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MG-160, a membrane sialoglycoprotein of the medial cisternae of the rat Golgi apparatus, binds basic fibroblast growth factor and exhibits a high level of sequence identity to a chicken fibroblast growth factor receptor

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SUMMARY

We report the primary structure of MG-160, a 160 kDa membrane sialoglycoprotein residing in the medial cisternae of the Golgi apparatus of rat neurons, pheochromocytoma (PC-12), and several other cells. The cDNA encodes a polypeptide of 1,171 amino acids with an M_r of 133,403. An intralumenal cleavable signal peptide is followed by a Pro-Gln-rich segment and 16 contiguous, approx. 60-residue-long, regularly spaced cysteine-rich segments showing sequence identities ranging from 15 to 35%. The lumenal domain is followed by a single membrane spanning domain and a short carboxy-terminal cytoplasmic tail. The protein contains 5 potential NXT glycosylation sites.

The sequence of MG-160 shows no homologies with enzymes and other membrane proteins of the Golgi apparatus. MG-160 displays a so far unique feature for a membrane protein of the Golgi apparatus: namely, an upstream open reading frame (uORF), encoding 58 amino acids, located in front of the major open reading frame (ORF). Most vertebrate mRNAs containing uORF or AUG codons in front of the major ORF encode growth factors and cell surface receptors (Geballe and Morris 1994). In that regard a 90% identity between the primary structure of MG-160 and a receptor for acidic and basic fibroblast growth factors (CFR), isolated from chicken embryos (Burrus et. al., 1992), may be relevant.

Immunoreactivity for MG-160 has been detected in the Golgi apparatus of neural and other cells of 2-day-old chicken embryos and adult chicken; furthermore, recombinant human basic fibroblast growth factor (bFGF) binds MG-160 purified from rat brain. MG-160 shows no sequence similarity with members of the family of fibroblast growth factor receptors (FGFR) involved in signal transduction. These findings are consistent with the hypothesis that MG-160 is involved in the traffic and processing of endogenous or autocrine FGFs. This is the first example of an intrinsic membrane protein of the Golgi apparatus which binds a growth factor and may be involved in its regulation.

Key words: membrane, protein, MG-160, Golgi apparatus

INTRODUCTION

The importance of the Golgi apparatus complex in the transport, processing and targeting of proteins destined for secretion, lysosomes and plasma membrane secretion, and processing of plasma membrane has emerged from morphological, biochemical and molecular studies (Mellman and Simons, 1992; Rothman and Orci, 1992). Also, the Golgi apparatus is involved in the adsorptive and receptor-mediated endocytosis of lectins, certain toxins and physiological ligands or receptors (Gonatas et al., 1977, 1980; Kornfeld, 1992). Recent studies have investigated the structure and function of enzymes in the organelle, the 'molecular machinery' of vesicular transport, and signals specifying retention of molecules within the organelle or targeting them to distal sites (Kleene and Berger, 1993; Paulson and Colley, 1989; Machamer, 1993).

Using an organelle-specific antibody we previously identified and isolated MG-160, an acidic (pI 4.7), Endo-H resistant, intrinsic membrane sialoglycoprotein which resides predominantly in the medial cisternae of the Golgi apparatus of rat brain neurons, astrocytes, adenohypophysis and cultured rat pheochromocytoma (PC-12) (Louvard et al., 1982; Gonatas et al., 1989: Croul et al., 1990). The localization of MG-160 in the medial cisternae of the Golgi apparatus, established by

immunoperoxidase ultrastructural immunocytochemistry, was further confirmed by its resistance to Endo-H digestion, and by its redistribution in brefeldin-A-treated PC-12 cells within the rough endoplasmic reticulum and nuclear envelope and not within the *trans*-Golgi network (TGN), which was redistributed around the centriole (Gonatas et al., 1989; Johnston et al., 1994). By light microscopic immunocytochemistry, MG-160 was localized in rat kidney, pancreatic islets, parathyroid, thyroid and adrenal tissue (Gonatas et al., 1989).

MG-160 migrated on SDS-PAGE as a 160 kDa band under reducing conditions and at 130 kDa under non-reducing conditions, consistent with the presence of intrachain disulfide bonds. MG-160 was shown to contain Asn-linked carbohydrates by its shift in molecular mass after digestion with the enzyme peptide:*N*-glycosidase F. The protein contained sialic acid residues as indicated from staining with *Limax flavus* lectin and by its susceptibility to neuraminidase digestion. Protease digestions of intact Golgi vesicles from brain neurons revealed long intralumenal and/or intramembrane domains and a short cytoplasmic segment. The protein yield per gram of rat brain was 0.9 microgram and represented 3% of the Golgi proteins (Gonatas et al., 1989).

In PC-12 cells treated with brefeldin A, MG-160 is redistributed into the rough endoplasmic reticulum and nuclear envelope, acquires resistance to Endo-H, but fails to be sialylated. These and other experiments provide evidence for the existence, under physiological conditions, of a retrograde transport pathway between the *trans*-Golgi network (TGN) and the Golgi cisternae (Johnston et al., 1994).

To further define the structure of MG-160 we cloned and sequenced the cDNA for this protein. The deduced amino acid sequence is consistent with the earlier biochemical study (Gonatas et al., 1989). However, to our surprise, the amino acid sequence of MG-160 is 90% identical to CFR, a cysteine-rich 'receptor' for fibroblast growth factors identified in the chicken (Burrus and Olwin, 1989; Burrus et al., 1992). Subsequently, we confirmed that recombinant human basic fibroblast growth factor (bFGF) binds to membrane fractions from rat brain and to MG-160 isolated from rat brain. Also, we detected immunoreactivity for MG-160 exclusively in the Golgi apparatus of chicken embryos early during development. MG-160 was not found on cell surfaces of PC-12 cells or chicken embryos, while exogenous bFGF was internalized only in the lysosomal-endosomal compartment of PC-12 cells.

The functional implications of these findings remain to be clarified. It is unlikely that MG-160 is involved in signal transduction for three reasons: first, the protein does not display the characteristic molecular features of the family of receptors to FGFs involved in signal transduction (Lee et al., 1989; Dionne et al., 1990; Keegan et al., 1991); second, the bulk of the protein is within the lumen of the Golgi cisternae; and third, exogenous bFGF is not internalized in the Golgi apparatus of PC-12 cells.

These findings raise the possibility that MG-160 controls the traffic, processing and export of endogenous FGFs and, indirectly, the regulation of the expression of cell surface receptors to FGFs. These studies strongly suggest that the Golgi apparatus plays a role in the regulation of the export of FGFs. In that regard, the conclusion of this study is consistent with the finding by Kiefer et al. (1993), who observed that FGF3 is retained in the Golgi apparatus of certain cells.

MATERIALS AND METHODS

Isolation of MG-160

The purification of MG-160 has been previously described in detail (Gonatas et al., 1989). Briefly, a 10% (w/v) homogenate from rat brain cerebral cortex in 0.32 M sucrose containing protease inhibitors was subjected to differential centrifugation. The microsomal pellet obtained after centrifugation at 100,000 g_{av} for 60 minutes was treated with 10 mM triethanolamine (TEA), 1% Nonidet P-40 (NP-40), 0.5% deoxycholate, 0.15 M NaCl, pH 8, and applied to two columns in tandem. The first was a mouse IgG coupled to Affi-Gel 10 (Bio-Rad, Hercules, CA, 94547), and the second affinity column consisted of 4 mg of monoclonal antibody (mAb, 10A8) per ml of resin. The mAb column was washed with 0.1% TEA, 0.1% NP-40, 1 M NaCl, pH 8, and the protein was eluted with 50 mM triethylamine, 0.1% NP-40 and 0.15 NaCl, pH 11. The eluted fraction was neutralized immediately with 0.1 M K_2HPO_4, concentrated by ultrafiltration using an Amicon YM 30 filter and precipitated with 7 volumes of acetone. Analysis of the isolated protein by SDS-PAGE and Coomassie staining revealed a single 160 kDa protein.

Peptide sequence analysis

MG-160 destined for proteolytic cleavage was reduced and *S*-carboxyamidomethylated by the method of Stone et al. (1989). The alkylation mixture containing *S*-carboxymethylated MG-160 was then subjected to trypsin digestion and the peptides were separated by reverse phase HPLC on a Hewlett-Packard HPLC equipped with a 1040 diode array detector, using a Vydac 2, 1 mm × 150 mm C18 column. Peptide fractions were applied directly to a polybrene precycled fiber filter placed in a reduced volume reaction microcartridge. The samples were subjected to automated Edman degradation on an ABI model 477A protein sequencer and the resultant phenylthiohydantoin amino acid fractions were manually identified using an online ABI model HPLC and Shimadzu CR4A integrator (Lane et al., 1991).The following peptides were sequenced: T4 (RGGAGPGGTGGGGWK, amino acid sequences 90-103 in Fig. 1), T24 (STISEIKECAEEPVGK, 174-189), T29 (ALNEACESVIQTACK, 494-508), T35 (LNDRIEMWSYAA, 1104-1115), T49 (LICGFMDDCKNDINLLK, 225-241), T54 (LLELQYFISR, 538-547), T56 (ACEPIIHNFCHDVA, 687-700), and T63 (LDPQLQLHCSDEIANLCA, 992-1010).

Library screening, cDNA cloning and sequencing of rat MG-160

Partially degenerate oligonucleotide probes were designed and synthesized, based on the amino acid sequences of the peptides, T24, T35, T49, and T56 (Kierchhausen et al., 1987). The synthetic oligonucleotides were prepared on a Biosearch 8700 synthesizer at the DNA core facility of the Mount Sinai Medical Center in New York City. The oligonucleotides were 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (GIBCO-BRL, Grand Island, NY 14072-0068) and used to screen a λZAP rat brain cortex cDNA library (Stratagene, La Jolla, CA, 92037) as described by Short et al. (1988) and Sambrook et al. (1989). Duplicate filters were hybridized at 42°C for 24 hours in a solution containing 40% formamide, 5× Denhardt's solution, 0.1% SDS, 6× SSC, 100 μg/ml salmon sperm DNA and 6×10^6 cpm/ml of labeled probe. Filters were washed three times at room temperature and then twice in 1× SSC, 0.1% SDS at 42°C. From 500,000 recombinants, three positive clones were identified. The Bluescript SK(−) plasmids containing the clones of interest were excised from the λZAP phages according to the Stratagene protocol and both strands were sequenced with the dideoxy chain termination method using T3 and T7 primers on exonuclease-III-generated nested deletions or custom made primers (Sequenase, USB, Cleveland OH, 44122; Erase-a-Base, Promega Biotec, Madison, WI 53711-5399) (Henikoff, 1987; Sanger et al., 1977). One of the clones (cl. 1),

extended from nucleotide position 2657 to the 3′-end position 5585 (Fig. 1). A second clone (cl.11) extended from 2025 to 4197. A 283 bp DNA fragment (2025-2308, Fig. 1) from the 5′-end of clone 11 was amplified using PCR with *Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT 06859), labeled with [^{32}P]dCTP by the random priming method using the Klenow fragment of DNA polymerase (Boehringer-Mannheim, Indianapolis, IN 46250), and used to screen 6×10^5 recombinants from a λZAP rat hypothalamus cDNA library (Stratagene) (Saiki et al., 1988). The following six positive clones were isolated and sequenced: clone 35, positions 342-4170 of the cDNA; cl.7D, 1-2560; cl. 36, 326-2481; cl. 4x, 886-2324; cl. 15, 1560-2557; cl. 2, 938-2557. RT-PCR was performed with the Gene Amp RNA-PCR kit (Perkin-Elmer), from rat brain poly(A)$^+$, using specific primers in order to amplify two DNA fragments from the 5′-end of the MG-160 mRNA (positions 112 to 431 and 298 to 621 of the cDNA. These fragments were subcloned into the PCR II vector using the TA cloning kit (Invitrogen, San Diego CA 92121) and sequenced.

Peptide synthesis and generation of antisera

Peptides were synthesized in the Protein Chemistry Facility of the University of Pennsylvania according to standard methods using an Applied Biosystems 430A synthesizer (Merrifield, 1963; Stewart et al., 1976; Barany and Merrifield, 1980). The amino acid sequences of the purified peptides were confirmed by Edman degradation. A peptide (CKQMIKRFCPEADSKTML, amino acids 876 to 893, Fig. 1) was coupled to keyhole hemocyanin using glutaraldehyde (Avrameas and Ternynck, 1971). Antisera were raised by immunizing female New Zealand White rabbits according to protocols previously described (Louvard et al., 1982; Gonatas et al., 1987).

RNA methods

Total RNA from rat brain and PC-12 cells was isolated by the guanidinium isothiocyanate method and electrophoresed in a 1% agarose, 2.2 M formaldehyde gel and transferred to a nylon membrane (Sambrook et al., 1989; Chomozynski and Sacchi, 1987). Hybridization was in 50% formamide at 42°C for 18 hours with the cDNA insert from clone 11 that was gel purified, random primed and used at a concentration of 4×10^6 cpm/ml. The final wash was at 55°C with 0.2× SSC, 0.1% SDS. Poly(A)$^+$ RNA used in cDNA synthesis was obtained by two passages through an oligo(dT)-cellulose column (Stratagene).

Polyacrylamide gel electrophoresis (PAGE), western blotting, and immunostaining of tissue sections

These procedures were performed according to the methods of Laemmli (1970) and Towbin et al. (1979) and by the immunoperoxidase method, as described previously (Gonatas et al., 1989).

Culture of PC 12 cells

The rat pheochromocytoma PC 12 line, a gift from L. Greene, was cultured according to a previously described method (Tischler and Greene, 1978). The cells were not exposed to nerve growth factor.

Ligand affinity blotting

MG-160 derived from rat brain (1 to 2 μg) or a carbonate-extracted microsomal pellet was mixed with Laemmli sample buffer without reducing agent and resolved on a 7.5% SDS-polyacrylamide gel (Gonatas et al., 1989; Howell and Palade, 1982). Following transfer to nitrocellulose, the membrane was blocked for 1 hour with 3% milk powder in 10 mM Tris-HCl, pH 7. 4, 0.15 M NaCl, 0.1% Tween-20 (TBST). The blot was then incubated at room temperature for 1 hour with 100 pM ^{125}I-bFGF, (specific activity 36 TBq/mmol, Amersham, Indianapolis, IN) using the same blocking buffer in the presence or absence of a 1000-fold molar excess of non-radioactive bFGF. After 3 washes with TBST, autoradiograms were made with Kodak X-OMAT AR film with a Dupont Cronex lighting-plus intensifying screen at −70°C. Biotin-labelled bFGF, purchased from Boehringer Mannheim, was also used for ligand blotting and immunocytochemistry. For ligand blotting, the nitrocellulose membrane with transferred MG-160 was blocked for one hour at room temperature with 3% gelatin in TBST and probed with 1.0 μg/ml biotin-labelled bFGF. After three washes with TBST the membrane was incubated for 30 minutes with avidin conjugated to alkaline phosphatase purchased from Vector (Burlingame, CA 94010), and stained with the NBT BCIP kit from Promega.

Immunocytochemistry

For the detection of surface staining of human recombinant basic fibroblast growth factor (bFGF) and of MG-160, unfixed frozen sections of chicken embryos were used. For endocytosis of bFGF, PC-12 cells were incubated with the ligand prior to fixation and immunostaining. For intracellular localizations of internalized bFGF and antibodies against MG-160, chicken embryos or PC-12 cells were fixed in 1% paraformaldehyde in buffered physiological saline (PBS), permeabilized with 50% ethanol in PBS and 0.5% Triton-X 100 in PBS, and immunostained with the avidin-biotin-peroxidase method as detailed previously (Gonatas et al., 1989; Croul et al., 1990). The monoclonal antibody 10A8, initially used for the detection and purification of MG-160 from rat tissues, and a polyclonal antiserum against immunoaffinity-purified MG-160, which detects the protein in the Golgi apparatus of chicken and other species, were used (Gonatas et al., 1989; Croul et al., 1990). Biotinylated recombinant human fibroblast growth factor (bFGF), purchased from Boehringer Mannheim (Indianapolis, IN 46250-0414) was used for the detection of surface or intracellular binding sites in chicken embryos, and in the internalization study in PC-12 cells; bFGF was detected by immunocytochemistry with the avidin-biotin-peroxidase method (Vector Vecstastain, ABC method).

Ultrastructural immunocytochemistry

The methods of Tokuyasu (1980, 1989) were used. Briefly, adult rats were perfused with PBS and a fixative of freshly made 4% paraformaldehyde and 0.1% glutaraldehyde. Brains, spinal cords and trigeminal ganglia were removed, cut into 0.3-0.5 cm^3 pieces, and fixed at room temperature for 3-4 more hours. Subsequently, tissues were washed in PBS containing 50 mM NH_4Cl, and cryoprotected in 5% sucrose until use. Tissues were then infiltrated with poly(vinylpyrrolidone), cryosectioned in a Cryo-Nova ultramicrotome, immunostained with monoclonal antibody 10A8, stained with OsO_4 and uranyl, and embedded in poly(vinyl alcohol). The goat anti-mouse IgG complexes with colloidal gold (15 nm) were purchased from EY Laboratories (San Mateo, CA 94401).

RESULTS

Degenerate oligonucleotides, designed from sequences of tryptic peptides from purified MG-160, were used to screen a λZAP cDNA library from rat brain cerebral cortex. Clone 11 extending from nucleotide 2025 to 4197, identified three peptide sequences, T56, T63, T35, verifying the authenticity of the clone. On the basis of sequence information from clone 11, a 283 bp PCR probe was generated corresponding to nucleotides (nt) 2025 to 2308 and used to screen a λZap cDNA library from rat hypothalamus. Seven clones were isolated and sequenced. The cDNA for MG-160 was derived from sequences of at least two separate overlapping clones, with the exception of the 5′-end (1-326), which was deduced from only one clone (cl. 7D, 1-2560). To confirm that the 5′-end of the cDNA indeed corresponded to the mRNA of the protein, two RT-PCR reactions were carried out with rat brain poly(A)$^+$

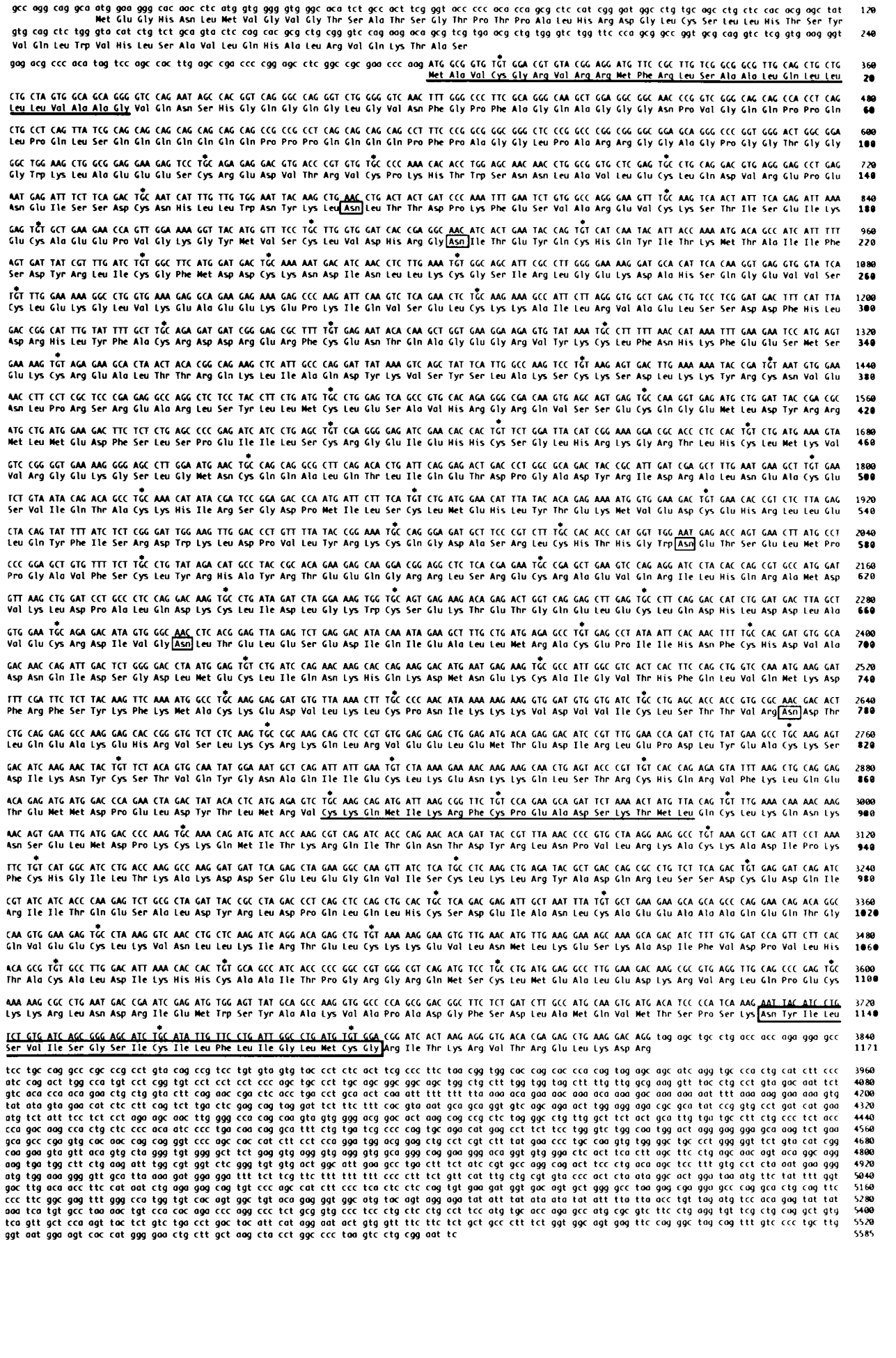

Fig. 1. Nucleotide and deduced amino acid sequence of MG-160; 5′ and 3′ untranslated regions are shown in lower case. Along the length of the right margin on the top, plain text numerals represent nucleotides, while below, bold numerals represent amino acids. Note that upstream from the principal open reading frame (ORF) starting at nucleotide 301, there is a short open reading frame (uORF), starting at nucleotide 13, encoding 58 amino acids, which, to avoid confusion with the principal protein, has not been numbered. The signal peptide at the amino terminus is underlined with a bold line. Cysteine residues are marked with an asterisk; putative NXT sites for asparagine-linked sugars are boxed. The membrane-spanning domain is enclosed in a box. Underlined by a thin line at amino acid residues 876-893 is the peptide, which was synthesized for the generation of a polyclonal antiserum which reacted by western blotting and immunocytochemistry with MG-160 (see Figs 5, 6).

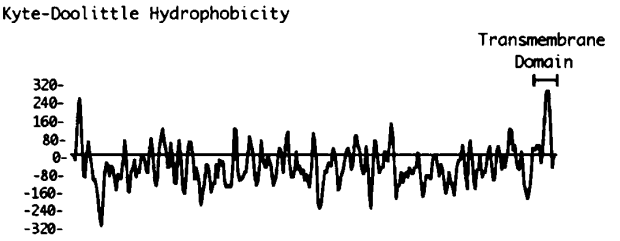

Fig. 2. Hydrophobicity calculated according to Kyte and Doolittle (1982). Hydrophobic regions are above the horizontal line. At the far left of the plot is the region of the signal peptide. At the far right side of the plot, the transmembrane domain is indicated by a bracket.

mRNA as a template and sets of internal primers. Two fragments corresponding to nucleotide positions 112-431 and 298-621 of the cDNA were amplified, subcloned and sequenced, and found to be identical to the 5′ region of cl. 7D.

The eight overlapping clones create a cDNA sequence of 5585 bp that contained all 8 unambiguously sequenced tryptic peptides from the purified protein. A minor open reading frame, encoding 58 amino acids has been identified in front of the major reading frame (Fig. 1). The major open reading frame of 3513 bp has an initiation codon ATG with A at −3 and G at +4, which agrees with the consensus sequence for eukaryotic translation initiation (Kozak, 1991). The start site is followed by 25 residues composed of a positively charged amino terminus, and a hydrophobic stretch followed by polar amino acids characteristic of a signal peptide. The sequence Ala-Ala-Gly conforms to the −3, −1 rule for naturally occurring signal peptidase cleavage sites (Von Heijne, 1986). A Kyte and Doolittle (1982), hydrophobicity plot revealed two hydrophobic regions, the amino-terminal signal sequence and a stretch of 22 amino acids near the carboxy terminus that serves as a transmembrane domain (Figs 1,2).

The signal peptide is followed by a Pro-Gln-rich segment (Fig. 1, residues 31-99). The protein contains 68 cysteine residues, one within the signal peptide and two within the intramembranous domain. The remaining 65 cysteines are arranged in 16 approx. 60-residue repeats, each of which contains four cysteines, with the exception of unit three, which contains an additional cysteine (Fig. 3). The 16 cysteine-rich repeats show 15-35% identity (R. D. Doolittle, personal communication). The cysteine-rich segments are followed by a single 22-residue membrane-spanning domain and a short, 13-residue, cytoplasmic carboxy-terminal tail.

In addition, 1772 bp were derived from the 3′ non-coding region but no polyadenylation signal was located. Clone 11, which extends from nt 1959 to 4131 in the cDNA sequence was used to probe total RNA from rat brain and PC-12 cells (Fig. 4). A single transcript of approximately 10 kb was visualized, suggesting that a large part of the messenger RNA is not translated (Fig. 4).

The cDNA for MG-160 codes for 1171 amino acids and predicts a molecular mass of 133,403, which, if one takes into account that the protein is significantly glycosylated, agrees with the 160 kDa estimated from its mobility in SDS-PAGE.

In agreement with biochemical data that showed carbohydrate moieties, the deduced amino acid sequence has 5 putative NXT glycosylation sites (Fig. 1).

To validate that the cDNA sequence encodes MG-160, an antiserum against a synthetic peptide representing a region near the 3′-end of the DNA coding sequence (amino acids 876-893, Fig. 1) was raised in a rabbit. Crude microsomes or MG-160, purified by immunoaffinity chromatography, from rat brain were probed with the antiserum against the above synthetic peptide or with an antiserum against MG-160 raised in a rabbit immunized with immunoaffinity-purified MG-160 (Croul et al., 1990) (Fig. 5A,B,C). All antisera reacted with prominent 160 kDa bands. In addition, in crude rat brain microsomes the antiserum against the synthetic peptide reacted with fainter 130 kDa and 80 kDa bands (Fig. 5A). The 130 kDa band may correspond to the unglycosylated precursor of MG-160, or to cross-reacting antigens.

In order to confirm by immunocytochemistry that the antiserum against the synthetic peptide reacts with the Golgi apparatus, sections of rat cerebellum were stained with monoclonal antibody 10A8, an antiserum against immunoaffinity purified MG-160, and the antiserum against the synthetic peptide. Identical staining patterns typical of the Golgi

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 1     GGWKLAEEESCREDVTRV  CPKHTWSNNLA       VLECL        QDVREPENEISSDCNHLLWNYKLNLTTD
 2     PKFESVAREVCKSTISEIKECAEEPVGKGY        MVSCL          VDHRGNITEYQCHQYITKMTAIIFSD
 3     YRLICGFMDDCKNDINLLK CGSIRLGEKDA HSQGEVVSCLEKGLVKEAEEKEPKIQVSELCKKAILRVAELSSDD
 4     FHLDRHLYFACRDDRERF  CENTQAGEGR        VYKCL          FNHKFEESMSEKCREALTTRQKLIAQD
 5     YKVSYSLAKSCKSDLKKYR CNVENLPRSRE ARLSYLLMCL        ESAVHRGRQVSSECQGEMLDYRRMLMED
 6     FSLSPEIILSCRGEIEHH  CSGLHRKGR         TLHCL        MKVVRGEKGSLGMNCQQALQTLIQETDPG
 7     YRIDRALNEACESVIQTA  CKHIRSGDPM        ILSCL          MEHLYTEKMVEDCEHRLLELQYFISRD
 8     WKLDPVLYRKCQGDASRL  CHTHGWNETSELMPPGAVFSCL   YRHAYRTEEQGRRLSRECRAEVQRILHQRAMD
 9     VKLDPALQDKCLIDLGKW  CSEKTETGQE          LECL          QDHLDDLAVECRDIVGNLTELESED
10     IQIEALLMRACEPIIHNF  CHDVADNQIDS    GDLMECL          IQNKHQKDMNEKCAIGVTHFQLVQMKD
11     FRFSYKFKMACKEDVLKL  CPNIKKKVD         VVICLSTTVRNDTLQEAKEHRVSLKCRKQLRVEELEMTED
12     IRLEPDLYEACKSDIKNY  CSTVQYGNAQ        IIECL           KENKKQLSTRCHQRVFKLQETEMMD
13     PELDYTLMRVCKQMIKRF  CPEADSKT          MLQCL          KQNKNSELMDPKCKQMITKRQITQNTD
14     YRLNPVLRKACKADIPKF  CHGILTKAKDDSELEGQVISCL          KLRYADQRLSSDCEDQIRIITQESALD
15     YRLDPQLQLHCSDEIANL  CAEEAAAQEQT    GQVEECL          KVNLLKIRTELCKKEVLNMLKESKAD
16     IFVDPVLHTACALDIKHH  CAAITPGRGR        QMSCL       MEALEDKRVRLQPECKKRLNDRIEMWSYA
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Fig. 3. Alignment of the 16 cysteine-rich segments. Cysteine and lysine residues are highlighted. Each of the segments contains four cysteines, except for unit 3, which contains an extra one. The 16, approx. 60-residue segments are 15% to 35% identical (by R. F. Doolittle, personal communication).

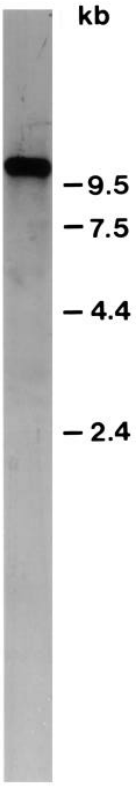

Fig. 4. Northern blot; total RNA from rat brain (10 μg), prepared as described under Materials and Methods, was denatured with formamide, fractionated on a 1% agarose gel containing formaldehyde, transferred to a nylon membrane, and hybridized with clone 11 (2025 to 4197, Fig. 1), labeled with [^{32}P]dCTP by random priming. Hybridization was carried out under stringent conditions, and washings were carried out with 0.2× SSC at 55°C.

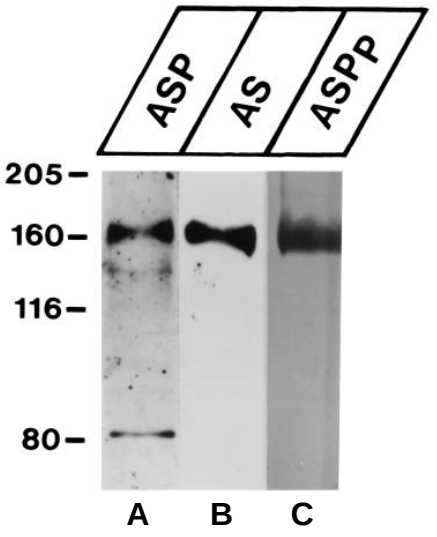

Fig. 5. Western blots of crude microsomes from rat brain (20 μg) were probed with a 1:50 dilution of the polyclonal antiserum against the synthetic peptide (ASP), or with the polyclonal antiserum against MG-160, purified by immunoblotting. The microsomes were electrophoresed in 8% PAGE-SDS; arrows indicate apparent molecular masses. In lane (C) labeled ASP_P, 1 μg of MG-160 was electrophoresed as above and probed with a 1:50 dilution of the polyclonal antiserum against the synthetic peptide as in lane ASP.

apparatus of Purkinje cells were observed with all three reagents used (Fig. 6A,B,C) (Gonatas et al., 1987).

Recently, Burrus and Olwin (1989) and Burrus et al. (1992), employing a FGF affinity column, isolated from membranes of chicken embryos an unusual cysteine-rich polypeptide with high affinity for FGF (CFR). The amino acid sequence of CFR, the chicken receptor for acidic and basic fibroblast growth factor, is about 90% identical to that of MG-160. In order to examine the relationship between CFR and MG-160, we performed ligand blotting experiments. MG-160 purified from adult rat brain was electrophoresed under non-reducing conditions, transferred to nitrocellulose and probed with ^{125}I-bFGF or biotinylated bFGF. By autoradiography, a single 130 kDa band was detected, while in the presence of excess unlabeled bFGF no radioactivity could be seen, indicating the specificity for MG-160 (Fig. 7). Also, biotinylated bFGF stained purified MG-160 (Fig. 7). Binding was also detected, only at 130 kDa, when crude microsomes from adult rat brain were electrophoresed under non-reducing conditions and probed with ^{125}I-bFGF(not shown). In the chicken, immunoreactivity for MG-160 is found only in the Golgi apparatus of notocord, neural tube and other cells early during embryonic development (Fig. 8) (Croul et al., 1990). In contrast, bFGF was localized only on areas rich in extracellular matrix (Fig. 9). In order to examine whether exogenous bFGF is internalized in the Golgi apparatus, and thus interacts with MG-160, we studied in PC-12 cells the endocytosis of biotinylated bFGF. By light microscopic cytochemistry bFGF was found only in small granules, consistent with endosomes and/or lysosomes (Fig. 10). Lastly, to confirm the localization of MG-160 in the Golgi apparatus, we examined by post-embedding immunocytochemistry the ultrastructural localization of the monoclonal antibody 10A8, which has been used for the detection and isolation of MG-160 (Gonatas et al., 1989). In ultrathin frozen sections of the nervous system of adult rats, 10A8 was localized uniquely in the Golgi apparatus (Fig. 11).

DISCUSSION

Most sequenced proteins of the Golgi apparatus, including the medial Golgi enzyme β-1,2-*N*-acetylglucosaminyltransferase I, are enzymes sharing common topological features: namely, they are type II proteins with an NH_2-terminal cytoplasmic tail, a single membrane-spanning domain and intralumenal stem followed by the catalytic and COOH-terminal domains (Kleene and Berger, 1993; Paulson and Colley, 1989; Sarkar et al., 1991; Singer, 1990). It has been reported that the transmembrane and flanking sequences of the type II Golgi enzyme β-1,2-*N*,*N*-acetylglucosaminyltransferase I specify its localization in the medial Golgi cisternae (Burke et al., 1992). It would be interesting to investigate the mechanism for the retention of MG-160 within the medial Golgi and compare it with the above medial Golgi enzyme.

Resident proteins of the Golgi apparatus, without a known enzyme function, include TGN38, ERGIC-53, p63, and the two human autoantigens encoding the 95 and 160 kDa proteins (Luzio et al., 1990; Schindler et al., 1993; Schweizer et al., 1993; Fritzler at al. 1993). TGN-38, a protein of the *trans*-Golgi network, recycles between cell surfaces and TGN; like TGN, MG-160 is a type I protein with an intralumenal NH_2 terminus, a cleavable signal peptide and a short cytoplasmic tail at the COOH terminus (Luzio et al., 1991). Interestingly, TGN38 and MG-160 display a relatively proline-rich region at the amino terminus (Fig. 1).

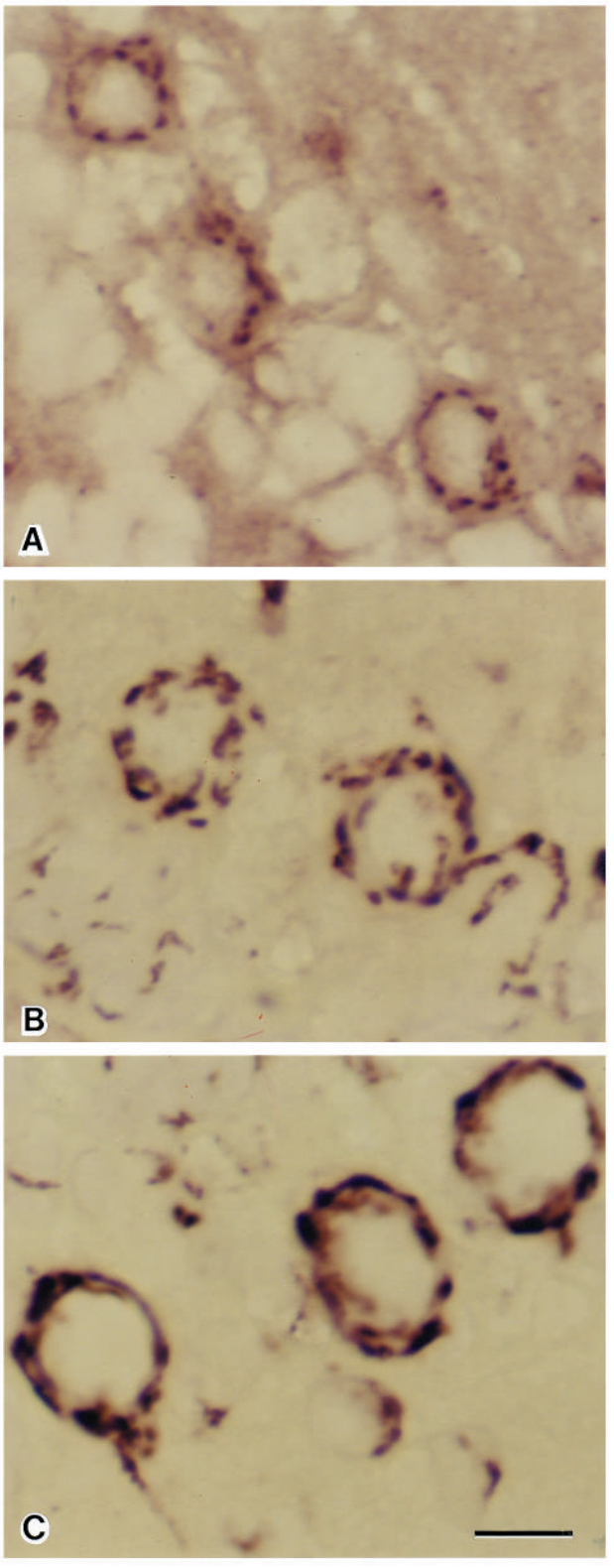

Fig. 6. Rat cerebellum was fixed in Carnoy's solution (60% ethanol, 30% chloroform, 10% glacial acetic acid) and embedded in paraffin. Sections (5 μm thick) were rehydrated and immunostained with a 1:100 dilution of the antiserum against the synthetic peptide (A), a 1:10 dilution of the supernatant of hybridoma 10A8 (B), and with a 1:4000 dilution of an antiserum against MG-160, purified by immunoaffinity chromatography (C). The magnification in all three photomicrographs is identical (×1,300); bar, 10 μm. Note that all three photomicrographs show the typical perinuclear staining corresponding to the Golgi apparatus of Purkinje neurons. As expected, the staining with the anti-peptide antiserum illustrated in A, although typical for the Golgi apparatus, is weaker than the stains obtained with mAb 10A8, or the polyclonal antiserum against MG-160.

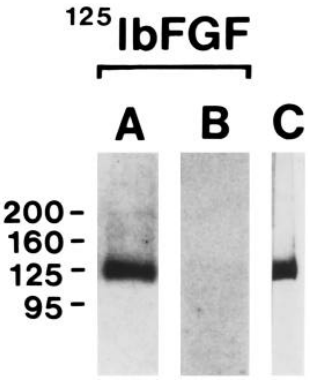

Fig. 7. Ligand blot. MG-160 (1 μg) was electrophoresed, transferred to a nitrocellulose membrane and incubated with 100 pM ^{125}I-bFGF in the presence (B), or absence (A) of 100 nM unlabelled bFGF. It was then subjected to autoradiography. In C, 1 μg of MG-160 was electrophoresed and transferred to a nitrocellulose membrane, incubated with 6 μg/ml of biotinylated bFGF and stained with avidin-biotin-peroxidase.

Unlike any other membrane protein of the Golgi apparatus, MG-160 displays a AUG codon and a short ORF in front of the major ORF (Fig. 1). It has been proposed that these upstream open reading frames modulate the translation of eukaryotic mRNAs. Interestingly, most of proto-oncogenes, and genes for growth factor and their receptors contain short upstream reading frames (Geballe and Morris, 1994).

MG-160 is abundantly present in rat neurons and PC12 cells (Gonatas et al., 1989). Also, ongoing in situ hybridization studies in chicken embryos have established that mRNA for the chicken homologue of MG-160 is expressed early in development at the primary streak/neural groove stage and abundantly in the nervous system (not shown). This finding is consistent with the abundant immunocytochemical detection of MG-160 (Fig. 8).

It has been well documented that neurons produce and respond to FGFs (Rydel and Greene, 1987; Walicke, 1988; Ferguson at al. 1990). For example, in cultures of PC-12 cells, acidic and basic fibroblast growth factors promote neuronal differentiation and the stable outgrowth of neurites (Rydel and Greene, 1987). Also, basic and acidic fibroblast growth factors have trophic effects on neurons cultured from multiple regions of the rat central nervous system (Walicke, 1988). In vivo, in retinal ganglion cells basic fibroblast growth factor undergoes receptor-mediated endocytosis and orthograde axonal transport to the lateral geniculate body (Ferguson et al., 1990). Lastly, immunocytochemical and in situ hybridization studies indicated that neurons synthesize and transport FGFs (Rifkin and Moscatelli, 1989; Burgess and Maciag, 1989; Sensenbrenner, 1993). Therefore, the expression of MG-160 in the Golgi apparatus of rat brain neurons and PC-12 cells may be related to trophic and differentiation activities of fibroblast growth factors.

On the basis of sequence analysis and functional studies, MG-160 does not share any significant features with the already known membrane proteins of the Golgi apparatus.

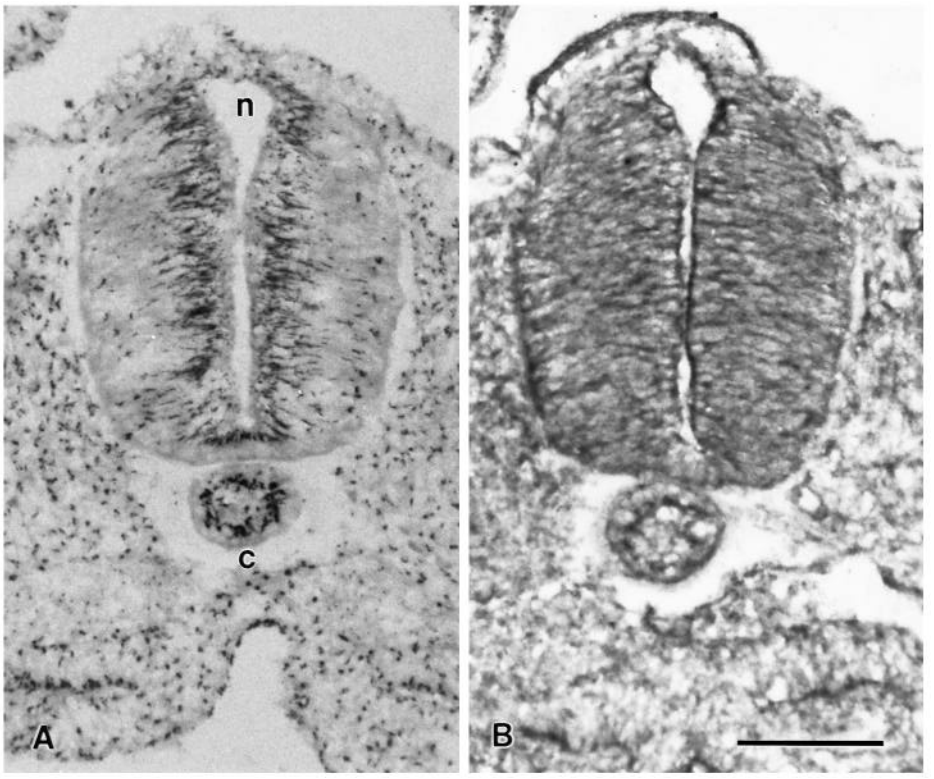

Fig. 8. Frozen sections from a 3-day-old chicken embryo were immunostained with a polyclonal antiserum against MG-160, raised in a rabbit (A), or with normal rabbit serum (B). In A, note prominent staining of the Golgi apparatus in cells of the notocord (c), and neural tube (n). In B, only background staining is seen. ×190. Bar, 100 μm.

Most likely, MG-160 isolated from rat brain and CFR isolated from membranes of chicken embryos are autologous (Burrus and Olwin, 1989; Burrus et al., 1992). This conclusion is supported by the high degree of identity of the primary structure of MG-160 with CFR, and the findings that MG-160 binds bFGF, and is expressed in chicken tissues (Figs 7, 8). Although both CFR and MG-160 bind FGFs, the primary structure of these proteins is entirely different from the FGF family of receptors, which is characterized by an extracellular domain of three Ig-like regions, a single transmembrane domain and a cytoplasmic segment containing a tyrosine kinase domain with a typical kinase insert (Lee et al., 1989; Dionne et al., 1990; Keegan et al., 1991). Therefore it is unlikely that CFR and MG-160 function as signal transducers, although indirectly they may control signal transduction, as receptors to the basic fibroblast growth factor are down-regulated by endogenous FGFs (Moscatelli, 1988).

The recognition that MG-160 binds FGF suggests that the Golgi apparatus is involved in the traffic of FGFs (Fig. 7). It is unlikely that MG-160 encounters exogenous FGFs. Biotiny-

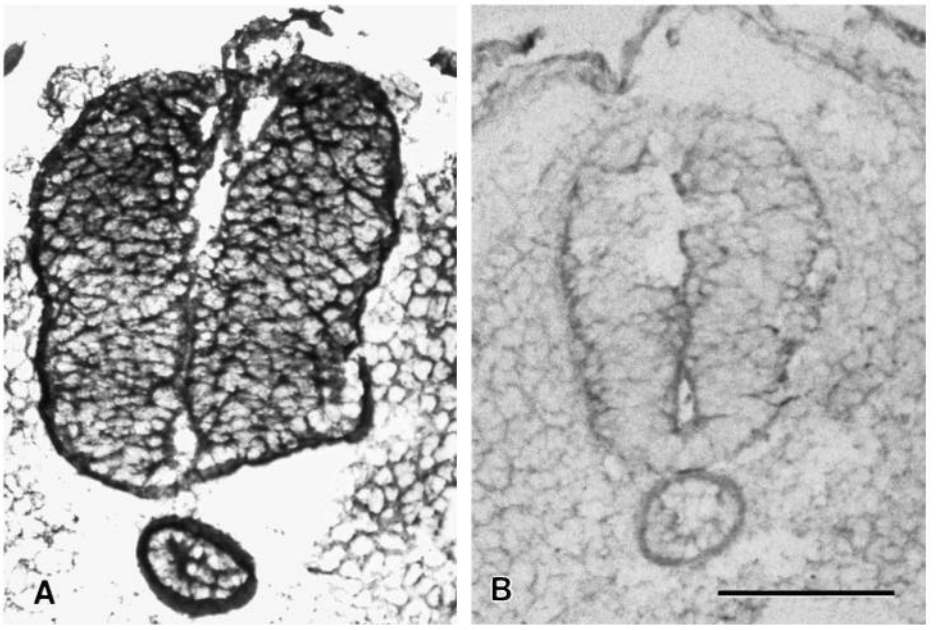

Fig. 9. Surface staining of bFGF in chick embryo. (A) Frozen sections of a 3-day-old chicken embryo were blocked for endogenous avidin and biotin, incubated with biotinylated bFGF (8 μg/ml), washed, fixed, permeabilized, and immunostained with avidin-biotin-peroxidase. Note strong stain around the notocord and neural tube, which probably is due to the binding of bFGF with extracellular matrix. None of the controls gave a surface stain; control sections were incubated with biotin only (2 mg/ml), undiluted supernatant of monoclonal antibody 10A8 used in the identification and isolation of MG-160, biotinylated IgG, polyclonal antiserum against MG-160, and culture medium. (B) Section incubated with biotinylated IgG. ×184. Bar, 100 μm.

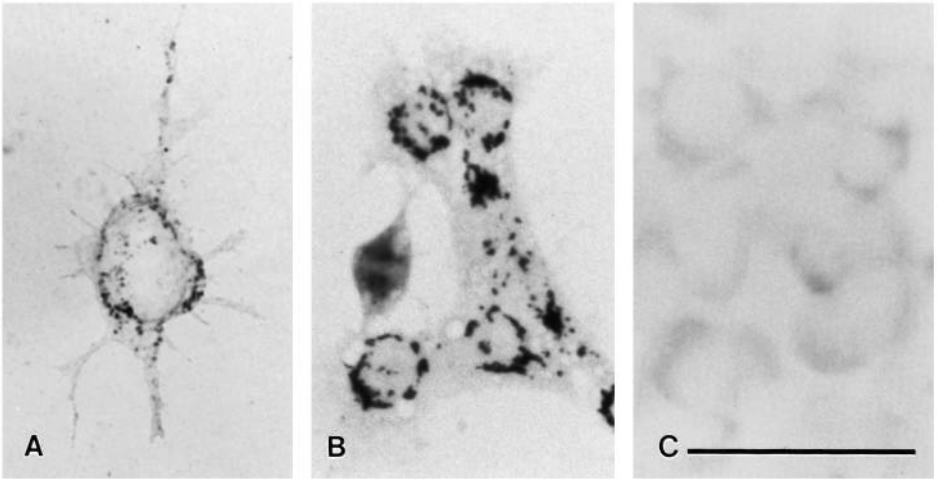

Fig. 10. (A) In PC-12 cells, exogenous biotinylated human basic fibroblast growth factor is internalized in punctate structures in the perikaryon and processes probably corresponding to endosomes-lysosomes; cells were incubated for 3 hours at 37°C with 10 μg/ml biotinylated bFGF, washed, fixed with 1% paraformaldehyde, permeabilized with 50% ethanol, incubated with avidin-biotin-peroxidase and stained for peroxidase with VIP as substrate according to Vector. In sharp contrast in B, the Golgi apparatus in PC-12, cells immunostained with monoclonal antibody 10A8, appears in the form of the typical perikaryal-perinuclear densities, while processes are not stained. In C, control, PC-12 cells were treated as in A, but instead of bFGF biotinylated IgG (10 μg/ml) was used. ×300. Bar, 100 μm.

lated bFGF binds only on the extracellular matrix of chicken embryos and is internalized exclusively in a lysosomal-endosomal compartment of PC-12 cells (Figs 9, 10). MG-160 has not been detected on surfaces of PC12 cells and there is no evidence that the protein recycles between the Golgi apparatus and plasma membranes (Gonatas et al., 1989). However, these experiments have not formally excluded the possibility of a very rapid rate of endocytosis and recycling of MG-160 between the plasma membrane and the Golgi apparatus. Alternatively, MG-160 may be involved in the processing of endogenous or autocrine FGFs.

The acid and basic growth factors lack cleavable signal sequences and are, presumably, released from cells without passing through the classical secretory pathway involving the rough endoplasmic reticulum and the Golgi apparatus (Abraham et al., 1986; Jaye et al., 1986; Kuchler, 1993). However, other members of the FGF family possess signal sequences and are processed through the Golgi apparatus (Kiefer et al., 1993). In that regard, recent evidence favors the hypothesis that certain of these factors are indeed released from cells by novel secretory mechanisms (Mason, 1994).

The abundant expression of MG-160 in the Golgi apparatus of neurons and other cells, especially early in development, strongly suggests that the protein plays important roles in the

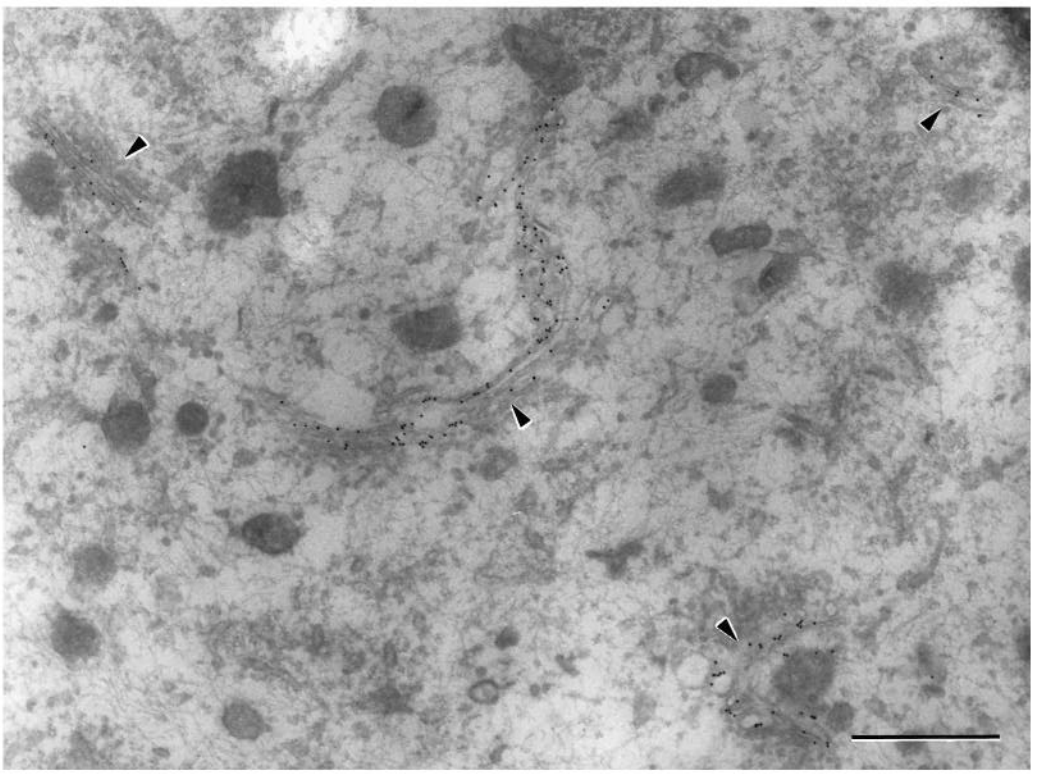

Fig. 11. Ultrathin frozen section of adult rat trigeminal ganglion neuron immunostained, according to Tokyuasu (1980, 1989), with monoclonal antibody 10A8, used in the isolation and sequencing of tryptic digests of MG-160 (Gonatas et al., 1989). Arrowheads point to four different groups of cisternae of the Golgi apparatus. Colloidal gold is exclusively seen over cisternae of the organelle. ×24,000. Bar, 1 μm.

biogenesis and function of the Golgi apparatus (Fig. 8). It remains to be elucidated whether the principal function of MG-160 is related to FGF binding, or MG-160 is a bifunctional protein, like the (CI)MPR, which mediates the targeting of lysosomal enzymes and binds insulin-like growth factor II (Kornfeld, 1992).

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