to the plasma membrane. The cytoplasmic domain of the MG160 protein does not contain a motif analogous to the SDYQRL of TGN38, and unlike TGN38, the cell surface ‘isoform’ of MG160 does not undergo endocytosis into the Golgi apparatus.

The type I medial Golgi MG160 protein is virtually identical to two functionally unrelated molecules: the ESL-1 of murine myeloid cell plasma membranes, which serves as ligand for E-selectin of endothelial cell surfaces, and the CFR, a chicken cysteine-rich receptor of certain fibroblast growth factors (Burrus et al., 1992; Gonatas et al., 1995; Steegmaier et al., 1995; Mourelatos et al., 1996).

The ESL-1 protein mediates the binding of murine myeloid cells to endothelial cells expressing surface E-selectin (Steegmaier et al., 1995). Furthermore, it has been confirmed that ESL-1 is found in both the Golgi apparatus and on cell surfaces of murine myeloid cells, which is not unexpected considering the identity of ESL-1 with the medial Golgi protein MG160 (Steegmaier et al., 1997). It is noteworthy that in the lymphoma cell line K46, 80% of the total labeling of the cell surface ESL-1 was on microvilli (filopodia), in contrast to 69% labeled by a control antigen (Steegmaier, 1997). The striking analogy between the subcellular distributions of ESL-1 in mouse myeloid cells and the deletion mutants of MG160 in the filopodia of CHO cell transfectants described here, suggests that the proposed proteolytic cleavage of the cytoplasmic domain of ESL-1 may result in the partial transport of the protein from the Golgi to the plasma membrane of myeloid cells.

The cysteine-rich Fibroblast Growth Factor Receptor (CFR) was also found recently in the Golgi apparatus of cell transfectants (Zuber et al., 1997). This finding is not surprising considering that the Golgi protein MG160 is virtually identical to CFR and ESL-1 (Mourelatos et al., 1996). It was proposed that CFR is involved in the intracellular trafficking of FGFs and in the regulation of cellular responses to FGF (Zuber et al., 1997). However, it is not yet known whether the CFR protein is physiologically found on the plasma membrane of certain cells and whether its presumed regulation of FGFs is performed by a protein localized in the GA the plasma membrane or both.

The functional relationship between the tyrosine kinase-containing FGF receptors involved in signal transduction and the cysteine-rich FGF binding protein CFR is a matter of considerable interest. According to a recent study, a segment of 450 residues of the CFR, CFR290-740, binds FGF-2 with an affinity indistinguishable from that of the full-length molecule. Furthermore, the binding of FGFs to CFR and to the tyrosine-kinase FGF receptors (FGFR) is mutually exclusive, since similar regions of FGF-2 interact with both CFR and FGFR, but only the FGFRs required heparan sulfate proteoglycans for FGF binding (Zhou et al., 1997). It is noteworthy that MG160 expressed in the yeast Pichia pastoris did not require complex carbohydrates and sialic acid for basic fibroblast growth factor binding, suggesting that the polypeptide backbone of MG160, rather than its carbohydrate moieties, is responsible for FGF binding (Chen and Gonatas, 1997).

The homologies between MG160, a Golgi protein, and CFR and ESL-1, suggest that MG160 has different functions depending on Golgi or plasma membrane localization. The hypothesis that a protein may be found in two locations, with different functions in each location, is not novel. Isoforms of the Golgi protein β-1,4-galactosyltransferase (GalTase) are present: the Golgi apparatus or on cell surfaces (Strous, 1986; Lopez et al., 1989; Youakim et al., 1994). When localized in the Golgi apparatus, GalTase is involved in the biosynthesis of glycoproteins and glycolipids, while at the cell surface, GalTase probably functions as an adhesion molecule in a variety of processes (Shur, 1993). In that regard, the presence of MG160 in filopodia suggests that the protein may be involved in cell to cell or cell to extracellular matrix contacts of migrating cells (Figs 3E,F, 4E,F, 6C) (Huttenlocker, 1995).

In summary, this study shows that the entire C-cytoplasmic domain of MG160 is required for its efficient and exclusive retention in the Golgi apparatus. A number of minor deletions of the short C-terminal cytoplasmic domain of the protein, including the removal of the last arginine1171, allowed its partial transport to the plasma membrane and filopodia. The transport of MG160 to the plasma membrane would expose its large, carbohydrate-rich, intralumenal Golgi domain to the extracellular space and mediate the binding of the protein to E-selectin to bFGF, and perhaps to other soluble or membrane-bound molecules.

It remains to be determined whether the mechanism for the transport of MG160 to the plasma membrane, demonstrated in this study of CHO cells transfected with cDNAs containing deletions in the cytoplasmic domain, is relevant to the physiological traffic and function of the protein in murine myeloid and perhaps in other cells.

Studies on possible interactions between the CD of MG160 and other cytoplasmic or peripheral membrane proteins may disclose associations mediating its exclusive retention in the Golgi apparatus or its transport to the plasma membrane.
REFERENCES


