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Autotaxin is an *N*-linked glycoprotein but the sugar moieties are not needed for its stimulation of cellular motility

M. L. Stracke,* A. Årestad, M. Levine, H. C. Krutzsch and L. A. Liotta

Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bldg 10/Rm 2A33, Bethesda, MD 20892, USA. Tel: (+1) 301 496 1843; Fax: (+1) 301 480 0853.

Autotaxin is a 125kD autocrine motility factor that stimulates both random and directed motility in producing the human A2058 melanoma cell line. The recently cloned autotaxin has been demonstrated to bind strongly and specifically to concanavalin A (con A). In this study, we show that the oligosaccharide side chains on autotaxin are exclusively asparagine linked, since *N*-glycosidase F, but not neuraminidase or *O*-glycosidase, decreases the protein molecular mass to 100–105kD, which is the calculated molecular mass of the deduced autotaxin polypeptide. Furthermore, removal of oligosaccharide side chains by *N*-glycosidase F can be performed under mild conditions that retain motility-stimulating activity, suggesting that the oligosaccharide side chains are not necessary for autotaxin to activate its receptor. Finally, when melanoma cells are treated with inhibitors of carbohydrate processing, such as *N*-methyl-1-deoxynojirimycin, 1-deoxymannojirimycin and swainsonine, they still secrete a motility-stimulating autotaxin. Therefore, the carbohydrate side chains on autotaxin are not necessary to stimulate motility; however, they may still play a role in folding, secretion or maintenance of the active conformation of the protein.

Key words: autocrine, glycosylation, motility factor.

Introduction

Tumour cell motility is a critical component of invasion and metastasis, but regulation of this motility is still poorly understood. At least some tumour cells secrete autocrine motility factors (AMFs) that stimulate motility in the producing cells. Like the analogous autocrine growth factors, these AMFs also allow tumour cells independence from the host milieu in this important component of the metastatic cascade. One AMF, autotaxin (ATX), has recently been purified to homogeneity and cloned from the human melanoma cell line, A2058.[1,2] As secreted by A2058 melanoma cells, ATX is a 125kD glycoprotein that binds to the lectin concanavalin A and is eluted by α-methylmannopyranoside. However, the calculated molecular mass of the deduced ATX polypeptide is approximately 100kD,[1] suggesting that as much as 20% of the molecular mass of the secreted protein may be carbohydrate.

A number of studies have shown that some tumour cell glycoproteins possess oligosaccharide side chains that are more branched[3–6] and more highly sialylated at their termini[7–10] than normal cell glycoproteins. These tumour-related alterations have been correlated with both invasiveness and metastatic potential. For example, extensive β1–6 branching of *N*-linked oligosaccharides has been correlated with increased metastatic potential in human breast carcinomas,[4–6] human oesophageal squamous carcinomas[11,12] and human colon carcinomas.[5] Likewise, in granulocytes from patients with chronic myeloid leukaemia, aberrant sialylation has been shown to result in decreased binding and faulty signal transduction by the chemotactic peptide, fMLP.[13]

Pharmacological agents which inhibit processing of asparagine-linked oligosaccharide chains have been used to elucidate further the functional roles of these side chains on tumour cell glycoproteins. For example, *N*-methyl-1-deoxynojirimycin (NMdNM) inhibits glucosidase I and II, resulting in high-mannose side chains with terminal glucose moieties.[14] The mannose analogue 1–deoxymannojirimycin (dMAN) inhibits Golgi mannosidase I, which yields high-mannose side chains without terminal glucose groups.[15] Another reagent, swainsonine (Swn), inhibits the Golgi-based α-mannosidase II, resulting in hybrid carbohydrate chains rather than highly branched, sialylated chains.[16] Swn pretreatment of B16-F10 murine melanoma cells or the murine lymphoid tumour cell line MDAY-D2 has been shown to inhibit the formation of pulmonary metastases after intravenous injection[17,18] and invasion of human melanoma cells into reconstituted basement membranes is inhibited by pretreatment with either Swn or dMAN.[19] Colon carcinoma cells pretreated with dMAN have altered integrin α- and β-chains with decreased adherence to extracellular matrix proteins.[20]

*To whom correspondence should be addressed

ATX is a glycoprotein that stimulates tumour cell motility through a presumed cell-surface receptor. Thus, ATX must be targeted for secretion, maintain an active configuration in solution and bind to its appropriate receptor. In the study reported, we have examined the size and nature of the carbohydrate chains on ATX. Furthermore, since oligosaccharide side chains can affect invasiveness and metastatic potential, we also studied the effect of altering the oligosaccharide composition of ATX to determine whether or not these oligosaccharide units are necessary for its motility-stimulating activity.

Materials and methods

Materials

Ethylene glycol (biotechnology grade) was from Fisher Biochemicals (Pittsburg, PA, USA). Peptides *N*-glycosidase F (PNGase F), *O*-glycosidase and neuraminidase (*Arthrobacter ureafaciens*) came from Boehringer-Mannheim (Indianopolis, IN, USA). 1-Deoxymannojirimycin (dMAN), *N*-methyl-1-deoxynojirimycin (NMdNM) and swainsonine (Swn) were from Oxford GlycoSystems (Rosedale, NY, USA). Biotinylated concanavalin A, horseradish peroxidase (HRP)-conjugated streptavidin and HRP-conjugated goat anti-rabbit immunoglobulin were purchased from Pierce Chemicals (Rockford, IL, USA). Affi-Gel 10 affinity resin was from Bio-Rad (Richmond, CA, USA). Polyvinylpyrrolidone-free polycarbonate membranes and the microchemotaxis chambers were from NeuroProbe (Cabin John, MD, USA).

Cell culture

The human melanoma cell line A2058, originally isolated by Todaro *et al.*,[21] was maintained as previously described by Liotta *et al.*[22]

Production of autotaxin

For these experiments, partially purified ATX was utilized as chemoattractant. The production of ATX has been previously described in detail.[1] In brief, after ammonium sulphate precipitation, the A2058 conditioned medium was sequentially fractionated through Phenyl Sepharose CL 4B (Pharmacia-LKB Biotechnology, Piscataway, NJ, USA), agarose-bound Con A (Vector Laboratories, Burlingame, CA, USA) and ZORBAX BioSeries-WAX (Mac Mod, Chadds Ford, PA, USA) columns. The final pooled active fraction was dialysed into 50 mM Tris (pH 7.5)–20% (v/v) ethylene glycol and stored at 5°C.

Production of anti-ATX peptides

Anti-peptide antibodies that recognized the peptide ATX-102[1] were generated as described previously.[2,23] A degree of specificity was demonstrated by the fact that antibody binding was inhibited by ATX-102 peptide but not by other peptides.[2]

Cell motility assays

Partially purified ATX was dialysed into 0.1% (w/v) bovine serum albumin in Dulbecco's phosphate-buffered saline (DPBS) containing calcium and magnesium using Centricon-30 ultrafiltration units (Amicon, Danvers, MA, USA). The assay to determine motility was performed in triplicate using a 48-well microchemotaxis chamber (Neuroprobe, Cabin John) as described previously in detail.[24,25] The Nucleopore membranes used in these modified Boyden chambers were fixed and stained by Diff-Quik (American Scientific Products, McGaw Park, IL, USA). Chemotaxis was quantitated by reading the stained membranes with a 2202 Ultroscan laser densitometer (LKB Instruments, Gaithersburg, MD, USA). Densitometer units (wavelength = 633 nm) have been shown[25,26] to be linearly correlated to the number of cells per high-power field (HPF). Typically, unstimulated motility corresponded to 5–10 cells/HPF and highly responding cells to 70–100 cells/HPF above unstimulated background (i.e. 75–110 total cells/HPF).

Gel electrophoresis

Protein samples were analysed by SDS-PAGE in a Tris/glycine buffer system as described by Laemmli,[27] using prepared 8–16% gradient gels purchased from Novex (Encinitas, CA, USA). Samples were diluted in SDS-containing sample buffer with or without 5% β-mercaptoethanol and boiled for 3–5 min before loading. Prestained high molecular weight standards (BRL, Gibco, MD, USA) were run concurrently in order to estimate molecular weights. After electrophoretic separation, gels were either used for Western blotting or stained using Coomassie blue G-250 as previously described.[28] In this staining protocol, which ordinarily requires no destaining step, as little as 10 ng of protein can be visualized.

Western blot analysis

Immunoblots were performed either as previously described [29,30] with 4-chloro-1-naphthol as developing agent or used with enhanced chemiluminescence (ECL). Both systems used anti-ATX 102 peptide as primary antibody. For ECL, the manufacturer's protocol (Amersham Life Sciences, Arlington Heights, IL, USA) was followed. Primary antibody was anti-ATX-102 peptide diluted 1:1,500. Secondary antibody was HRP-conjugated goat anti-rabbit immunoglobulin diluted 1:2,500 (for 4-chloro-1-naphthol colorimetric development) or 1:25,000 (for ECL). The completed ECL immunoblot was exposed to Hyperfilm-ECL (Amersham Life Sciences), for 30 s to 15 min, then developed in an X-omat film developer.

In blots with biotinylated concanavalin A, the proteins were separated by SDS-PAGE and transferred by electrophoresis onto an Immobilon membrane. The membrane was incubated for 1 h at room temperature in blocking buffer [10 mM Tris, 0.15 M sodium chloride and 30 mg/ml bovine serum albumin (BSA), pH 7.4], then rinsed three times in washing buffer (10 mM Tris, 0.15 M sodium chloride, 1 mg/ml BSA, ph 7.4). It was then incubated for 1 h in the biotinylated concanavalin A solution diluted 1:10,000 in the same washing buffer. After three more rinses, the membrane was incubated for 1 h at room temperature in HRP-conjugated streptavidin diluted 1:2,000 in washing buffer, then rinsed twice with washing buffer and once with Tris-buffered saline. It was then developed using 4-chloro-1-naphthol as described previously.[30]

Enzymatic deglycosylation of ATX

ATX that was to be treated with PNGase F was first dialysed into 0.2 M sodium phosphate–10% (v/v) ethylene glycol pH 7.0, using Centricon-30 ultrafiltration units. Varying concentrations of PNGase F were added to the ATX and incubated for 16–18 h at 37°C. Complete digestion appeared to occur at concentrations of enzyme above 30 units/ml. For comparison, the experiments were repeated in the presence of 0.1 M β-mercaptoethanol or 0.1% (w/v) SDS plus 0.5% (v/v) Nonidet P40. ATX that was to be treated with neuraminidase or *O*-glycosidase was dialysed into 20 mM sodium phosphate, 0.1 M calcium acetate, and 10% (v/v) ethylene glycol (pH 7.2). Neuraminidase was added to a final concentration of 2 U/ml. For treatment with neuraminidase alone, this mixture was incubated for 16–18 h at 37°C. Since *O*-glycosidase requires the removal of terminal sialic acid residues for efficient deglycosylation, ATX was preincubated with neuraminidase for 30–60 min at 37°C, then *O*-glycosidase was added at a concentration of 125 mU/ml and incubated for 16–18 h at 37°C. The treated ATX was then dialysed into 50 mM Tris with 20% ethylene glycol for storage at 5°C.

Treatment of ATX with *N*-glycosylation altering agents

A2058 cells were split into four 150-cm^2 flasks and incubated until just subconfluent in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). Then, the medium was replaced with fresh 10% FCS/DMEM to which had been added DPBS for control, 1 mM dMAN, 1 mM NMdNM or 10 μM (1.7 μg/ml) Swn. Concentrations of these pharmacological agents were similar to those used to inhibit *N*-glycan-processing enzymes in melanoma cells[19,31] and other carcinoma cells.[32] On the next day, each flask was washed twice with DPBS then 20 ml of DMEM supplemented with 0.01% (w/v) BSA was added. The same concentration of each agent was added to the appropriate equilibrated flask and incubated for approximately 24 h, after which the medium from each treatment group was collected, concentrated, washed into DPBS and stored at 5°C. When concanavalin A binding to each of the treatment groups and the control was tested as described above, differences were found in both the number and intensity of bands for each group. This indicates that each agent was incorporated into A2058 cells and exerted a measurable effect on the processing of *N*-linked sugar moieties. Cells from each flask were trypsinized and counted. There was no loss of viability or reduced cell number in any of the treatment groups compared with control cells.

Results

Effect of PNGase F on ATX

ATX binds to Con A–agarose beads and is eluted with buffer containing 0.5 M α-methylmannopyranoside, indicating that ATX is likely to contain mannose residues. Such mannose sugar residues are most characteristic of *N*-linked oligosaccharides. In order to verify that ATX contained asparagine-linked oligosaccharides, we treated it with the endoglycosidase, PNGase F, which cleaves high-mannose, hybrid and complex *N*-linked oligosaccharides at the asparagine residue.

Partially purified ATX was treated with 60 U/ml of enzyme under a variety of increasingly denaturing conditions and then separated by PAGE (Figure 1). Lane 1 shows untreated material, and the 125-kD band (arrow) is autotaxin. When this material is treated overnight with PNGase F, under very mild conditions, the size of the 125-kD band decreases to approximately 100–105 kD. Addition of 0.1M β-mercaptoethanol (lane 2) or 0.5% Nonidet P40 (lane 3) to the ATX sample has no effect on the size of the resultant protein band. Even complete denaturation of ATX

Figure 1. ATX treatment with *N*-glycosidase F Partially purified ATX was treated with 60 U/ml *N*-glycosidase F at 37°C for 16 h under increasingly denaturing conditions. The treated ATX samples were separated by SDS-PAGE run under reducing conditions and stained with Coomassie blue G-250. Lane 1 contains untreated ATX (arrow) with no enzyme added. Lane 2 contains the reaction mixture run under non-denaturing conditions (50 mM Tris/10% ethylene glycol, pH 7). Lanes 3 and 4 have added 0.1 M β-mercaptoethanol and 0.5% Nonidet P40 respectively. Lanes 5 and 6 contain the reaction mixtures in which the ATX sample was first boiled for 3 min in 0.1% SDS with (lane 6) or without (lane 5) 0.1 M β-mercaptoethanol, then had 0.5% Nonidet P-40 added to prevent enzyme denaturation. The enzyme can be detected as an approximately 44 kD band in lanes 2–6.

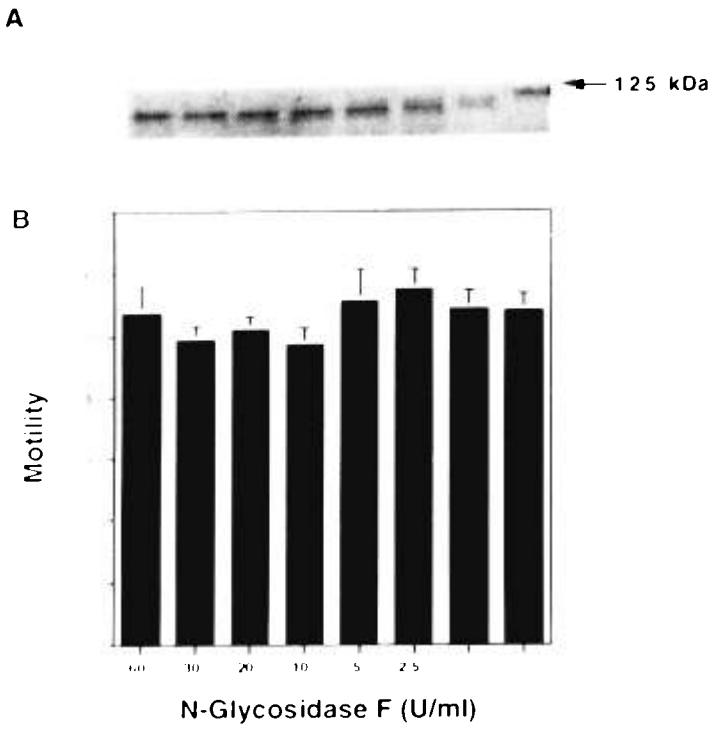

Figure 2. Effect of varying concentrations of *N*-glycosidase F on ATX molecular weight and motility-stimulating activity. Partially purified ATX was treated with various concentrations (range 0–60 U/ml, shown on horizontal axis) of *N*-glycosidase F at 37°C for 16 h. A shows the effect of the different treatments on ATX molecular weight. At concentrations of enzyme > 30 U/ml, the deglycosylation reaction appears to be complete. B shows the effect of the identical reaction mixtures on motility-stimulating capacity (immediately below the corresponding protein band of A). There is no significant difference between any of the treatment groups.

by boiling the sample for 3 min in 0.1% SDS with (lane 5) or without (lane 4) mercaptoethanol, followed by addition of 0.5% Nonidet P40 to maintain enzymatic activity, has no effect on the final size of deglycosylated protein, indicating that the deglycosidation reaction was complete under the milder conditions.

Because these results showed that ATX contains *N*-linked oligosaccharide groups, it becomes important to determine if these sugar moieties are necessary for stimulation of motility. The partially purified ATX sample was treated with varying concentrations of PNGase F (0.1–60U/ml) under mild (non-denaturing) conditions. Analysis of the resulting digest by polyacrylamide gel electrophoresis is shown in Figure 2A. As this figure shows, the digestion was incomplete using from 0.1 to 10 U/ml of enzyme and resulted in a smear of protein between 100 and 125kD. However, at higher concentrations of enzyme, cleavage of *N*-linked oligosaccharides from ATX appears to be complete. When these different digestion products were compared for their capacity to stimulate motility (Figure 2B), there was no significant difference between groups.

Western blots of deglycosylated ATX

In order to confirm that the 125 kD protein band was ATX, the deglycosylation was repeated using anti-ATX 102 affinity-purified antibody to localize the protein on an immunoblot. In addition to *N*-glycosidase F, the effect of neuraminidase, which removes terminal sialic acid groups from oligosaccharides, and *O*-glycosidase, which removes *O*-linked sugar groups once terminal sialic acids have been cleaved off, were investigated. The results are shown in Figure 3A.

Untreated ATX is shown in lane 1. After treatment with PNGase F (lane 2), the molecular weight decreases to approximately 105kD. However, treatment with neither neuraminidase alone nor neuraminidase plus *O*-glycosidase significantly decreases the molecular weight of ATX. This indicates that the oligosaccharide side chains on ATX are predominantly or exclusively attached via *N*-linkage. Further evidence that ATX lacks *O*-linked sugar moieties came from experiments in which trifluormethanesulphonic acid was used to hydrolyse all glycan groups from ATX (GlycoFree Chemical Deglycosylation Kit, Oxford GlycoSystems, Rosedale, NY, USA). The resulting protein was again approximately 105kD in molecular weight, the same size or slightly larger than after treatment with *N*-glycosidase alone (data not shown).

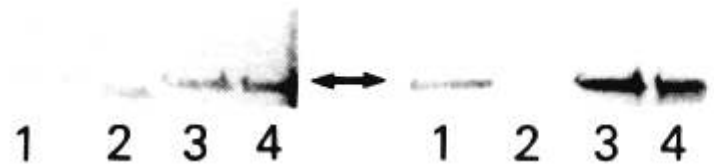

Figure 3. Effect of ATX digestion with deglycosylating enzymes as detected by immunoblot versus concanavalin A binding. Partially purified ATX was untreated (marked by arrow, lane 1), treated with 30 U/ml *N*-glycosidase F treated with 2 U/ml neuraminidase (lane 3) or pretreated for 30 min with neuraminidase then with 125 mU/ml *O*-glycosidase (lane 4). Each treatment group was then separated by SDS-PAGE run under reducing conditions and transferred electrophoretically to Immobilon membranes. In A, an immunoblot was performed with anti-peptide ATX-102 antibody as primary antibody and HRP-conjugated anti-rabbit immunoglobulin as secondary antibody. In B, the membrane was treated first with biotinylated concanavalin A, then with HRP-conjugated streptavidin. Both membranes were colorimetrically developed with 4-chloro-1-naphthol. Note than *N*-glycosidase F treatment removes concanavalin A-binding capacity.

Effect of deglycosylation on ATX binding to Con A

An identical Immobilon blot to Figure 3A was allowed to react with biotinylated Con A in order to localize mannose-containing glycoproteins (Figure 3B). As seen in lane 1, there are several protein bands in the untreated sample, including the 125kD ATX band. After treatment with PNGase F, the 125kD band disappeared (lane 2). Treatment with neuraminidase alone (lane 3) or neuraminidase plus *O*-glycosidase had no effect on the 125kD band, though other bands showed variable digestion (data not shown). These data provide further evidence that *N*-glycosidase F treatment completely deglycosylated ATX.

Effect of inhibitors of processing enzymes on ATX

In order to see if secretion or proper folding of ATX was dependent on oligosaccharide processing, A2058 cells were cultured in the presence of NMdNM, dMAN or Swn, which modify various oligosaccharide-processing pathways. Conditioned medium from each of these treatment groups was compared with that of untreated, control cells with respect to their molecular weight (Figure 4A) and their capacity to stimulate motility (Figure 4B). As seen by immunoblot using anti-peptide ATX-102 antibody, all treatment groups resulted in a reactive band of similar molecular mass (approximately 125kD). These agents affect primarily the terminal sugar moieties on the glycan chain, and ATX is a large glycoprotein with at most four glycosylated asparagines. Differences in the molecular mass of ATX after treatment were therefore very difficult to detect.

Cells treated with each agent continued to produce conditioned medium that stimulated motility. Cells treated

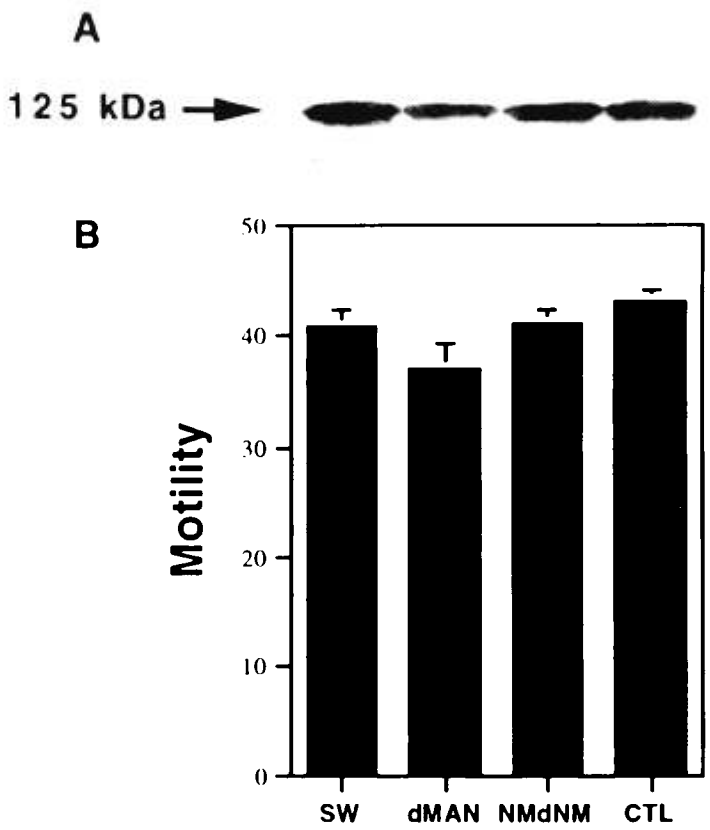

Figure 4. Effect of inhibitors of *N*-glycosylation processing enzymes. A2058 cells were split evenly into four flasks and grown to 75% confluence. Fresh medium alone (CTL) or fresh medium containing 1 mM dMAN, 1 mM NMdNM or 10 μM Swn (SW) was added and incubated with the cells for 24 h. Then the flasks were washed with DPBS, and each treatment was repeated with DMEM containing 0.1 mg/ml bovine serum albumin. One day later the conditioned media were collected, concentrated 20-fold and dialysed into DPBS. In A, the individual treatment groups were separated by SDS-PAGE run under reducing conditions and transferred electrophoretically to an Immobilon membrane. An immunoblot was performed using anti-peptide ATX-102 antibody as primary antibody and HRP-conjugated anti-rabbit immunoglobulins. The blot was developed with Amersham ECL reagents. In B, the capacity of the identical treatment groups (as shown above) to stimulate motility was assayed.

with NMdNM showed a slightly less intense band, suggesting slightly less efficient secretion of ATX when the sugar side chains are incompletely processed. Motility-stimulating activity was also proportionally slightly decreased. In addition, cells from each treatment group responded in the same way as untreated control cells when stimulated chemotactically by partially purified ATX (data not shown).

These data suggest that glycosylation may play a role in targeting ATX for secretion but is not necessary for its recognition by its receptor.

Discussion

In this study, we have shown that the carbohydrate side chains on ATX are exclusively asparagine linked. These

oligosaccharides are removed by PNGase F but not by sialidase or *O*-glycosidase. In addition, the molecular weight of ATX is the same after removal of *N*-linked sugars or after acid hydrolysis of all carbohydrate side chains. Recent full-length sequencing of the cDNA clone for ATX yielded a predicted 915 amino acid sequence with four potential sites for *N*-glycosylation.[2] The molecular mass of the deglycosylated ATX is approximately equal to the calculated mass of the ATX polypeptide predicted from the cDNA clone. Neither alteration of the carbohydrate side chains by inhibitors of processing enzymes nor complete removal of the sugar side chains caused loss of motility-stimulating activity.

Several growth hormone receptors have proven to require glycosylation for proper function. For example, receptors of basic fibroblast growth factor lose their ability to bind hormone after deglycosylation.[33] Insulin receptors, but not type I insulin-like growth factor receptors, have reduced affinity for their ligand if the cells are unable to synthesize complex carbohydrate side chains.[34] In the case of the epidermal growth factor receptor, glycosylation is required for activation and intracellular translocation, but not for ligand binding.[35]

However, the requirement for glycosylation is not invariant, and certain ligands do not need oligosaccharide side chains to recognize their receptors. For example, kFGF is the 22-kD product of the oncogene *Kfgf*, which is related to the fibroblast growth factors. In order for kFGF to stimulate cellular transformation, the protein must be secreted, but *N*-linked glycosylation appears to be irrelevant.[36] Similarly, scatter factor/hepatocyte growth factor (SF/HGF) is a heterodimeric, basic glycoprotein that stimulates chemotaxis and scattering activity in a variety of responder cells.[37] Like ATX, SF/HGF binds to the lectin concanavalin A and contains exclusively *N*-linked oligosaccharide side chains. However, glycosylation is required neither for secretion of the protein nor for stimulation of scattering activity.

Thus, like kFGF and SF/HGF, the ATX oligosaccharide side chains may play a role in proper folding of the protein, may target the molecule for secretion or may help to maintain the secreted protein in active form. However, the *N*-linked sugars do not appear to be necessary for ATX to bind to and activate its receptor.

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