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# Role of Carbohydrate Modification in the Production and Secretion of Human Granulocyte Macrophage Colony-Stimulating Factor in Genetically Engineered and Normal Mesenchymal Cells†

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ABSTRACT: Colony-stimulating factors (CSFs) are a group of acidic glycoproteins which stimulate the proliferation and differentiation of hematopoietic progenitor cells in vitro and stimulate hemopoiesis in vivo. Human GM-CSF contains two N-linked carbohydrate side chains of the complex acidic type and several sites of O-linked carbohydrate clustered on serine and threonine residues near the N-terminus of the molecule. Previous studies have failed to detect a significant functional role for the carbohydrate modification characteristic of human GM-CSF. Using permanent cell lines and transient expression systems which produce moderate to high levels of native or carbohydrate-deficient forms of the growth factor, the role of carbohydrate modification in the biosynthesis and secretion of GM-CSF was studied. Unlike a number of other secreted glycoproteins, the transit time and secretory efficiency of several carbohydrate-deficient mutants of GM-CSF are indistinguishable from those of the native growth factor in BHK, 293, COS, and ldlD cells. Furthermore, normal human endothelial cells and fibroblasts, which normally produce the growth factor, can synthesize and secrete GM-CSF that lacks all forms of carbohydrate modification. These studies help to point out the range of roles played by carbohydrate modification in the biosynthesis, assembly, and secretion of glycoprotein hormones.

Granulocyte macrophage colony-stimulating factor (GM-CSF) is an acidic glycoprotein required for the survival, proliferation, and differentiation of hematopoietic progenitor cells in semisolid culture systems and participates in the functional activation of mature blood cells (Metcalf, 1986; Clark & Kamen, 1987). Recently, recombinant human (hu) GM-CSF was shown to stimulate hematopoiesis in vivo and is thus a useful therapeutic agent in the recovery of bone marrow function following cytotoxic therapy (Groopman et al., 1987) and in a number of conditions of bone marrow failure (Vadhan-Raj et al., 1987).

The primary structure of hu GM-CSF contains 144 amino acids, including a 17-residue secretory leader sequence, resulting in a predicted molecular mass of 14 465 daltons for the mature polypeptide. When analyzed by a number of physicochemical means, natural and recombinant hu GM-CSF is quite heterogeneous. Initial studies using inhibitors of N-linked glycosylation, such as tunicamycin or 2-deoxyglucose, suggested that much of the size and charge heterogeneity characteristic of the CSFs is due to variable degrees of sialic acid containing glycosylation (Tsuneoka et al., 1981). This has now been clearly demonstrated using site-directed mutants of human (Donahue et al., 1986; Kaushansky et al., 1987) and murine (Miyajima et al., 1986) GM-CSF.

The functional role of the carbohydrate modification of hu GM-CSF is not clearly understood. Several recent studies have suggested that forms which lack some or all of the carbohydrate modification normally present on GM-CSF are fully active in a number of biological assays (Sparrow et al., 1985; Kaushansky et al., 1987; Moonen et al., 1987). In fact, most reports suggest that hu GM-CSF which lacks N-linked carbohydrate has a significantly enhanced specific activity in vitro when compared to the native recombinant growth factor (Kaushansky et al., 1987; DeLamarter et al., 1987; Moonen et al., 1987). In addition, although the initial tissue distribution

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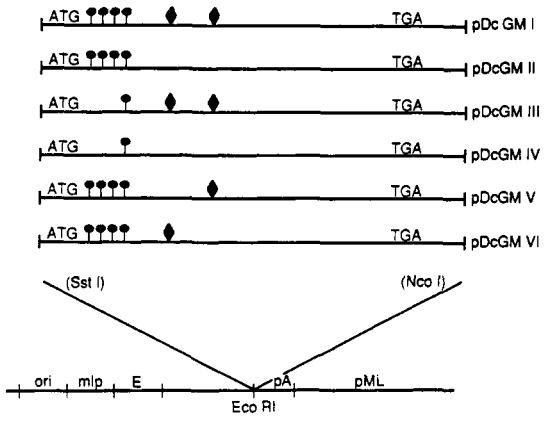


FIGURE 1: Native and carbohydrate-deficient GM-CSF expression vectors. GM-CSF expression vectors [derived from the plasmid vector pML (Lusky & Botchan, 1981)] contain the major late promoter (mlp) of adenovirus, the origin of replication (ori), the enhancer (E), and the polyadenylation signal (pA) of SV 40 and contain either the native human GM-CSF cDNA (cGM I) or one which has an Asn to Gln or a Ser to Ala mutation(s) in the codon(s) for $Asn_{44}$, $Asn_{54}$, $Ser_{22}$, $Ser_{24}$, or $Ser_{26}$ (Kaushansky et al., 1987; it should be noted that this reference incorrectly identified the three Ser residues as $Ser_{20}$, $Ser_{22}$, and $Ser_{24}$). Asn-linked carbohydrate sites (♦), Ser- or Thr-linked carbohydrate sites (●), and the initiation (ATG) and termination (TGA) codons are shown.

phase of GM-CSF is modestly affected, the circulatory survival and therapeutic efficacy of the growth factor are not significantly altered by the elimination of N-linked or O-linked carbohydrate (Donahue et al., 1986; Kaushansky, 1989).

A number of recent reports have suggested that carbohydrate modification is critically involved in the biosynthesis, assembly, or processing of a number of secreted glycoproteins (Machamer et al., 1985; Dube et al., 1988; Matzuk & Boime, 1988; Dorner et al., 1987). To understand the role of carbohydrate modification in the biosynthesis and secretory processing of hu GM-CSF, we have established and studied permanent BHK- and ldlD-derived cell lines which secrete moderate to large amounts of either native GM-CSF or one of three carbohydrate-deficient mutants of the molecule, and transient expression of six different carbohydate-deficient forms of the growth factor in several transformed cell lines. Our results suggest that neither N-linked nor O-linked carbohydrate modification is required for the biosynthesis or secretion of hu GM-CSF in these cell lines. To test the hypothesis that carbohydrate modification is required for the biosynthesis or secretion of the growth factor in cells which normally produce the protein, human dermal fibroblasts (HDFs) and human umbilical vein endothelial cells (HUVEs) were also studied. These results confirm those found in the transformed cell lines, and along with other studies help to point out the diverse roles played by carbohydrate modification in the biosynthesis, assembly, and secretion of glycoprotein hormones.

## Materials and Methods

*Cell Lines.* Several carbohydrate-deficient GM-CSF cDNA expression vectors were generated by site-directed mutagenesis as previously described (Kaushansky et al., 1987) and are shown in Figure 1. BHK cells were obtained from ATCC (Rockville, MD), and the ldlD cells were kindly provided by Monty Krieger (Massachusetts Institute of Technology, Cambridge, MA). Expression vectors were cotransfected into BHK cells along with an expression vector containing the resistant form of the dihydrofolate reductase gene ($DHFR^R$) by the calcium phosphate method (Graham & Van der Eb, 1973). Expression vectors were transfected into ldlD cells by the same method (except that the glycerol shock was omitted) along with an expression vector encoding the neomycin resistance ($neo^R$) gene. Primary transfectants were split into culture medium containing methotrexate or G418, and individual colonies were isolated. Levels of secreted GM-CSF were determined by immunoassay (ELISA; Brown et al., 1990), and for the $DHFR^R$-containing cell lines, they were amplified by successive passage through increasing concentrations of methotrexate. BHK cell lines were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics; ldlD cell lines were grown in 50% DMEM and 50% Ham's F12 medium supplemented with either 5% FCS or, for elimination of O-linked carbohydrate on secreted proteins, with the $ITS^+$ serum substitute (Collaborative Research, Bedford, MA). For pulse labeling, 100-mm plates of confluent cell lines or cells transiently expressing each of the carbohydrate-deficient mutants were washed twice in PBS and incubated in 7 mL of DMEM devoid of cysteine supplemented with 1% dialyzed FCS, antibiotics, and 50 $\mu$Ci/mL [$^{35}$S]cysteine. Following a 2–4-h labeling, the cells were washed twice and incubated in regular culture medium.

*Transient Transfection of Cell Lines and Primary Mesenchymal Cells.* Each of the GM-CSF expression vectors was transfected into BHK, simian COS or human kidney cell line 293 cells by the calcium phosphate method when 30% confluent (Graham & Van der Eb, 1973). Tenth–twelfth-passage primary human dermal fibroblasts (HDFs) and second–fourth-passage human umbilical vein endothelial cells (HUVEs) were harvested when 80% confluent and transfected by electroporation. A total of $2 \times 10^6$ cells were trypsinized, washed in PBS, and resuspended in 1 mL of HBS electroporation buffer [21 mM Hepes (pH 7.05)/137 mM NaCl/5 mM KCl/0.7 mM $Na_2HPO_4$/6 mM glucose], and electroporated. The conditions of electroporation were 1000 V/cm and 60 $\mu$F (Cellporator, BRL, Gaithersberg, MD) at room temperature for HDFs and at 4 °C for HUVEs. The cells were allowed to recover for 10 min, and following transfection, the cells were divided into three plates, and each was incubated for varying lengths of time in regular culture medium. As a control for transfection efficiency, cells were cotransfected with the plasmid vector RSV$\beta$-gal (Bornstein & McKay, 1988).

*Analysis of GM-CSF Production.* Conditioned culture medium was harvested from transiently transfected cells and from metabolically labeled cell lines after incubation for 1–96 h. Cell monolayers were trypsinized, washed twice with PBS, and then divided for $\beta$-galactosidase and GM-CSF determinations. For GM-CSF ELISA (Brown et al., 1990), conditioned culture medium or the cell pellet [lysed in 10 nM Tris (pH 7.5)/5 mM EDTA/0.15 M NaCl/1% deoxycholate/1% Triton X-100/0.1% $NaDodSO_4$] was diluted for assay. For the purposes of this study, we determined that the ELISA detects nonglycosylated forms of GM-CSF 2-fold more efficiently than the native form of the growth factor. The reported values reflect this difference. For determination of $\beta$-galactosidase activity, cell pellets were lysed by three successive freeze–thaw cycles, the cell debris was removed by centrifugation, and dilutions of the supernatant were assayed as previously described (Bornstein & McKay, 1988). Aliquots from metabolically labeled cells were immunoprecipitated with a polyvalent rabbit antiserum to human GM-CSF and Staph A. This antiserum recognizes all forms of GM-CSF equally well. The immunoprecipitates were size-fractionated by electrophoresis through 12% polyacrylamide gels (Laemmli,

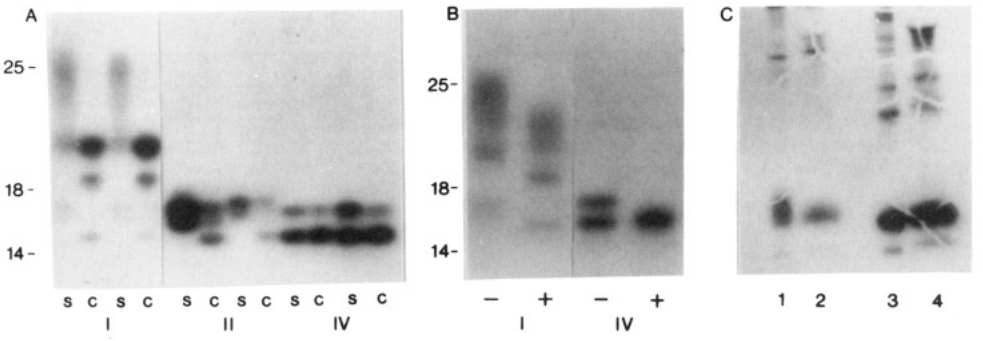


FIGURE 2: Metabolic labeling of native and two carbohydrate-deficient forms of human GM-CSF. (A) Three forms of GM-CSF (cGM I, cGM II, and cGM IV), each produced in two independent BHK-derived cell lines, were metabolically labeled by exposing the cells to a 4-h pulse of [$^{35}$S]cysteine. Cell-associated (c) and secreted (s) GM-CSF was immunoprecipitated with a specific polyvalent antibody and Staph A, size-fractionated by polyacrylamide gel electrophoresis, and exposed to X-ray film overnight. Size markers (kDa) are shown on the left. This experiment has been repeated twice with similar results. Panel B shows an autoradiogram of metabolically labeled cGM I and cGM IV before (−) and after (+) treatment with neuraminidase and *O*-glycanase. Panel C shows an autoradiogram of immunoprecipitated cGM II protein produced by ldlD cells grown in regular culture medium (lanes 1 and 2) or ITS$^+$ medium (lanes 3 and 4) which eliminates O-linked glycosylation. The proteins in lanes 2 and 4 were treated with neuraminidase and *O*-glycanase.

1970); the gels were soaked in Enlightening enhancer (New England Nuclear, Boston, MA) and exposed to film with intensifying screens for 1–3 days. The relative intensities of the autoradiogram bands were evaluated by computer-assisted densitometry using exposures in the linear range of photographic densities.

*Enzymatic Treatment of Metabolically Labeled GM-CSF.* Aliquots of metabolically [$^{35}$S]cysteine-labeled GM-CSF were denatured and reduced in 1% $NaDodSO_4$ and 0.75 M 2-mercaptoethanol and treated with 2 units/mL neuraminidase (Boehringer-Mannheim, Indianapolis, IN) at 22 °C for 60 min in the presence of 20 mM Tris–maleate (pH 6.0)/10 mM D-galactono-γ-lactone/1 mM calcium acetate and then with 25 milliunits/mL *O*-glycanase (Genzyme, Cambridge, MA) in the above buffer supplemented with 0.5% NP-40/0.05% $NaDodSO_4$/1 mM dithiothreitol/0.5 mM PMSF and finally analyzed by gel electrophoresis.

## RESULTS

*Permanent Cell Lines.* Several cell lines producing native GM-CSF or forms of the molecule which lack N-linked or O-linked carbohydrate modification were screened by ELISA for levels of secretion. Nine cell lines which secreted either high (approximately 1 mg/L) or low levels of GM-CSF or its mutants were selected for further study. Following a 2–4-h pulse with [$^{35}$S]cysteine, the culture medium and cell lysates were immunoprecipitated and size-fractionated by polyacrylamide gel electrophoresis. When analyzed by computer-assisted densitometry, three independent BHK-derived cell lines which produce native GM-CSF (cGM I) secreted 37–53% of the their growth factor in the 2–4-h culture period. The results from two of these cell lines are shown in Figure 2. Similarly, 46–61% of the GM-CSF lacking N-linked carbohydrate (cGM II) and 47–59% of the GM-CSF lacking N-linked and most of the O-linked carbohydrate (cGM IV) were secreted from six additional cell lines (three producing cGM II and three producing cGM IV) in $^{35}$S pulse experiments.

It is also clear from the data in Figure 2 that most of the size heterogeneity of native GM-CSF results from variable degrees of N-linked carbohydrate modification, as this degree of heterogeneity is not present in the forms which lack N-linked carbohydrate. In addition, as very little of this heterogeneous highest molecular weight form of native GM-CSF was detected in the cell lysates, this modification is likely the last step to occur in the processing and secretion of human GM-CSF. However, an additional site(s) of O-linked carbohydrate is (are) present on human GM-CSF.

Also apparent in Figure 2 is that the cGMs IV produced and secreted by the BHK-derived cell lines have two distinct electrophoretic mobilities. Amino acid sequence analysis of purified cGM IV revealed that the peak for $Thr_{27}$-phenylthiohydantoin is reduced in intensity from that expected for unmodified threonine. This suggested that a proportion of the molecules of cGM IV might contain O-linked carbohydrate at this position. The presence of residual O-linked carbohydrate on cGM IV was confirmed when metabolically labeled protein was treated sequentially with neuraminidase and *O*-glycanase (Figure 2B). The electrophoretic mobility of the deglycosylated material was reduced to that of the more rapidly migrating bands of GM-CSF, in close agreement with the predicted size of the unmodified polypeptide. An additional mutant of human GM-CSF was then produced which contained a Thr to Ala substitution at this position. This mutation partially but incompletely eliminated the residual heterogeneity in the preparation of the growth factor (data not shown). These data thus identify $Thr_{27}$ as containing additional O-linked carbohydrate and add to the previously known sites of such modification of human GM-CSF.

An alternate approach to eliminate all of the O-linked carbohydrate on native and on N-linked carbohydrate-deficient forms of human GM-CSF was thus undertaken. When grown in the absence of serum, the Chinese hamster ovary (CHO) cell derivative ldlD fails to add O-linked carbohydrate to secreted glycoproteins (Matzuk et al., 1987). Stable cell lines derived from ldlD cells were produced which secrete native human GM-CSF or the N-linked deficient form, cGM II. When grown in the absence of serum, the secretory rate of native GM-CSF was reduced by 1%, that of cGM II by 8%. Thus, elimination of all of the carbohydrate failed to substantially reduce the secretory rate of human GM-CSF from this CHO cell line derivative. The complete elimination of O-linked carbohydrate was confirmed in these cells by immunoprecipitation and enzymatic treatment of $^{35}$S metabolically labeled cGM II (Figure 2C).

To analyze the transit time of the various forms of GM-CSF, pulse–chase experiments were performed. Following a 2-h pulse, several of the cell lines were incubated with regular culture medium and harvested 15 min to 2 h later. The culture medium and cell lysates were immunoprecipitated and size-fractionated. Densitometry of the autoradiograms revealed that 13–20% of the native GM-CSF was secreted within 15

Table I: Secretory Efficiency of Carbohydrate-Deficient Mutants of Human GM-CSF in COS Cells and Dermal Fibroblasts[a]

| | level secreted (pg/mL) | | | | | |
|---|---|---|---|---|---|---|
| | COS cells | | | fibroblasts | | |
| | 1 d | 2 d | 3 d | 1 d | 2 d | 3 d |
| cGM I (native) | 780 ± 10 | 990 ± 50 | 4200 ± 10 | 8 ± 10 | 11 ± 16 | 8 ± 7 |
| cGM II | 140 ± 10 | 970 ± 45 | 2200 ± 40 | 22 ± 16 | 20 ± 10 | 46 ± 9 |
| cGM III | 450 ± 15 | 1200 ± 200 | 3700 ± 260 | 11 ± 14 | 18 ± 8 | 75 ± 4 |
| cGM IV | 120 ± 20 | 2400 ± 50 | 7500 ± 80 | 12 ± 9 | 20 ± 10 | 35 ± 6 |
| cGM V | 440 ± 80 | 1500 ± 70 | 4700 ± 200 | 5 ± 7 | 19 ± 7 | 34 ± 10 |
| cGM VI | 1000 ± 90 | 3300 ± 70 | 3800 ± 10 | 6 ± 4 | 6 ± 9 | 11 ± 6 |

[a] The level of GM-CSF in conditioned culture medium of COS cells or HDFs transfected with a number of GM-CSF expression vectors was determined by ELISA 1–4 days (d) following transfection. The results represent the mean of four experiments corrected for the cell number and relative level of β-galactosidase activity present in each culture and, in the case of the fibroblasts, for the low level of GM-CSF present in sham-transfected control cultures.

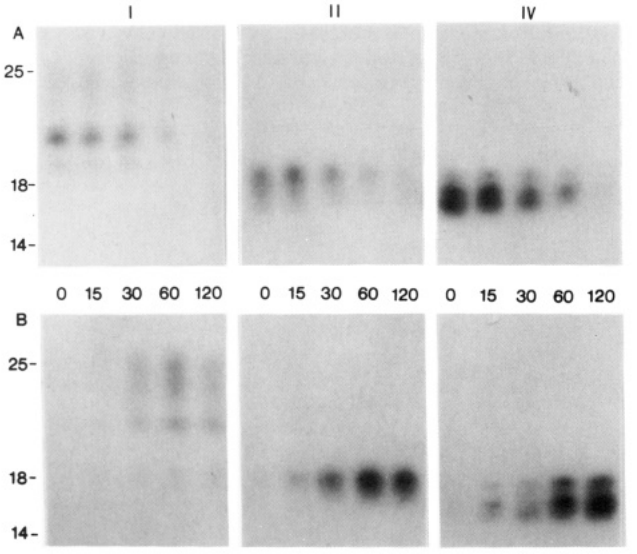


FIGURE 3: Pulse–chase labeling of native and carbohydrate-deficient forms of human GM-CSF. (A) Cell lines which produce native GM-CSF (I) or GM-CSF which lacks N-linked (cGM II, panel II) or N-linked and most of the O-linked carbohydrate (cGM IV, panel IV) were labeled with [$^{35}$S]cysteine for 2 h, washed, and then incubated with regular medium for 15 min to 2 h. GM-CSF present in the cell extracts (A) and culture supernatants (B) were immunoprecipitated and analyzed by gel electrophoresis, autoradiography, and digital densitometry. This experiment has been repeated 3 times with similar results.

min, compared to 15–30% for cGM II and 10–24% for cGM IV. Greater than 95% of all forms of GM-CSF were secreted from the cell within 2 h of synthesis. An example of one of these experiments is shown in Figure 3.

The cloned cell lines we developed for this study were chosen on the basis of their high levels of secretion of GM-CSF. It was thus possible that a selection bias toward those cells which efficiently secrete any form of protein was introduced. We next tested the efficiency of *transient* expression of GM-CSF in a number of cell lines. As shown in Table I, no substantial differences were noted between the amount of native or mutant GM-CSF which was secreted in the first 24–72 h, with the exception of the cGM IV mutant which was initially poorly secreted but which lead to the secretion of an increased level of immunoreactive protein by 2 and 3 days. Similar results were obtained for transient expression in BHK and 293 cells (data not shown).

Finally, to determine whether secretion was independent of carbohydrate modification in cells which normally synthesize the growth factor, the secretory efficiencies of a number of carbohydrate-deficient mutants of GM-CSF were compared to that of the native molecule in human dermal fibroblasts (HDFs). As shown in Table I, HDFs were capable of producing as much or more carbohydrate-deficient GM-CSF as the native protein. Similar results were obtained with human umbilical vein endothelial cells (data not shown).

## Discussion

The role of carbohydrate modification in the biological function of human GM-CSF is not clearly understood. Carbohydrate modification of several glycoproteins including immunoglobulins (Nose & Wigzell, 1983), gonadotropins (Ryan et al., 1988), and the basic fibroblast growth factor receptor (Feige & Baird, 1988) is required for certain aspects of their function. In contrast, a number of secreted and membrane-bound glycoproteins, including human GM-CSF, are fully functional in the absence of their characteristic carbohydrate modification. In this study, we have investigated the role of carbohydrate modification in the biosynthesis and processing of the growth factor.

Using both established cell lines engineered and selected to secrete native GM-CSF and forms deficient in all of the N-linked sites and four sites of O-linked carbohydrate, and transient expression of these growth factors in transformed cells, little effect of carbohydrate modification was found on the secretory efficiency or transit time of GM-CSF. However, as none of the transformed cell lines investigated in this study normally produces GM-CSF, and each has been selected for its ability to secrete high levels of foreign protein, we also tested the hypothesis that carbohydate modification might be required for the efficient processing or secretion of the growth factor by cells which normally produce the molecule.

Human fibroblasts, endothelial cells, monocytes, and T-lymphocytes produce GM-CSF in response to a number of mediators of the inflammatory response (Kaushansky et al., 1988; Fibbe et al., 1988). We transfected normal human fibroblasts and endothelial cells to analyze the requirements for efficient secretion of GM-CSF in these normal cell types. Again, carbohydrate-deficient forms of GM-CSF were secreted at levels very similar to those expressed by the cells producing normal GM-CSF, and at levels similar to that produced by normal mesenchymal cells in response to inflammatory mediators.

It was previously suggested that all of the O-linked carbohydrate was present on $Ser_{22}$, $Ser_{24}$, and $Ser_{26}$ (Donahue et al., 1986; Kaushansky et al., 1987). Our present results demonstrate that a proportion of molecules are additionally modified at $Thr_{27}$ to contain O-linked carbohydrate, at least when produced in BHK cells. As the presence of this site of O-linked carbohydrate modification could have influenced our initial results, we produced an additional mutation in which $Thr_{27}$ was replaced with Ala. Although the transit time of this form of GM-CSF was virtually identical to that of the native polypeptide sequence, this mutation also failed to eliminate

all carbohydrate heterogeneity, suggesting the presence of at least one additional site of O-linked carbohydrate. As there are no reliable predictors for which Ser or Thr residues will be modified, further mutagenesis will be required to identify this last site(s) of O-linked modification of human GM-CSF. Because of these findings, ldlD cells, which have a reversible defect in their capacity to add O-linked carbohydrate to secreted glycoproteins, were used to produce GM-CSF-secreting cell lines and were analyzed for secretory efficiency. The complete elimination of N-linked and O-linked carbohydrate failed to substantially reduce the secretion of GM-CSF from these cells, providing strong evidence against a role for carbohydrate in the secretory efficiency of human GM-CSF.

In addition to these conclusions, our findings may have additional implications for the folding and tertiary structure of GM-CSF. It is clear that improperly folded proteins are not secreted from mammalian cells (Lodish, 1988) and are likely bound in the endoplasmic reticulum by binding proteins (Dorner et al., 1987). The efficient biosynthesis and secretion of carbohydrate-deficient forms of human GM-CSF by a number of cell types imply that the tertiary structure of the protein is not dependent upon its carbohydrate modification. Additional evidence for this conclusion comes from studies comparing the recombinant human GM-CSF produced in bacterial and in animal cells (Wingfield et al., 1988). Both forms demonstrated identical kinetic patterns of urea-induced unfolding, leading these authors to conclude that carbohydrate played no role in the stability of human GM-CSF. A number of recent studies have investigated the role of carbohydrate modification in the biosynthesis of secreted glycoproteins. Dube and co-workers have shown that elimination either of two of the three potential N-linked carbohydrate side chains or of the single site of O-linked carbohydrate modification of human erythropoietin prevents secretion of the molecule (Dube et al., 1988). In a second study which supports the concept of carbohydrate side chain specificity, Matzuk and Boime demonstrated that deficiency of one of the two N-linked carbohydrate side chains of the $\alpha$ subunit of human chorionic gonadotropin (HCG) leads to rapid degradation of the molecule but that elimination of the other side chain alters the formation of HCG $\alpha$–$\beta$ dimers (Matzuk & Boime, 1988). Using permanent cell lines which secrete a number of native or mutant recombinant plasma proteins, Dorner and coworkers showed that elimination of N-linked carbohydrate results in their association with heavy-chain binding protein (BiP, Dorner et al., 1987), a protein located in the rough endoplasmic reticulum responsible for the elimination of excess or denatured secretory proteins (Bole et al., 1986). In contrast, processing of the $\beta$-adrenergic receptor (George et al., 1986) and a number of glycoproteins secreted by a canine kidney cell line (Pan et al., 1987) is independent of carbohydrate modification. Thus, the secretion of human GM-CSF resembles that of these latter glycoproteins and suggests that no general rule may be applied to the role of carbohydrate in the biosynthesis, processing, or secretion of glycoproteins.

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# Inhibition of Protein Kinase C by Annexin V†

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ABSTRACT: Annexin V is a protein of unknown biological function that undergoes $Ca^{2+}$-dependent binding to phospholipids located on the cytosolic face of the plasma membrane. Preliminary results presented herein suggest that a biological function of annexin V is the inhibition of protein kinase C (PKC). In vitro assays showed that annexin V was a specific high-affinity inhibitor of PKC-mediated phosphorylation of annexin I and myosin light chain kinase substrates, with half-maximal inhibition occurring at approximately 0.4 $\mu$M. Annexin V did not inhibit epidermal growth factor receptor/kinase phosphorylation of annexin I or cAMP-dependent protein kinase phosphorylation of the Kemptide peptide substrate. Since annexin V purified from both human placenta and recombinant bacteria inhibited protein kinase C activity, it is not likely that the inhibitor activity was associated with a minor contaminant of the preparations. The following results indicated that the mechanism of inhibition did not involve annexin V sequestration of phospholipid that was required for protein kinase C activation: similar inhibition curves were observed as phospholipid concentration was varied from 0 to 800 $\mu$g/mL; the extent of inhibition was not significantly affected by the order of addition of phospholipid, substrate, or PKC, and the core domain of annexin I was not a high-affinity inhibitor of PKC even though it had similar $Ca^{2+}$ and phospholipid binding properties as annexin V. These data indirectly indicate that inhibition occurred by direct interaction between annexin V and PKC. Since the concentration of annexin V in many cell types exceeds the amounts required to achieve PKC inhibition in vitro, it is possible that annexin V inhibits PKC in a biologically significant manner in intact cells.

Annexins are a family of $Ca^{2+}$ binding proteins that undergo reversible $Ca^{2+}$-dependent binding to phospholipids that are located on the cytosolic face of the plasma membrane (Crompton et al., 1988; Crumpton & Dedman, 1990). They are abundant intracellular proteins, and several different annexin gene products are expressed in all mammalian cells examined to date. The exact physiological functions of the annexins are not yet known. Proposed roles include regulation of membrane traffic and exocytosis (Drust & Creutz, 1988; Ali et al., 1989; Nakata et al., 1990; Sarafian et al., 1991), mediation of cytoskeletal–membrane interactions (Gerke & Weber, 1984; Powell & Glenney, 1987; Ikebuchi & Waisman, 1990), mitogenic signal transduction (De et al., 1986; Pepinsky & Sinclair, 1986; Haigler et al., 1987), transmembrane channel activity (Pollard & Rojas, 1988; Rojas et al., 1990), inhibition of blood coagulation (Funakoshi et al., 1987; Iwasaki et al., 1887; Grundmann et al., 1988; Tait et al., 1988; Hauptmann et al., 1989), and inhibition of phospholipase $A_2$ (Pepinsky et al., 1986, 1988; Wallner et al., 1986). It is not yet known whether different annexins perform different biological functions. It is, of course, also possible that an individual annexin performs different biological functions in different tissues as has been observed for the $Ca^{2+}$ binding protein calmodulin.

It is now important to determine which of the proposed functions of annexins represent actual physiological activities. The inhibition of blood coagulation and phospholipase $A_2$ activity in vitro appears to occur by $Ca^{2+}$-dependent sequestration of phospholipid in the reactions, thereby raising doubts about the physiological significance of these observations (Haigler et al., 1987; Davidson et al., 1987; Funakoshi et al., 1987). These inhibitor studies emphasize the importance of clearly distinguishing between phospholipid sequestration and direct affects when investigating new functions of the annexins.

Protein kinase C (PKC) is a key element in the signal transduction pathway by which a number of extracellular effectors modulate intracellular activity [see Nishizuka (1988) for a review]. Cellular PKC is activated by $Ca^{2+}$-dependent binding of the kinase to phosphatidylserine and diacylglycerol on the cytosolic face of the plasma membrane. Although PKC shares certain $Ca^{2+}$ and phospholipid binding properties with the annexins, there is no structural similarity between the two gene families. Several annexins are good substrates for PKC in in vitro reactions (Gould et al., 1986; Weber et al., 1987; Schlaepfer & Haigler, 1988), and others which have not been studied to date have amino acid sequences that appear to

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