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EVIDENCE FOR THE GLYCOSYLATION OF THE GRANULOCYTE COLONY-STIMULATING FACTOR RECEPTOR

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Summary: The granulocyte colony-stimulating factor receptor (G-CSFR) was overexpressed in WEHI-3B D^+ myelomonocytic leukemia cells by the transfection of an expression plasmid containing the murine G-CSFR cDNA. Two different forms of the G-CSFR were observed in these cells by western blotting. Metabolic labeling and cell surface labeling demonstrated that the majority of the G-CSFR exists in a non-mature form and is presumably present in the cytoplasm as a 115-kDa protein. A relatively small portion of the G-CSFR is present as the fully mature form on the cell surface as a 150-kDa protein; this form of the G-CSFR binds to granulocyte colony-stimulating factor (G-CSF). Both the mature and non-mature forms of the G-CSFR appear to be N-glycosylated, as determined by glycanase digestion and inhibition of glycosylation by tunicamycin. Glycosylation of the G-CSFR may be of importance for the transport of the receptor to the cell surface.

Granulocyte colony-stimulating factor receptor (G-CSFR) is a cell surface protein which plays an important role in the regulation of the proliferation and differentiation of granulocyte progenitor cells, as well as in the survival and function of mature granulocytes through its interaction with granulocyte colony-stimulating factor (G-CSF) (1-4) Molecular analysis of the structure of the G-CSFR demonstrates that it consists of an immunoglobulin-like domain, a cysteine-rich ligand binding domain, three fibronectin type III modules, a membrane proximal WSxWS motif, a transmembrane domain and a cytoplasmic domain (5, 6). Different forms of the human G-CSFR, resulting from alternative splicing of G-CSFR mRNA, have been identified (7). Sequence analysis of the G-CSFR cDNA has shown that the extracellular domain of the murine G-CSFR has 13 putative sites of N-glycosylation (5). No direct experimental evidence exists, however, for glycosylation of the receptor. In addition, the role of glycosylation of the G-CSFR in the transduction of growth and differentiation signals and in the transport of the G-CSFR

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Abbreviations: G-CSF, granulocyte colony-stimulating factor; G-CSFR, G-CSF receptor; PBS, phosphate-buffered saline (50 mM sodium phosphate, 150 mM NaCl, pH 7.2); SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TBST, Tris-buffered saline with Tween-20 (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20); digitonin buffer, 1% digitonin, 10 mM triethanolamine, pH 7.8, 0.15 M NaCl, 10 mM iodoacetamide, 1 mM PMSF, 1 mM EDTA, 2 mM *O*-phenanthroline, 1 μg/ml of leupeptin, 1 μg/ml of pepstatin, 1 μg/ml of aprotinin.

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to the cell surface is unknown. To study the biochemical and molecular mechanisms by which various biological effects are exerted through the binding of G-CSF to its receptor, we have established a series of clones from WEHI-3B D^+ myelomonocytic leukemia cells, transfected with a G-CSFR expression plasmid, which exhibit high levels of the receptor. We have found that the predominant role of the G-CSFR in these WEHI-3B D^+ clones is to mediate differentiation (8). In this report, we present evidence for the post-translational glycosylation of the G-CSFR and speculate on its role in the transport of the G-CSFR to the cell surface.

MATERIALS AND METHODS

Cell culture: WEHI-3B D^+ and WEHI-3B D^- leukemia cells and a G-CSFR transfected clone (D+GR6) of WEHI-3B D^+ described previously (8) were maintained in suspension culture in X-VIVO-15 serum-free medium (BioWhittaker, Wakersville, MD) at 37°C in a humidified atmosphere of 95% air/5% CO_2. When necessary, 10 μM $CdCl_2$ was included in the medium to stimulate the expression of the G-CSFR, which was under the control of the human metallothionein promoter.

Western blotting: Cells were collected by centrifugation at 300g for 5 min and resuspended at a level of 10^7 cells/ml in PBS containing protease inhibitors (2 mM PMSF, 2 mM EDTA, 2 mM *O*-phenanthroline, 1 μg/ml of leupeptin, 1 μg/ml of pepstatin, 1 μg/ml of aprotinin), mixed with an equal volume of 2x loading buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol). The extract was sonicated to fragment genomic DNA, boiled for 5 min, and subjected to 7.5-15% gradient SDS-PAGE. Proteins were then transferred onto a nitrocellulose membrane with a SemiPhor Semi-dry transfer unit (Hoffer, San Francisco, CA). The membranes were blocked with 10% dry milk in TBST for 1 h at 37°C, rinsed twice with TBST-M (TBST containing 5% dried milk) for 10 min each, incubated with rabbit polyclonal anti-G-CSFR antibody (1:500 diluted in TBST-M) produced in this laboratory by the methodology described by Fukunaga et al. (9) for 1 h at 37°C, and washed with four changes of TBST-M for a total of 1 h. The membrane was then switched to room temperature (about 25°C) and incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG for 1 h, and washed for 2 h with TBST-M (six changes). The immunoreactive proteins were visualized by the enhanced chemiluminescence method (Amersham, Arlington Heights, IL).

Metabolic labeling with [^{35}S]methionine and immunoprecipitation: One x 10^7 exponentially growing cells were washed twice with pre-warmed serum-free and methionine-free RPMI 1640 medium, resuspended in medium at 5 x 10^6 cells/ml, incubated at 37°C for 15 min, pelleted, resuspended and labeled in 0.5 ml of RPMI 1640 medium containing [^{35}S]-methionine (1 mCi/ml) and 10% fetal bovine serum. Cells were then lysed with 200 μl of digitonin buffer by rotating cultures at 4°C for 15 min. Lysates were centrifuged at 10,000g for 30 min, precleared with killed, fixed *S. aureus* for 1 h at 4°C, and incubated with anti-G-CSFR antibody (1:200 dilution) overnight. Protein A-Agarose was then added and the incubation was continued for 2 h with rotation. The G-CSFR-antibody-protein A-Agarose complex was collected by centrifugation and washed with digitonin buffer six times. The G-CSFR was released by boiling for 5 min in 1x loading buffer and separated by SDS-PAGE. Gels were dried and subjected to autoradiography.

Cell surface labeling with Na[^{125}I]: Cells ($1x10^7$) were collected, washed with PBS three times and resuspended in 100 μl of 50 mM Tris-HCl, pH 7.4, and 0.15 M NaCl. Two iodobeads (Pierce, Rockford, IL) were washed twice with 1 ml of PBS, dried, then incubated with 1 mCi of Na[^{125}I] in 90 μl of 50 mM Tris-HCl, pH 7.4, and 0.15 M NaCl for 5 min. The cell suspension was then added and the mixture incubated for 30 min at room temperature. After labeling, cells were washed three time with PBS by centrifugation at 3000 rpm in an Eppendorf 5415C centrifuge for 2 min, and resuspended in 0.5 ml of digitonin buffer. Extracts were then subjected to immunoprecipitation and SDS-PAGE as described above.

Chemical cross-linking of [^{125}I]rhG-CSF to the G-CSFR: Mutant rhG-CSF (Tyr1,3-rhG-CSF; a gift from Kirin Brewery Co. Ltd., Japan) was iodinated with Na[^{125}I] following previously published procedures (10). [^{125}I]rhG-CSF was incubated with D+GR6 cells and cross-linked to the G-CSFR with disuccinimidyl suberate and disuccinimidyl tartarate as described previously (8).

Digestion of glycosylated proteins: N-Glycanase and O-glycanase were obtained from Genzyme (Cambridge, MA) and neuraminidase from Boehringer Mannheim (Indianapolis, IN). Proteins were digested by the enzymes following the manufacture's procedures.

RESULTS AND DISCUSSION

WEHI-3B D$^+$ leukemia cells express a low level of G-CSFRs, as determined by northern hybridization and binding of G-CSF to its receptor on the cell surface (5, 8, 11). High levels of expression of the G-CSFR, as measured by specific binding of rhG-CSF to cells and by chemical cross-linking experiments, were obtained by transfection of these cells with the expression plasmid pGR7-2 (8). The high level of G-CSFR expression in clone D+GR6 was demonstrated by western blotting using a rabbit polyclonal anti-G-CSFR antibody produced in this laboratory, by the procedure of Fukunaga et al. (9) (Figure 1). Two specific bands of 150 kDa and 115 kDa

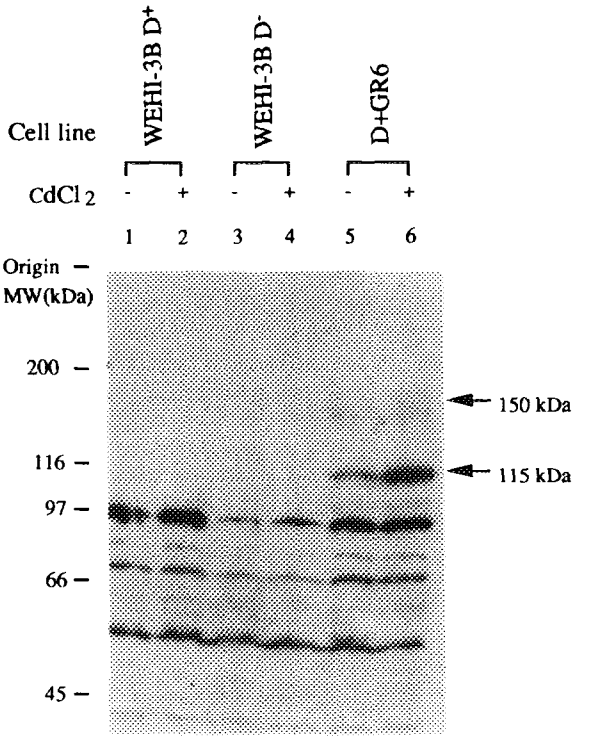

Figure 1. Overexpression of the G-CSFR in transfected WEHI-3B D$^+$ cells as determined by Western blotting. Cells were grown in serum-free medium in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of 10 μM $CdCl_2$. Total cellular proteins were separated by SDS-PAGE. WEHI-3B D$^+$, lanes 1 and 2; WEHI-3B D$^-$, lanes 3 and 4; D+GR6, lanes 5 and 6.

were detected in the G-CSFR overexpressing clone D+GR6 by the antibody (lanes 5 and 6). The 150 kDa species is the form that binds to G-CSF, as shown by the finding that the G-CSF-receptor complex is about 170 kDa, determined by chemical cross-linking experiments, given that the rhG-CSF is 20 kDa. The 115 kDa species is presumed to be a non-mature form of the G-CSFR present in the cytoplasm, since it was not detected by cell surface labeling (Figure 2A). The 115 kDa band is the major species of the G-CSFR formed, which indicates that most of the overexpressed receptor exists in an immature form. It is not clear whether the immature G-CSFR has any function in these cells. The level of expression of the G-CSFR in WEHI-3B D^+ parental cells was suffiently low that it could not be detected under the conditions employed (Figure 1, lanes 1 and 2). G-CSFR negative WEHI-3B D^- cells, used as a negative control, as expected did not exhibit detectable G-CSFR (Figure 1, lanes 3 and 4).

To determine whether post-translational modification of the overexpressed G-CSFR occurs, D+GR6 cells were metabolically labeled with [^{35}S]-methionine. Newly synthesized G-CSFR was immunoprecipitated with the anti-G-CSFR antibody and analyzed by autoradiography.

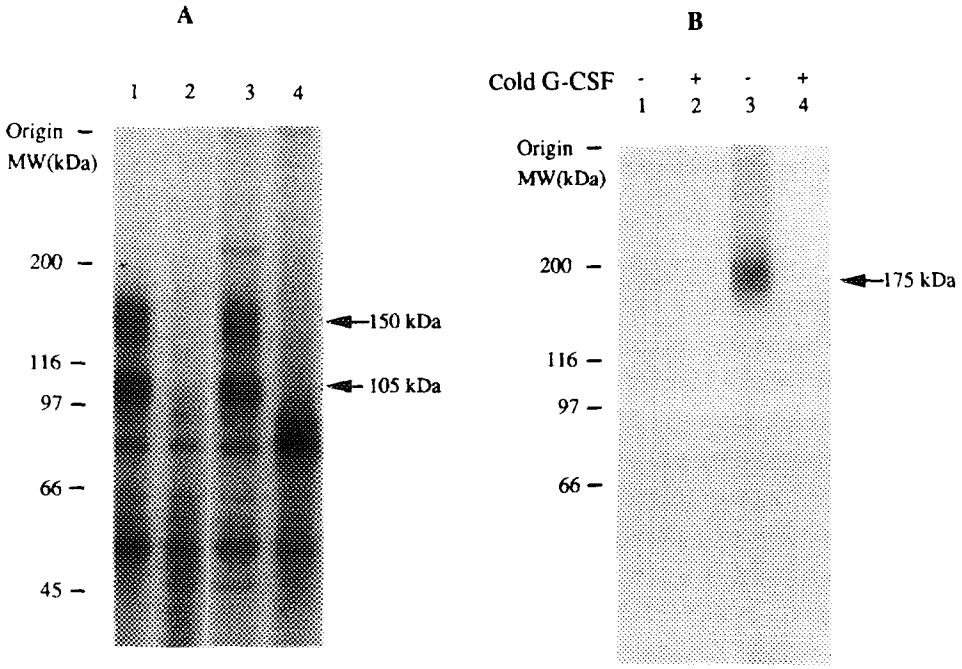

Figure 2. (A) Detection of cell surface G-CSFR. D+GR6 cells were cultured in the absence (lanes 1, 3 and 4) or presence (lane 2) of tunicamycin for 2 days to block glycosylation. Cell surface proteins were labeled with Na[^{125}I] and immunoprecipitated with anti-G-CSFR antibody. The immunoprecipitates were incubated without (lane 3) or with N-glycanase (lane 4). (B) Determination of the species of the G-CSFR that binds to G-CSF by chemical cross-linking of [^{125}I]rhG-CSF to the receptor. WEHI-3B D^+ and D+GR6 cells were incubated with 2 nM [^{125}I]rhG-CSF in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of a 100-fold excess of unlabeled rhG-CSF. [^{125}I]rhG-CSF was then cross-linked to its receptor with disuccinimidyl suberate and disuccinimidyl tartarate and subjected to SDS-PAGE. WEHI-3B D^+, lanes 1 and 2; D+GR6, lanes 3 and 4.

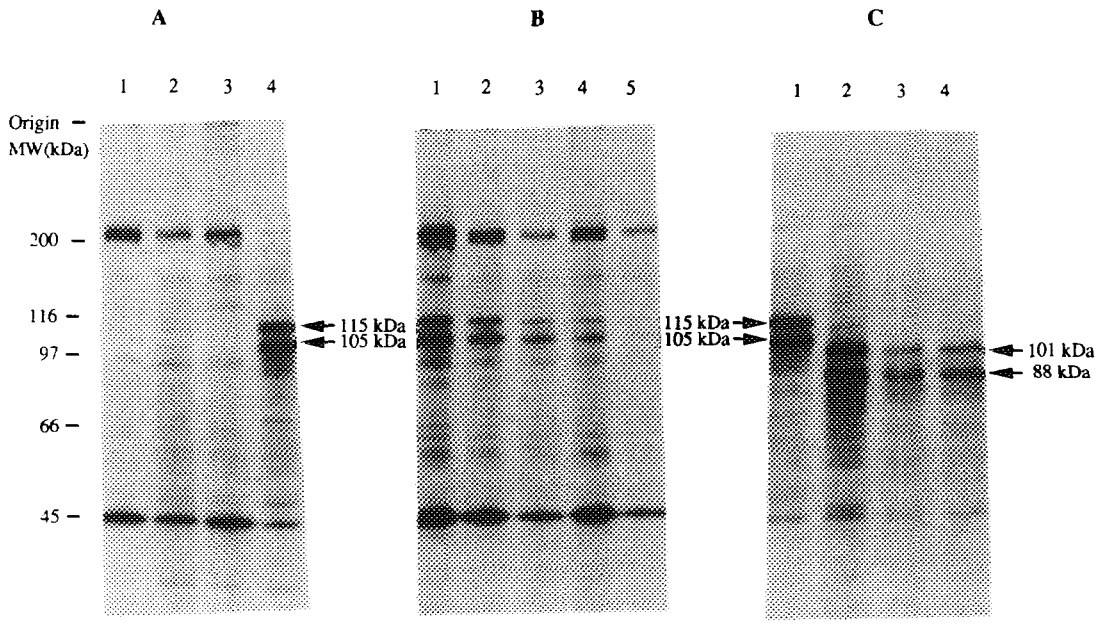

Figure 3. Evidence for the glycosylation of the G-CSFR. The G-CSFR was metabolically labeled with [^{35}S] methionine and immunoprecipitated with a polyclonal anti-G-CSFR antibody. (A) Comparison of the expression of the G-CSFR in parental WEHI-3B D$^+$ cells (lanes 1 and 2) and the G-CSFR transfected D+GR6 clone (lanes 3 and 4). Lanes 1 and 3, pre-immune serum; lanes 2 and 4, anti-G-CSFR antibody. (B) Pulse-chase labeling of the G-CSFR. D+GR6 cells were labeled for 15 min with [^{35}S] methionine (lane 1), then chased with excess cold methionine for 30, 60, 90 and 180 min (lanes 2, 3, 4 and 5, respectively). (C) Digestion of the G-CSFR isolated by immunoprecipitation. Lane 1, undigested; lane 2, N-glycanase; lane 3, N-glycanase and neuraminidase; lane 4, N-glycanase, neuraminidase and O-glycanase.

Two specific proteins of about 115 kDa and 105 kDa (Figure 3A, lane 4) were detected by the antibody, but not by the pre-immune serum. The 115 kDa immunoprecipitated protein is the major band detected by western blotting (Figure 1, lane 4). The identity of the 105 kDa protein is unknown. It is conceivable that it is a different form of the G-CSFR or a G-CSFR-associated protein co-precipitated by the antibody.

The expression of endogenous G-CSFR in parental WEHI-3B D$^+$ cells was very low and was barely detectable by the antibody under the conditions employed (Figure 3A, lane 2). The size of the endogenous G-CSFR was the same as the exogenous expressed G-CSFR. When the G-CSFR was labeled for 15 min with [^{35}S]-methionine and then chased with unlabeled methionine, the 115 kDa and 105 kDa proteins disappeared gradually over 3 h, with no apparent conversion of these proteins into higher species being observed (Figure 3B). These findings suggested that these proteins were being degraded, with a half-life of about 1.5 h.

To obtain evidence for the post-translational glycosylation of the G-CSFR, newly synthesized G-CSFR was isolated by immunoprecipitation, treated with enzymes that produce deglycosylation and analyzed by SDS-PAGE (Figure 3C). The molecular weights of both the 115 kDa and 105 kDa proteins were decreased by digestion with N-glycanase, but were not affected by digestion with neuraminidase or a combination of neuraminidase and O-glycanase, suggesting that

the protein is N-glycosylated and probably not O-glycosylated. After N-glycanase digestion new bands appeared at 101 kDa and 88 kDa. Although the 88 kDa species is closer to the predicted molecular weight of the murine G-CSFR, estimated to be 90 kDa by cDNA sequence analysis, it is difficult to determine which of these two proteins is the G-CSFR. These findings also imply that the G-CSFR is glycosylated immediately after being synthesized, since no bands of these relatively low molecular weight proteins were detected, even when cells were labeled for only 15 min.

To provide further evidence for the glycosylation of the G-CSFR, D+GR6 cells were grown in the presence of the N-glycosylation inhibitor tunicamycin for 2 days at concentrations of from 0.1 to 0.3 μg/ml; at these levels of tunicamycin the growth of D+GR6 cells was not markedly affected. Therefore, cellular extracts were prepared and analyzed by western blotting (Figure 4). The intensity of the G-CSFR band at 115 kDa decreased with increasing concentrations of tunicamycin and a new smaller molecule appeared, whose intensity increased as the level of tunicamycin was increased. These findings provide confirming evidence that the G-CSFR is N-glycosylated.

Since only a small portion of the G-CSFR exists as the mature ligand-binding form and the metabolic labeling with [^{35}S]-methionine revealed that the major form of the G-CSFR existed as a non-mature form, cell surface proteins were labeled with Na[^{125}I] to ascertain whether the mature

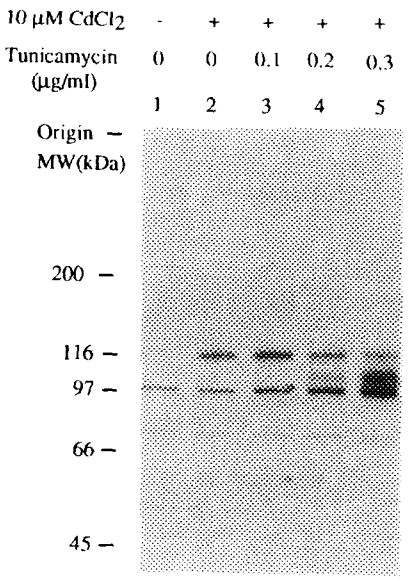

Figure 4. Effects of tunicamycin on the glycosylation of the G-CSFR. D+GR6 cells were grown in the absence or presence of various concentrations of tunicamycin for 2 days and subjected to Western blotting as described in Materials and Methods.

cell surface form of the G-CSFR was glycosylated. The ^{125}I-labeled G-CSFR was immunoprecipitated, digested with N-glycanase and analyzed by SDS-PAGE and autoradiography (Figure 2A). Two proteins were precipitated by the antibody (lanes 1 and 3). The 150 kDa protein corresponds to the form of the G-CSFR that binds to G-CSF, as determined by chemical cross-linking (Figure 2B); the identity of the 105 kDa protein is unknown. After N-glycanase digestion both species became smaller and a newly formed band of about 83-92 kDa, close to the predicted unmodified molecular weight of murine G-CSFR (Figure 2A, lane 4), was observed. When D+GR6 cells were treated with 0.3 μg/ml of tunicamycin for 2 days before cell surface labeling with Na[^{125}I], little or no specific proteins were precipitated by the antibody, suggesting that the relatively low amount of mature G-CSFR present on the cell surface is due to the lack of glycosylation of the receptor. These findings support the concept that glycosylation of the G-CSFR is necessary for the transport of the receptor to the cell surface.

The G-CSFR can mediate both growth-stimulatory and differentiation-promotion signals. In WEHI-3B D$^+$ cells, the G-CSFR is involved primarily in controlling differentiation (8). In FDC-P1 cells, the G-CSFR primarily stimulates proliferation (9). Since both FDC-P1 and WEHI-3B D$^+$ cells overexpress the same transfected G-CSFR gene, it is important to determine how these different physiological signals are transduced by the same receptor in these different cell lines. Studies on the glycosylation and other post-translational modifications of the G-CSFR and the role of these modifications in the transport of the receptor to the cell surface and in ligand binding may assist in the elucidation of the mechanism by which different signals are transduced.

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