N-Glycosylation of the Human Granulocyte-Macrophage Colony-stimulating Factor Receptor α Subunit Is Essential for Ligand Binding and Signal Transduction*

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The α subunit of the receptor for human granulocyte-macrophage colony-stimulating factor (GM-CSF) is a glycoprotein containing 11 potential N-glycosylation sites in the extracellular domain. We examined the role of N-glycosylation on α subunit membrane localization and function. Tunicamycin, an N-glycosylation inhibitor, markedly inhibited GM-CSF binding, GM-CSF-induced deoxyglucose uptake, and protein tyrosine phosphorylation in HL-60(eos) cells but did not affect cell surface expression of the α subunit as detected by an anti-α subunit monoclonal antibody. In COS cells expressing the α subunit and treated with tunicamycin, N-unglycosylated α subunit was expressed and transported to the cell surface but was not capable of binding GM-CSF. High affinity binding in COS cells expressing both α and β subunits was also blocked by tunicamycin treatment. These studies indicate that N-linked oligosaccharides are essential for α subunit ligand binding and signaling by the human GM-CSF receptor.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a hematopoietic growth factor that promotes the proliferation and maturation of myeloid progenitor cells and enhances the function of mature granulocytes and mononuclear phagocytes (1). GM-CSF exerts its effect via its cognate receptor, granulocyte-macrophage colony-stimulating factor receptor (GM-CSF-R). The GM-CSF-R is composed of an α subunit that binds GM-CSF with low affinity ($K_d = 1-10$ nM) (2–4) and a β subunit that has no intrinsic GM-CSF-binding capacity but associates with the α subunit to form a high affinity receptor with a $K_d$ of 10–50 pM (5, 6). The high affinity receptor signals for proliferation and functional activation via protein phosphorylation pathways (7–11). We recently found that the isolated α subunit signals for glucose uptake through a protein phosphorylation-independent pathway (12).

The α subunit of the human GM-CSF receptor is an 84-kDa glycoprotein that readily binds lectins and has 11 potential N-glycosylation sites in the cDNA-determined amino acid sequence, all located in the extracellular domain (13). The calculated molecular mass of the α subunit based on amino acid sequence is 40 kDa. The difference between the apparent and calculated molecular mass (44 kDa) is in part due to N-glycosylation. It is not known what role, if any, the N-linked carbohydrates present in the extracellular domain play in the function of the α subunit. We used the N-glycosylation inhibitor tunicamycin to probe the role of N-glycosylation in the function and surface expression of the GM-CSF-R in the HL-60(eos) cell line, which expresses a high affinity GM-CSF-R, and in COS cells transfected with α subunit cDNA alone or both α and β subunit cDNA. Our results indicate that N-glycosylation of the α subunit is essential for ligand binding and signaling by the human GM-CSF-R.

**EXPERIMENTAL PROCEDURES**

Cell Culture—A previously described eosinophilic subline of HL-60, HL-60(eos) (14, 15), was cultured in Iscove’s modified Dulbecco’s medium (IMDM) (pH 7.6) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.5 mM β-mercaptoethanol, 1% glutamine, and antibiotics. COS cells were cultured in IMDM supplemented with 10% FBS, 1% glutamine, and antibiotics.

GM-CSF Binding—COS cells were detached from culture dishes by incubation with EDTA and chondroitin sulfate (13). HL-60(eos) or suspended COS cells were incubated with varying concentrations of $^{125}$I-labeled GM-CSF (DuPont NEN) (13) in IMDM containing 0.3% bovine serum albumin overnight at 4 °C, and nonspecific binding was determined by addition to the incubation of 1.5 μM unlabeled human recombinant GM-CSF produced in Escherichia coli (a gift from Amgen, Thousand Oaks, CA). Cells were washed by centrifugation through FBS and counted in a γ spectrometer to determine GM-CSF binding.

Deoxyglucose Uptake—HL-60(eos) cells were glucose-starved for 2 h, treated with GM-CSF for 1 h, and incubated with 2 μCi/ml (12.5 μCi/mM) 2-deoxyglucose (DuPont NEN) and unlabeled 200 μM deoxyglucose (Sigma) for 10 min. Cells were washed, lysed, and counted by liquid scintillation (16, 17).

Phosphotyrosine Immunoblotting—HL-60(eos) cells were serum starved for 24 h, incubated with 0.01–10 nM GM-CSF for 5 min in IMDM containing 0.3% bovine serum albumin, lysed by ultrasonication, and immunoblotted with an antiphosphotyrosine monoclonal antibody (Upstate Biotechnology Inc.) using enhanced chemiluminescence (ECL) reagents (Amersham, Buckinghamshire, United Kingdom) (12).

GM-CSF Receptor Expression in COS Cells—Complementary DNA encoding the α or β subunit subcloned into the eukaryotic expression vector PMX (a gift from Genetics Institute, Inc., Boston) was transfected into COS cells using a DEAE-dextran method (18). Transfected COS cells were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS, 1% glutamine, and antibiotics with or without varying concentrations of tunicamycin (Sigma) for 2 days. Medium was replaced after 24 h.

Phosphotyrosine Immunoblotting—Whole cell lysates obtained by ultrasonication and membrane proteins obtained by ultracentrifugation were separated on 10% SDS-acrylamide gels and immunoblotted using the ECL protocol with a polyclonal antibody we developed against a bacterial fusion protein composing the C terminus of the α subunit.

Immunologic Detection of Cell Surface α Subunit—COS cells were detached with EDTA and chondroitin sulfate and incubated with varying amounts of monoclonal anti-α subunit antibody (Santa Cruz Bio-
Tunicamycin Inhibits GM-CSF Binding and Signaling without Affecting α Subunit Expression in HL-60(eos) Cells—To determine whether N-glycosylation plays a role in the function of the GM-CSF receptor, we treated HL-60(eos) cells with tunicamycin for 24 h and assessed GM-CSF binding. Tunicamycin treatment inhibited GM-CSF binding in a dose-dependent manner with maximal inhibition (85%) seen at 3 μg/ml (Fig. 1A). At 3 μg/ml tunicamycin, GM-CSF binding was markedly inhibited over the entire range of GM-CSF concentrations examined (Fig. 1B). Scatchard analysis of GM-CSF binding in untreated HL-60(eos) revealed a single class of binding sites with an affinity of 230 pM and a density of 650 sites/cell (Fig. 1C). Tunicamycin treatment induced a 2-fold decrease in binding affinity to 470 pM and a 3-fold decrease in the number of binding sites to 160 sites/cell. To exclude the possibility that inhibition of ligand binding by tunicamycin treatment was a result of decreased α subunit expression on the cell surface, we assessed the cell surface expression of α protein with an anti-α subunit monoclonal antibody. This is a blocking antibody that reacts with an extracellular epitope in the α subunit. Therefore, it can be used to determine if the protein is located on the cell surface. Absence of or decreased binding of the antibody to the cell surface of tunicamycin-treated cells must be interpreted with caution due to the monoclonal nature of the antibody. Tunicamycin did not alter the binding of the monoclonal anti-α antibody to the cell surface (Fig. 1D). Control experiments demonstrated absence of antibody binding to the surface of cells that do not express GM-CSF receptor α subunit (data not shown, see also Fig. 3, C and D). The data can be interpreted as indicating that tunicamycin treatment, although greatly affecting GM-CSF binding, did not alter the level of cell surface α subunit.

To correlate the effect of tunicamycin on GM-CSF binding with receptor function, we assessed glucose uptake and protein tyrosine phosphorylation in HL60(eos). In the absence of tunicamycin, GM-CSF-stimulated deoxyglucose uptake in a dose-dependent manner (Fig. 2A). An effect on deoxyglucose uptake was first seen at 10 pM with maximal stimulation (1.9-fold) seen at 10 nM GM-CSF. This dose response is consistent with our previous observations (7, 12). In tunicamycin-treated cells, deoxyglucose uptake was stimulated only at concentrations of GM-CSF greater than 1 nM (Fig. 2A). At 10 nM GM-CSF, deoxyglucose uptake was stimulated only 1.3-fold. In untreated HL60(eos), GM-CSF signaling resulted in tyrosine phosphorylation of several proteins migrating at 75, 60, 58, 55, and 42 kDa (Fig. 2B and data not shown) (9). The p42 protein comigrated with the p42 microtubule-associated protein kinase. Induction of protein phosphorylation by GM-CSF was dose dependent and was stimulated by GM-CSF concentrations as low as 10–30 pM. Treatment of HL-60(eos) with tunicamycin completely blocked GM-CSF-induced protein tyrosine phosphorylation. Cells were left untreated or pretreated with 3 μg/ml tunicamycin for 24 h and incubated with GM-CSF for 5 min; total cell lysates were then immunoblotted with an anti-phosphotyrosine antibody. Molecular standards (×10^3) are marked. The arrows indicate positions of the major tyrosine phosphorylation products in untreated cells. Microtubule-associated protein kinase was positioned by reprobing the blot with an anti-microtubule-associated protein kinase antibody (not shown).

Tunicamycin Treatment Causes Cell Surface Expression of N-Unglycosylated α Subunit in Transfected COS Cells—Since HL-60(eos) cells express a small number of high affinity GM-CSF receptors composed of both α and β subunits, we sought to distinguish the effects of tunicamycin on the two subunits. Using a COS cell expression system, we generated high level selective expression of isolated α subunit without the β subunit. Whole cell lysates of α-transfected COS cells revealed a spectrum of anti-α subunit immunoreactive bands ranging from 40 to 90 kDa (Fig. 3A). No anti-α immunoreactive bands

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**RESULTS**

**Tunicamycin inhibits GM-CSF binding in HL-60(eos) cells.**

**A.** Scatchard analysis of GM-CSF binding in untreated HL-60(eos) cells. **B.** Scatchard analysis of GM-CSF binding in tunicamycin-treated HL-60(eos) cells. **C.** Scatchard analysis of GM-CSF binding in tunicamycin-treated HL-60(eos) cells. **D.** Scatchard analysis of GM-CSF binding in tunicamycin-treated HL-60(eos) cells.
Membrane anti-
mock-transfected COS cells; monoclonal anti-
m were observed in mock-transfected cells. Treatment of the cells with increasing concentrations of tunicamycin (0.01–30 μg/ml) decreased the amount of anti-α immunoreactive proteins of higher molecular mass (50–90 kDa) and increased those of lower molecular mass (42 and 44 kDa). The simplest interpretation of these results is that the 42-kDa band corresponds to the totally N-unglycosylated α subunit. The 44-kDa band does not simply reflect the presence of residual N-glycosylation because the relative abundance of both bands did not change in cells treated with concentrations of tunicamycin from 0.1 to 30 μg/ml (Fig. 3A). It may result from other post-translational modifications such as O-glycosylation or phosphorylation (19).

None of the large anti-α immunoreactive proteins were present at tunicamycin concentrations of 0.1 μg/ml or higher, and only two bands of 42 and 44 kDa were present in these samples.

To examine whether the N-unglycosylated α protein, synthesized in tunicamycin-treated cells, was able to be transported to the cell surface, membrane fractions enriched in plasma membrane were prepared and immunoblotted with anti-α subunit serum. The membrane fraction from untreated α subunit-transfected COS cells revealed a spectrum of anti-α immunoreactive proteins ranging from 50 to 80 kDa (Fig. 3B, lane 5). Membrane anti-α immunoreactive proteins detected were only smaller species (40–46 kDa) (lane 6) in tunicamycin-treated cells. No membrane anti-α immunoreactivity was detected in mock-transfected cells (lane 4). The two major immunoreactive 70-kDa species present in total cell lysates (lanes 1–3) but not in membrane fractions (lanes 4–6) may be cytosolic proteins nonspecifically bound by the antisera, and their intensity of staining varied in different preparations. Since tunicamycin treatment resulted in a decreased size of the α subunits from 47–90 kDa to 40–46 kDa, we consider the former to be N-glycosylated forms and the latter N-unglycosylated forms. Densitometric analysis of the blot revealed that almost all of the anti-α immunoreactive proteins present in total cell lysates of untreated cells were N-glycosylated. Tunicamycin treatment drastically decreased the amount of N-glycosylated α protein and increased the N-unglycosylated form. Similarly, all of the anti-α immunoreactive proteins detected in the cell membrane were N-glycosylated; however, more than 95% were N-unglycosylated after tunicamycin treatment. Thus, tunicamycin profoundly reduced N-glycosylation of the α subunit.

Because the plasma membrane preparation in the above experiments may contain intracellular membrane fractions, we studied α subunit expression in unfixed transfected COS cells using a monoclonal anti-α subunit antibody, which allows detection of surface α protein only. 2 days after transfection, α-transfected COS cells bound the antibody in the same manner in the presence or absence of tunicamycin (Fig. 3C). Antibody binding increased with increasing concentration of antibody and was saturated at 0.3 μg/ml. In contrast, mock-transfected cells did not bind the antibody. Using a sub-saturating concentration (0.2 μg/ml) of antibody allowed us to track the time course of α subunit expression on the cell membrane. In untreated cells, antibody binding increased at 20 h, reached a maximum at 30 h, and declined slightly at 40 h after α subunit transfection (Fig. 3D). Tunicamycin delayed the appearance of antibody binding for 10 h; however, antibody binding at 40 h was equivalent to the maximal binding in untreated cells at 30 and 40 h. These experiments revealed that despite a slight alteration in the kinetics of cell surface expression, N-unglycosylated α subunit protein is efficiently transported to the cell surface.

Tunicamycin Abolishes GM-CSF Binding in COS Cells Expressing Either Low or High Affinity GM-CSF Receptor—Having established that tunicamycin inhibited N-glycosylation but did not affect cell surface expression of α subunit in COS cells, we examined whether such N-unglycosylated forms of the α subunit expressed on the COS cell surface bound ligand. Untreated α subunit-transfected COS cells bound 125I-labeled GM-CSF in a dose-dependent manner without saturation even up to 8 nM (Fig. 4A). Cotransfection of both α and β subunits gave higher binding over the entire range of GM-CSF concentrations tested. In contrast, mock-transfected cells only bound small amounts of GM-CSF and only at high concentrations. This base-line binding coincides with our previous finding that COS cells express a low level of monkey GM-CSF receptor α subunit (20). Scatchard analysis of GM-CSF binding data from α subunit-transfected cells revealed a single class of GM-CSF binding sites with a Kd of 9 nM and 3 × 105 sites per cell (Fig. 4B). This low nanomolar dissociation constant is characteristic of the low affinity GM-CSF receptor (4, 5). The high number of binding sites per cell is consistent with our ability to detect the
α protein in immunoblots of total cell lysates, Scatchard analysis of α and β cotransfected cells (Fig. 4C) revealed two binding components, one with a dissociation constant (Kd) of 280 pM and a density of 9 × 10^3 sites/cell and the other with a Kd of 8 nM and a density of 3 × 10^4 sites/cell. The binding sites with a Kd of 280 pM are consistent with the affinity of the GM-CSF binding sites present in HL-60(eos) and correspond to the high affinity GM-CSF receptor, although the Kd is 5-10-fold greater than the Kd measured on mature myeloid cells and their progenitors (7). Tunicamycin treatment (0.3 μg/ml) completely abolished GM-CSF binding in COS cells transfected with the α subunit alone and in cells cotransfected with both α and β subunits (Fig. 4A), suggesting that the N-uni-glycosylated α subunit is unable to bind to ligand even in the presence of the β subunit.

We sought to verify the results with tunicamycin using N-glycosidase F digestion. Although only a small amount of N-glycan was removed from the α subunit by N-glycosidase F as assessed by immunoblotting using the anti-α subunit antiserum, GM-CSF binding was decreased (data not shown).

**DISCUSSION**

N-glycosylation is a cotranslational modification found in most cell surface proteins, but the precise function of the carbohydrate on these proteins is not well understood (21). Evidence suggests that N-glycosylation may be required for protein folding and trafficking (22–25), ligand binding (26–30), or signaling (31–34). N-Glycosylation occurs on asparagine residues in the consensus sequence Asn-X-Ser/Thr, where X is any amino acid except proline or aspartic acid. Such glycosylation is initiated in the endoplasmic reticulum with an oligosaccharide core linked to asparagine via dolichol phosphate (35). The antibiotic tunicamycin inhibits the function of dolichol phosphate as an acceptor of N-acetyl glucosamine and thereby prevents N-glycosylation (35). We examined the role of N-glycosylation in the GM-CSF receptor and found that tunicamycin inhibited GM-CSF binding by decreasing the number of binding sites 3-fold and the affinity 2-fold in HL-60(eos) cells expressing the high affinity receptor. GM-CSF signal transduction as measured by glucose uptake and protein tyrosine phosphorylation was blocked by tunicamycin. In a previous report (36), it was concluded that tunicamycin treatment resulted in decreased cell surface expression of the GM-CSF receptor proteins as evidenced by decreased GM-CSF binding sites. Our data indicate that binding inhibition and lack of ligand-induced signaling did not result from abrogation of cell surface expression of α subunit, since α subunit expression on the cell surface was not affected by tunicamycin.

We investigated the role of N-glycosylation on the expression and function of the isolated α subunit in COS cells. α Subunit-transfected COS cells expressed abundant N-glycosylated α subunit on the cell surface. Tunicamycin reduced the molecular weight of the α subunits as shown by immunoblotting but did not affect their cell surface expression as measured by immunoblotting and antibody binding. These results indicate that N-glycosylation does not play a crucial role in the biosynthesis, stability, or cell surface targeting of the GM-CSF receptor α subunit. Tunicamycin, however, abolished GM-CSF binding in COS cells transfected with α subunit cDNA alone or cotransfected with both α and β subunit cDNA. Thus, N-unglycosylated α subunits present on the cell surface were unable to bind GM-CSF. Removal of oligosaccharides from α subunits in the membrane fraction by N-glycosidase F led to a decrease in both the molecular weight and GM-CSF binding capacity of the α subunit. Taken together, the results with N-glycosylation inhibition and N-endoglycosidase F digestion in cells either endogenously or exogenously expressing α subunit indicate that N-glycosylation of the α subunit is essential for ligand binding and signaling by the human GM-CSF receptor.

It is uncertain why N-glycosylation of α subunit is essential for ligand binding and signaling. N-Glycosylation may stabilize a conformation required for binding, or oligosaccharides may themselves be an essential part of the binding site. Our observations that α subunit devoid of N-glycosylation was still expressed on the cell surface and was recognized by a monoclonal antibody suggest no major conformational difference between the N-glycosylated and N-unglycosylated forms. Therefore, we propose that N-glycosylation does not function to support the overall conformation of α subunit but rather plays a critical role in maintaining the appropriate conformation of the binding site.

Tunicamycin reduced the number of GM-CSF binding sites 3-fold and decreased the receptor affinity 2-fold in HL-60(eos). Based on our data in COS cells indicating that N-unglycosylated α subunit does not bind GM-CSF, we reason that in HL-60(eos), tunicamycin blocked N-glycosylation and led to synthesis of N-unglycosylated α subunits, which did not bind GM-CSF. The turnover of previously synthesized N-glycosylated α subunit led to a decreased number of GM-CSF binding sites. The 85% decrease in binding sites caused by tunicamycin over 24 h suggests that the half-life of α subunit on the membrane is less than 10 h. The decreased affinity in HL-60(eos) resulting from tunicamycin treatment may be due to partially N-glycosylated forms of α subunit. This concept is supported by our observation that partial removal of N-glycosylation from α subunit by N-glycosidase F led to inhibition but not complete abrogation of GM-CSF binding.

GM-CSF binding in α- and β-cotransfected COS cells was abolished by tunicamycin, as it was in COS cells transfected with α subunit alone. The β subunit is a 120-kDa glycoprotein with an apparent molecular mass substantially larger than that (96 kDa) calculated on the basis of the amino acid sequence deduced from its cDNA (6). The β subunit contains three consensus N-glycosylation sites in the extracellular domain (6). Our experiments did not assess the contribution of N-glycosylation of β subunit in high affinity GM-CSF binding because unglycosylation of α subunit alone abolished all GM-CSF binding.

GM-CSF is a glycoprotein in which glycosylation is not required for its biologic activity. Unglycosylated GM-CSF produced by E. coli is fully active (37). On the other hand, we have demonstrated that GM-CSF receptor α subunit requires N-glycosylation for binding and signaling. Given that glycosylation may vary in pattern and extent among cells of different types (38–41), N-glycosylation of the α subunit may serve as a means to modulate cellular responsiveness to GM-CSF. Variations in binding affinity of GM-CSF receptors in different cells (42) could be explained by differences in glycosylation. Finally, the α subunits of interleukin-3 and interleukin-5 receptors, which share a common β subunit with GM-CSF receptor (43, 44), are also glycoproteins; the importance of N-glycosylation in GM-CSF receptor function may have implication for interleukin-3 and interleukin-5 receptors.

Acknowledgment—We thank Rong-Hua Zhang for excellent technical assistance.

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