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Depletion of Manganese within the Secretory Pathway Inhibits O-Linked Glycosylation in Mammalian Cells

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ABSTRACT: Proteins transiting the secretory pathway are posttranslationally modified by addition of oligosaccharides to asparagine N-linked and serine and threonine O-linked residues. The effects of divalent cation depletion on oligosaccharide processing of erythropoietin (EPO) and macrophage colony stimulating factor (M-CSF) were studied in Chinese hamster ovary cells. Treatment with A23187 did not inhibit M-CSF or EPO secretion but did inhibit addition of complex N-linked and O-linked oligosaccharides to both molecules. Similar results were obtained by treatment with thapsigargin, a potent inhibitor of the Ca^{2+}-activated microsomal ATPase, indicating that the effect was due to depletion of divalent cations within the secretory pathway. Whereas addition of extracellular calcium chloride did not reverse the inhibition in complex N-linked and O-linked glycosylation, addition of manganese chloride partially reversed both defects. These results are consistent with a specific manganese requirement within the secretory pathway for the processing of complex N-linked oligosaccharides and the addition of O-linked oligosaccharides. Since there are no known specific inhibitors of O-linked glycosylation, the use of ionophores should significantly facilitate studies on the requirement and role of O-linked oligosaccharides in protein structure and function.

Proteins that are destined for the secretory pathway are cotranslationally translocated into the lumen of the endoplasmic reticulum (ER)[1] where a number of modifications occur that facilitate protein folding. These modifications include signal peptide cleavage, disulfide bond formation, subunit assembly, addition of high mannose containing core oligosaccharides to asparagine residues, and other amino acid modifications such as γ-carboxylation of glutamic acid residues and hydroxylation of proline and aspartic acid residues. Exit from the lumen of the ER constitutes a rate-limiting step in intracellular protein transport and is dependent on the rate at which proteins properly fold and assemble (Lodish et al., 1983). The lumen of the ER contains a set of protein chaperones (Gething & Sambrook, 1992), such as GRP78, otherwise known as BiP (Munro & Pelham, 1986), GRP94 (Lee et al., 1984), calnexin (Hammond & Helenius, 1993), calreticulin (Michalak et al., 1992), and ERP72 (Dorner et al., 1990), that bind calcium and regulate calcium flux within the cell and also guide protein-folding reactions. The intralumenal calcium ion concentration can also affect protein-folding and -modification reactions. For example, depletion of intralumenal calcium inhibits N-linked oligosaccharide modification and secretion of specific proteins such as α1-antitrypsin (Kuznetsov et al., 1993; Lodish & Kong, 1990). In addition, depletion of intralumenal calcium can accelerate degradation within the ER (Wileman et al., 1991) and can promote dissociation of the α-subunit of the T-cell receptor from BiP and facilitate its secretion (Suzuki et al., 1991). As

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[1] Abbreviations: M-CSF, macrophage colony stimulating factor; EPO, erythropoietin; ER, endoplasmic reticulum; BiP, immunoglobulin binding protein; GRP78, glucose-regulated protein of 78 kDa; Endo-H, endoglycosidase H; O-Gly, *O*-glycanase; N-Gly, *N*-glycanase; GalNAc, *N*-acetylgalactosamine, CHO, chinese hamster ovary.

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proteins transit the ER into the Golgi apparatus, further modifications occur that include proteolytic processing of propeptides, addition of complex N-linked oligosaccharides, and addition of O-linked oligosaccharides to serine and threonine residues.

The sequential ordered pathway of N-linked oligosaccharide maturation provides information regarding the location of a protein within the secretory pathway. Whereas inhibition of core N-linked oligosaccharide addition can inhibit secretion of specific proteins, maturation of the high mannose containing core oligosaccharide to complex and hybrid type structures is associated with transport from the ER to the Golgi apparatus and is generally not required for protein secretion [for reviews, see Warren (1993) and Helenius (1994)]. However, these modifications do affect protein structure and function in biologically significant ways (Warren, 1993). The importance of proper N-linked oligosaccharide processing within specific polypeptides was determined primarily from the use of specific inhibitors of N-linked oligosaccharide addition and processing (Elbein, 1991). In contrast, there are no known inhibitors for O-linked glycosylation. Therefore, studies on the functional requirements for O-linked oligosaccharide addition require the use of specific mutant cell lines defective in the enzymes required for O-linked glycosylation or the use of specific glycosidases (Krieger et al., 1989; Umemoto et al., 1977). In the course of our studies on the role of intralumenal calcium on posttranslational modification and protein secretion, we observed that depletion of intralumenal manganese specifically inhibited O-linked glycosylation. In this report, we show that intralumenal manganese is required for O-linked oligosaccharide addition on erythropoietin (EPO) and macrophage colony stimulating factor (M-CSF).

EXPERIMENTAL PROCEDURES

Materials. [^{35}S]Methionine (>1000 Ci/mmol) and En^3-Hance were from New England Nuclear Corp. (Boston, MA). A23187, aprotinin, tunicamycin, galactose (Gal), and *N*-acetylgalactosamine (GalNAc) were purchased from Sigma Chemical Co. (St. Louis, MO). Deoxymannojirimycin (DMJ), N-glycanase (N-Gly), endoglycosidase H (Endo-H), and O-glycanase (O-Gly) were from Genzyme Corp. (Boston, MA). Neurominidase was from New England Biolabs (Beverly, MA). Thapsigargin was from L. C. Labs (Woburn, MA). Anti-M-CSF (HMT/4.4.10) and anti-EPO (1.4.1) monoclonal antibodies were kindly provided by Dr. E. Alderman, Genetics Institute (Cambidge, MA). Rabbit anti-mouse IgG antibody was from Southern Biotechnology Associates Inc. (Birmingham, AL).

Cell Culture. Mutant M-CSF-223 was constructed by site-directed mutagenesis to introduce a termination codon after residue 223 within the 4.0-kb M-CSF cDNA (Wong et al., 1987) contained within the expression vector pED (Kaufman et al., 1991). CHO cells that express the truncated form of M-CSF were derived by transfection of DHFR-deficient CHO cells and selection for DHFR gene amplification as described (Kaufman, 1990). A clonal line was obtained that was resistant to 2 μM methotrexate, and it was designated D2. CHO-D2 cells were maintained in complete α-medium containing 10% dialyzed heat-inactivated fetal bovine serum and 2 μM methotrexate in a 5% CO_2 atmosphere. The medium was supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, and 1 mM glutamine. For labeling experiments the same supplements plus 1% aprotinin and 2% fetal bovine serum were added to all media. The derivation, propagation, and characterization of the mutant CHO ldlD cell line that expresses EPO (clone ldlD-A1) were previously described (Wasley et al., 1991).

Radiolabeling and Immunoprecipitation. Cells were labeled with [^{35}S]methionine essentially as described (Dorner & Kaufman, 1990). Briefly, cells were labeled for 20 min with 200 μCi/mL [^{35}S]methionine, and then a chase was performed for 2 h by removing the label and adding complete medium. Cells were pretreated with A23187 (5 μM, a concentration that did not reduce cell viability), thapsigargin (10 μM), or tunicamycin (10 μg/mL) for 1 h or with DMJ (3 mM) for 3 h prior to labeling, and these agents were also present during the metabolic pulse-labeling and chase period. The concentration of 5 μM A23187 was optimized for the CHO-D2 cell line for minimal inhibition of protein synthesis with maximal inhibition of glycosylation. Treatment with 2.5 μM A23187 partially inhibited glycosylation, whereas 7 μM A23187 severely inhibited protein synthesis (greater than 80%). The ldlD-A1 cells displayed greater sensitivity to inhibition of protein synthesis upon treatment with 5 μM A23187. Thus, the narrow dose–response curve requires that the concentration of A23187 be optimized for different cell lines. Where indicated, $CaCl_2$ or $MnCl_2$ was added either during or immediately after the A23187 pretreatment. ldlD-A1 cells were labeled in the presence of galactose (10 μM) in the presence or absence of *N*-acetylgalactosamine (GalNAc) (100 μM) as previously described (Wasley et al., 1991). Samples of conditioned medium were immunoprecipitated with monoclonal antibodies directed against EPO or M-CSF. Immunoprecipitated proteins were electrophoresed on SDS–polyacrylamide gels and visualized after treatment with En^3-Hance. Samples were digested with N-Gly, Endo-H, and/or O-Gly with neuraminidase as described (Dorner & Kaufman, 1990).

RESULTS

M-CSF is a hematopoietic growth factor required for proliferation and differentiation of the monocytic lineage (Stanley & Heard, 1977). Two cDNA clones of 4.0 and 1.6 kb were isolated and expressed in several types of eukaryotic cells (Wong et al., 1987; Rettenmier & Roussel, 1988; Manos, 1988). The translation product from the 1.6-kb cDNA clone encodes a 256 amino acid polypeptide including a signal sequence, an extracellular biologically active growth factor domain, a transmembrane domain, and a short cytoplasmic tail (Kawasaki et al., 1985). The translation product from the 4-kb cDNA clone is identical to the product of the 1.6-kb cDNA with the inclusion of an additional extracellular 298 amino acids derived by alternative splicing (Wong et al., 1987). The product encoded by the 4-kb cDNA clone yields a cell-associated polypeptide that is efficiently cleaved to yield a glycosylated disulfide-linked homodimer with an apparent molecular weight of 85 000. The 43-kDa monomeric subunit is derived by proteolytic processing after residue 223 within the unique amino acid sequences encoded by the 4-kb cDNA clone and contains both N- and O-linked oligosaccharides (Rettenmier & Roussel, 1988; Kawasaki et al., 1985). For the studies described below we studied expression of a truncated form of M-CSF that was engineered to contain a termination codon after residue 223. The secretion and posttranslational processing of this truncated form of M-CSF (M-CSF-223) are indistinguishable from those of the product derived from the full-length 4-kb cDNA clone (A. J. Dorner, personal communication).

The effect of A23187 treatment on the secretion and processing of M-CSF was studied by labeling CHO-D2 cells

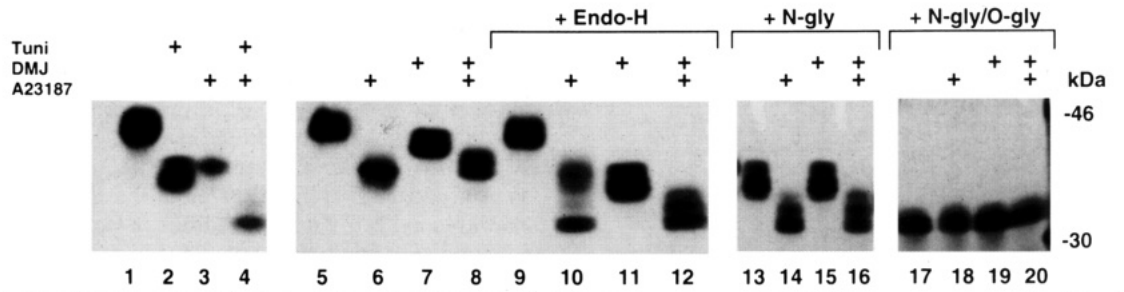

FIGURE 1: A23187 inhibits O-linked glycosylation of M-CSF. CHO-D2 cells that express M-CSF-223 were pretreated for 1 h with A23187 in the presence or absence of tunicamycin or DMJ as described in Experimental Procedures. Cells were then labeled with [^{35}S]methionine, and conditioned medium was harvested for immunoprecipitation of M-CSF as described in Experimental Procedures. Immunoprecipitated proteins were treated with N-Gly, Endo-H, or O-Gly prior to SDS–PAGE and autoradiography. Molecular mass markers are indicated on the right.

that express M-CSF-223 with [^{35}S]methionine. Conditioned medium was harvested for immunoprecipitation with anti-M-CSF antibody and analysis by SDS–PAGE under reducing conditions. M-CSF secreted from untreated CHO-D2 cells migrated as a heterogeneous smear at 43 kDa (Figure 1, lane 1). Inhibition of N-linked oligosaccharide addition by tunicamycin treatment yielded M-CSF migrating as a doublet at 38–40 kDa (Figure 1, lane 2). In the presence of A23187 the mobility of M-CSF was also increased, with M-CSF migrating at 40 kDa (Figure 1, lane 3). In the presence of both tunicamycin and A23187, the M-CSF migrated at 32 kDa. The reduced secretion of M-CSF in the presence of A23187 resulted from reduced protein synthesis, whereas protein secretion was not altered (data not shown). The increased mobility of M-CSF secreted in the presence of A23187 and tunicamycin was reminiscent of that of secreted mature M-CSF digested with both *N*-glycanase and *O*-glycanase (Rettenmier & Roussel, 1988), suggesting that both N-linked and O-linked oligosaccharides were absent on the secreted M-CSF.

Since depletion of intralumenal calcium by ionophore treatment can inhibit processing of N-linked oligosaccharides (Kuznetsov et al., 1993; Lodish & Kong, 1990) by inhibition of the ER α-1,2-mannosidase (Schutzbach & Forsee, 1990), we compared the oligosaccharides on M-CSF secreted in the presence of A23187 to those on M-CSF secreted in the presence of 1-deoxymannojirimycin, an inhibitor of the ER α-1,2-mannosidase (Fuhrmann et al., 1984; Bischoff et al., 1986). Treatment with A23187 yielded M-CSF migrating at 40 kDa compared to treatment with DMJ, which yielded M-CSF migrating at 44 kDa (Figure 1, lanes 6 and 7). Addition of both agents yielded M-CSF migrating close to that observed with A23187 alone (Figure 1, compare lanes 6 and 8). To confirm that the different molecular weight species detected are attributed to differences in N-linked and O-linked glycosylation, immunoprecipitated M-CSF was treated with endoglycosidase H (Endo-H), *N*-glycanase (N-Gly), or *O*-glycanase (O-Gly) with neuraminidase prior to SDS–PAGE. M-CSF secreted in the absence of DMJ and A23187 was resistant to Endo-H digestion (Figure 1, lane 9) but was sensitive when secreted in the presence of DMJ (Figure 1, compare lanes 7 and 11). Since complex and hybrid N-linked oligosaccharides are resistant to Endo-H digestion, these results are consistent with DMJ inhibition of trimming of mannose residues on the N-linked oligosaccharides. A portion of M-CSF secreted in the presence of A23187 was sensitive to Endo-H digestion, yielding a 32-kDa species and a resistant 40-kDa species (Figure 1, lane 10), indicating some inhibition of N-linked oligosaccharide processing. M-CSF secreted in the presence of both DMJ and A23187 was sensitive to Endo-H digestion and yielded primarily a species migrating at 32 kDa (Figure 1, lane 12), the same molecular weight as M-CSF secreted in the presence of tunicamycin and A23187 (Figure 1, lane 4). N-Gly digestion increased the mobility of M-CSF produced in the absence of DMJ and/or A23187, indicating the presence of N-linked oligosaccharides (Figure 1, lanes 13–16). N-Gly digestion reduced the molecular weight by approximately 3 kDa, irrespective of whether the M-CSF was secreted in the presence of A23187 and/or DMJ (Figure 1, lanes 13–16). Thus, the reduced molecular weight of M-CSF produced in the presence of A23187 was not due to differences in core N-linked oligosaccharide addition. Treatment with N-Gly and O-Gly with neurominidase yielded unglycosylated M-CSF migrating at 32 kDa, irrespective of whether the protein was produced in the presence of DMJ or A23187 (Figure 1, lanes 17–20). The 32-kDa M-CSF species is the same molecular weight as that observed upon treatment with both tunicamycin and A23187. N-Gly digestion yielded a portion of M-CSF from A23187-treated cells that migrated slightly slower than 32 kDa (Figure 1, lanes 14 and 16) that was reduced to 32 kDa upon O-Gly digestion (Figure 1, lanes 18 and 20). This suggests that O-linked oligosaccharide addition was partially inhibited in this experiment. These results are consistent with the conclusion that A23187 treatment inhibits both trimming of N-linked oligosaccharides, as previously described (Kuznetsov et al., 1993), and O-linked glycosylation.

Whether A23187 inhibited O-linked glycosylation by depleting intralumenal stores of divalent cations or by another mechanism was evaluated by studying the effect of thapsigargin, a potent inhibitor of the Ca^{2+}-activated microsomal ATPase, the presumptive Ca^{2+} pump (Thastrup et al., 1989). Treatment with either tunicamycin, A23187, or thapsigargin increased the mobility of M-CSF, although to slightly different extents (Figure 2, lanes 2, 3, and 4). Significantly, treatment with both tunicamycin and thapsigargin increased to mobility of the secreted M-CSF to 32 kDa, similar to that observed with treatment with A23187 and tunicamycin (Figure 2, lane 5). These results indicate that A23187 and thapsigargin both inhibit O-linked glycosylation and that intralumenal divalent cations may be required for O-linked glycosylation.

UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase catalyzes addition of the first GalNAc residue to either serine or threonine residues in a polypeptide. Since this enzyme has a strict requirement for manganese for *in vitro* activity (Sugiura et al., 1982; Elhammer & Kornfeld, 1986; Wang et al., 1989) and the ionophore A23187 does not exhibit selectivity between calcium and manganese (Weissman et al., 1980), we tested whether addition of manganese chloride could reverse the effect of A23187 treatment. Cells were treated with A23187 in the presence of $CaCl_2$ (5 mM) or $MnCl_2$ (0.1 and

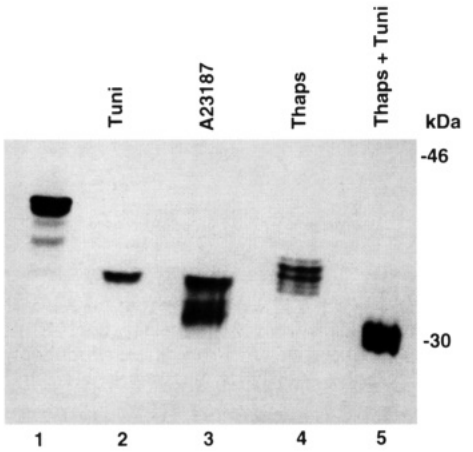

FIGURE 2: Thapsigargin inhibits O-linked glycosylation. CHO-D2 cells were pretreated with tunicamycin, A23187, or thaspigargin for 1 h and then labeled with [^{35}S]methionine in the presence of the same agents. Samples of conditioned medium were harvested and analyzed by SDS–PAGE and autoradiography.

1.0 mM). Whereas addition of $CaCl_2$ did not reverse the inhibition of oligosaccharide processing mediated by A23187 (Figure 3A, lane 4), addition of $MnCl_2$ partially restored oligosaccharide addition to the secreted M-CSF (Figure 3A, lanes 1–3).

To confirm that the reversal of M-CSF processing by $MnCl_2$ was due to O-linked oligosaccharide addition, the immunoprecipitated polypeptides were treated with O-Gly with neuraminidase and/or N-Gly prior to SDS–PAGE under reducing conditions. M-CSF produced in the presence of A23187 and $CaCl_2$ yielded a 40-kDa species (Figure 3B, lane 2), whereas in the presence of $MnCl_2$ a portion of polypeptides comigrated with those observed in the absence of A23187 (Figure 3B, lane 1). After treatment with N-Gly, both species of polypeptides were shifted to a lower molecular weight (Figure 3B, lane 10). In contrast, the higher molecular weight species was not detected after digestion with O-Gly and neuraminidase (Figure 3B, lane 7) or O-Gly with neuraminidase in the presence of N-Gly (Figure 3B, lanes 3–5). These results show that the protein synthesized in the presence of A23187 and $MnCl_2$ was reduced by O-Gly digestion, indicating that $MnCl_2$ addition restored O-linked glycosylation.

Since $MnCl_2$ addition partially restored O-linked glycosylation, we asked whether $MnCl_2$ addition also restored addition of complex N-linked oligosaccharides. CHO-D2 cells were treated with A23187 in the presence or absence of $MnCl_2$ or $CaCl_2$. M-CSF isolated from untreated cells was resistant to Endo-H digestion (Figure 3C, lanes 1 and 2). Treatment with DMJ yielded M-CSF that was susceptible to Endo-H digestion (Figure 3C, lanes 3 and 4). Addition of $MnCl_2$ or $CaCl_2$ had no effect on the oligosaccharide processing inhibited by DMJ (Figure 3C, lanes 5–8). Treatment with A23187 yielded M-CSF in which a significant portion of the polypeptides were sensitive to Endo-H (Figure 3C, lanes 9 and 10). Addition of $CaCl_2$ had no effect on the Endo-H sensitivity of M-CSF secreted in the presence of A23187 (Figure 3C, lanes 11 and 12). In contrast, addition of $MnCl_2$ restored Endo-H resistance to the majority of M-CSF species secreted in the presence of A23187 (Figure 3C, lanes 13 and 14). These results show that M-CSF secreted in the presence of A23187 and $MnCl_2$ comprised two species. One contained O-linked oligosaccharides and was resistant to Endo-H digestion, and the other did not contain O-linked oligosaccharides and was sensitive to Endo-H digestion.

To determine whether the effect of $MnCl_2$ depletion on O-linked oligosaccharide addition was unique to M-CSF, we evaluated the effect of A23187 treatment on oligosaccharide addition to erythropoietin expressed in the CHO mutant cell line ldlD, which is deficient in the UDP-galactose and UDP-*N*-acetylgalactosamine 4-epimerase (Kingsley et al., 1986). When these cells are propagated in the absence of GalNAc, they are unable to catalyze addition of GalNAc to serine and threonine residues because they are unable to produce UDP-GalNAc from UDP-*N*-acetylglucosamine. Previous studies have shown that a portion of EPO expressed in CHO cells has a single O-linked oligosaccharide attached to serine residue 126 (Wasley et al., 1991; Sasaki et al., 1987). The effect of A23187 treatment on EPO secreted from the ldlD CHO cells propagated in the presence and absence of GalNAc was studied. Immunoprecipitation of EPO from conditioned medium of cells labeled with [^{35}S]methionine in the presence of GalNAc and Gal detected a heterogeneous smear of EPO migrating between 32 and 45 kDa (Figure 4, lane 5), as previously characterized (Wasley et al., 1991). Treatment of the immunoprecipitated EPO with N-Gly prior to SDS–PAGE reduced the molecular weight to a primary species migrating at 22 kDa and a minor species migrating at 21 kDa (Figure 4, lane 6). The difference between these two species is attributed to the presence of one O-linked oligosaccharide on the 22-kDa species that is absent on the 21-kDa species (Wasley et al., 1991). EPO secreted from ldlD cells propagated in the absence of GalNAc was secreted as a spectrum of polypeptides migrating between 30 and 43 kDa (Figure 4, lane 1) and upon N-Gly digestion yielded primarily the 21-kDa species and a minor 22-kDa species (Figure 4, lane 2). EPO secreted in the presence of A23187 migrated with the same mobility irrespective of whether GalNAc was present (Figure 4, lanes 3 and 7), although the amount of EPO secreted in the presence of A23187 was reduced due to inhibition of protein synthesis in this experiment. In the presence of A23187 the secreted EPO migrated with a slightly lower molecular weight and was less heterogeneous compared to EPO secreted in the absence of A23187. N-Gly digestion of EPO expressed in the presence of A23187 generated only the single 21-kDa species, and not the 22-kDa species, indicating the absence of O-linked oligosaccharides on EPO irrespective of whether GalNAc was present (Figure 4, lanes 4 and 8).

DISCUSSION

Protein synthesis, folding, and secretion are regulated by calcium levels in the lumen of the ER (Sambrook, 1990). Depletion of ER intralumenal calcium inhibits protein synthesis, likely through signals that mediate phosphorylation of the α-subunit of the eukaryotic initiation factor 2 (Prostko et al., 1992). A number of low-affinity, high-capacity calcium binding proteins exist within the lumen of the ER that regulate free calcium levels and directly affect protein folding, assembly, and ER retention through their chaperonin-type functions. Depletion of divalent cations within the lumen of the ER inhibited secretion of selective proteins such as α1-antitrypsin, α1-antichymotrypsin, and complement C3, whereas it did not affect secretion of albumin (Kuznetsov et al., 1993; Lodish & Kong, 1990). The block to secretion may result from the inability of selective proteins to fold properly in the presence of reduced calcium or from alterations in chaperonin protein function in the presence of reduced calcium. For example, depletion of intralumenal ER calcium inhibited folding of the asialoglycoprotein receptor (Lodish et al., 1992) and oligomerization of rotavirus glycoproteins (Poruchynsky et al., 1991) and facilitated BiP release and secretion of a T-cell

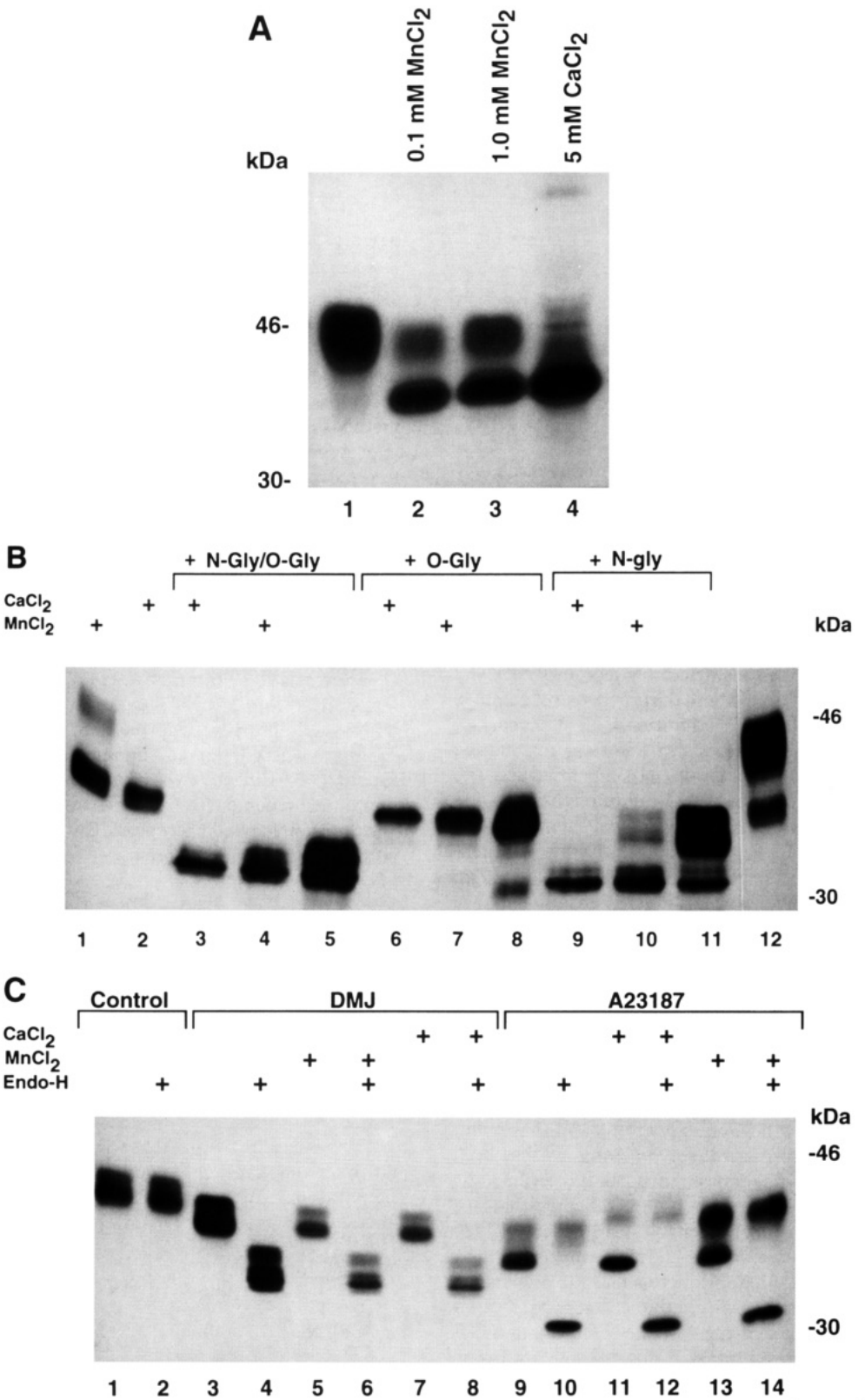

FIGURE 3: $MnCl_2$ restores O-linked glycosylation. CHO-D2 cells were treated with A23187 in the presence or absence of added $MnCl_2$ (1 mM, except lane 2, which was 0.1 mM) or $CaCl_2$ (5 mM). Cells were labeled with [^{35}S]methionine and the M-CSF in the conditioned medium was immunoprecipitated and analyzed by SDS–PAGE and autoradiography. Prior to SDS–PAGE, immunoprecipitated M-CSF was treated with N-Gly and/or O-Gly (panel B) or Endo-H (panel C) as described in Experimental Procedures. For lanes 2–4 in panel A, $MnCl_2$ and $CaCl_2$ were present during the [^{35}S]methionine labeling period only. For all other lanes, $MnCl_2$ and $CaCl_2$ were present during the pretreatment period as well as the [^{35}S]methionine labeling period. Panel B, lane 12, is without A23187.

receptor α-subunit (Suzuki et al., 1991). In this report we show that A23187 treatment did not affect the secretion of M-CSF or EPO expressed in CHO cells. Since both of these proteins are efficiently secreted from CHO cells and neither of these proteins detectably interacts with BiP (Dorner et al., 1989, 1992), A23187 treatment did not improve secretion of these molecules. In addition, in contrast to the secretion of α1-antitrypsin, complement C3, α1-antichymotrypsin, rotavirus glycoproteins, and the asialoglycoprotein receptor, the secretion of M-CSF and EPO did not require intralumenal

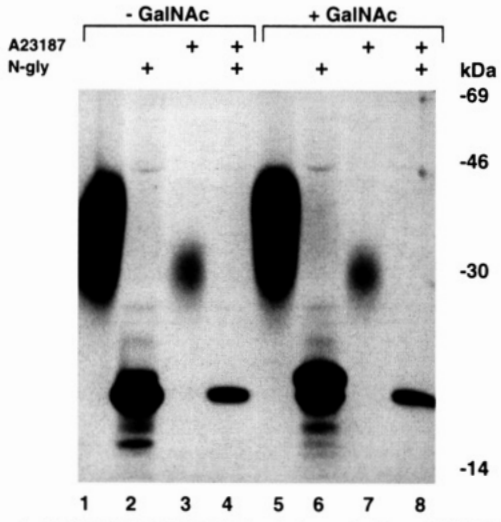

FIGURE 4: A23187 inhibits O-linked glycosylation of EPO. ldlD-A1 cells were pretreated with A23187 and then labeled with [^{35}S]-methionine in the presence or absence of GalNAc as described in Experimental Procedures. Samples of conditioned medium were harvested, and the EPO was analyzed by immunoprecipitation and SDS–PAGE. Where indicated, the immunoprecipitated EPO was digested with N-Gly prior to SDS–PAGE.

ER divalent cations for efficient secretion.

Depletion of divalent cations within the secretory pathway by A23187 treatment inhibited both the processing of complex N-linked oligosaccharides and the addition of oligosaccharides to serine and threonine residues. In addition, manganese chloride and not calcium chloride partially reversed the inhibition of complex N-linked and O-linked oligosaccharide addition. This suggests a unique requirement for manganese within the secretory pathway for oligosaccharide addition. The observation that thapsigargin also inhibited complex N-linked and O-linked oligosaccharide addition indicates that the calcium-activated microsomal ATPase possibly pumps manganese into the secretory pathway. Although the calcium-activated microsomal ATPase likely maintains calcium stores within the ER (Thastrup et al., 1989), it is not known what mechanisms maintain divalent cation stores within the Golgi compartment, the location where O-linked glycosylation occurs. We suspect that the mechanisms that maintain ER divalent cation stores are also responsible for maintaining divalent cation levels in the Golgi apparatus and that either A23187 thapsigargin disrupts these processes or that both may. The ability of manganese to partially reverse the inhibition in oligosaccharide processing indicates that the processing machinery was not irreversibly disrupted by A23187 treatment. At present we do not know the mechanism by which manganese is required for oligosaccharide processing. It is possible that manganese within the secretory pathway is directly required for the enzyme activities involved in oligosaccharide addition, consistent with observations on the *in vitro* requirement for manganese by these enzymes (Sugiura et al., 1982; Elhammer & Kornfeld, 1986; Wang et al., 1989). Alternatively, manganese may be required for the production of precursors for glycosylation, such as UDP-monosaccharides, or their transport into the lumen of the ER. The results reported here are the first to demonstrate a manganese-requiring step for oligosaccharide processsing *in vivo*.

Addition of manganese chloride to cells in the presence of A23187 partially reversed both the complex N-linked oligosaccharide addition and the O-linked oligosaccharide addition in an "all or none" manner as evidenced by the secretion of two populations of M-CSF. One population was fully processed with both complex N-linked and O-linked oligosaccharides, while another population had high-mannose Endo-H-sensitive N-linked oligosaccharides and lacked O-linked oligosaccharides. The two populations detected upon manganese chloride repletion could result from secretion from different cells within the culture or secretion from different compartments within the secretory pathway or from a common requirement for addition of N-linked and O-linked oligosaccharides, such as the synthesis of UDP-monosaccharide precursors.

Our studies show that manganese is uniquely required for O-linked glycosylation in intact mammalian cells. In contrast to the multitude of specific inhibitors of N-linked oligosaccharide addition available to dissect the role of N-linked oligosaccharides (Elbein, 1991), there are no known inhibitors of O-linked oligosaccharide addition. To date, only two methods exist to evaluate the role of O-linked glycosylation in protein structure and function: (1) the use of *O*-glycanase to remove O-linked oligosaccharides (Unemoto et al., 1977) and (2) the use of cell lines defective in enzymes in this biosynthetic pathway (Krieger et al., 1989). Thus, our understanding of the importance of O-linked glycosylation in protein structure and function is very limited. Previous studies demonstrated that O-linked glycosylation was not required for the secretion of human chorionic gonadotropin (Zanni et al., 1989), apolipoprotein E (Zanni et al., 1989), erythropoietin (Wasley et al., 1991), and coagulation factors V and VIII (Pittman et al., 1994). This is consistent with our observations on M-CSF and EPO reported here. To date there are few examples where O-linked oligosaccharides influence protein function. O-Linked glycosylation protects the low-density lipoprotein receptor from degradation (Kingsley et al., 1986) and the granulocyte–macrophage CSF from denaturation (Oheda et al., 1990), and it is required for cell surface expression of glycophorin A (Remaley et al., 1991) and the IL-2 receptor (Kozarsky et al., 1988). Recently it was shown that *O*-glycosylation of the P-selectin ligand increases binding to P-selectin (Sako et al., 1993). The ability to specifically inhibit O-linked glycosylation by depletion of manganese upon ionophore treatment provides a direct convenient approach to dissect the importance of this modification in protein folding, structure, and function.

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