The order of processing events in mouse mammary tumor virus envelope protein maturation: implications for the location of the glucocorticoid-regulated step

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Treatment of the W7MG1 mouse T lymphoma cell line with glucocorticoid stimulates directly or indirectly two observable steps in the processing of mouse mammary tumor virus (MMTV) envelope glycoprotein precursor Pr74: cleavage of Pr74 to yield the mature glycoprotein products gp52 and gp33, and processing of the N-linked oligosaccharides to endoglycosidase H (endo H)–resistant forms found on the mature products but not on the precursor. Therefore, the primary hormone-regulated event in this pathway must occur at or before the point where MMTV envelope proteins become endo H resistant. Pulse-chase analyses identified a novel endo H–resistant 80-kDa species (designated gp80) as a processing intermediate. Therefore, in contrast to conclusions drawn for the envelope proteins of several other retroviruses, proteolytic cleavage of MMTV envelope proteins occurs after acquisition of endo H resistance. Also, proteolytic cleavage cannot be the primary hormone-regulated step. Second, inhibition of mannosidase II by the drug swainsonine did not prevent Pr74 from being proteolytically processed, thus demonstrating that conversion of oligosaccharide chains from endo H–sensitive to –resistant forms was not a prerequisite for proteolytic cleavage. Therefore, the requisite hormone-regulated event in MMTV glycoprotein processing must precede both acquisition of endo H resistance and proteolytic cleavage. This places the regulated event in the endoplasmic reticulum or early Golgi.

Introduction

Many cellular proteins are targeted for transport through the endoplasmic reticulum and Golgi complex, where they will undergo posttranslational processing and sorting into distinct intracellular transport pathways (Burgess and Kelly,

1987; Pfeffer and Rothman, 1987; Lingappa, 1989). Most proteins transit through this system in a constitutive manner. However, it has been observed for some proteins that hormones can acutely influence posttranslational events, such as processing and subcellular localization. For example, formation of secretory granules and sorting of prolactin into them is induced by a combination of estradiol, insulin, and epidermal growth factor in the rat pituitary cell line, GH₃C₁ (Scammell *et al.*, 1986). Insulin can regulate the translocation of glucose transporters from a post-Golgi compartment to the plasma membrane in 3T3-L1 adipocytes (Blok *et al.*, 1988; Calderhead and Lienhard, 1988). In contrast, glucocorticoid induces the internalization of glucose transporters from the cell surface to an intracellular compartment in fibroblasts (Horner *et al.*, 1987). Glucocorticoid and estrogen have also been demonstrated to regulate the levels of specific glycosyl transferases involved in secondary glycosylation of N-linked oligosaccharide chains (Chilton *et al.*, 1988; Wang *et al.*, 1989). Thus, although processing and sorting of most proteins seem to occur constitutively, hormones selectively regulate these processes for specific proteins in specific cell types. Studies to date have been primarily descriptive in nature; the specific molecular mechanisms by which hormones control these systems remain to be elucidated.

We have previously shown that the complete processing of the mouse mammary tumor virus (MMTV) envelope glycoproteins in the mouse T lymphoma cell line, W7MG1, is dependent on glucocorticoid hormone (Rabindran *et al.*, 1987; Rabindran and Stallcup, 1987). The envelope precursor is expressed in the presence or absence of hormone as a 74-kDa polyprotein (Pr74). Only after treatment of the cells with glucocorticoid is a portion of the Pr74 population cleaved to yield the mature products, gp52 and gp33, which are the N- and C-terminal fragments, respectively, of Pr74. The mechanism by which glucocorticoid regulates this pathway and the identity of the primary hormone-regulated step are unknown.

The carbohydrate chain structures of glycoproteins provide valuable markers for determining the order of glycoprotein processing events. N-linked oligosaccharide chain cores are added cotranslationally to proteins in the lumen of the endoplasmic reticulum; residues of the core oligosaccharide chains are trimmed off and additional terminal sugars are subsequently added as the proteins pass through the endoplasmic reticulum and Golgi (Kornfeld and Kornfeld, 1985). The first four mannose residues are removed by mannosidase I in the endoplasmic reticulum and cis-Golgi. The final trimming step involves removal of two mannose residues in the medial Golgi by mannosidase II. Glycoproteins that have reached this compartment and been trimmed are distinguishable by their resistance to digestion with endoglycosidase H (endo H), which is unable to cleave off the carbohydrate chain if less than four mannose residues are present (Robbins *et al.*, 1977).

The MMTV envelope polyprotein precursor Pr74 has five N-linked oligosaccharide chains; three of these are in the gp52 domain and the remaining two are in the gp33 domain of Pr74 (Redmond and Dickson, 1983). Our initial characterization of this processing pathway included an analysis of the carbohydrate structures of these MMTV glycoproteins (Snider *et al.*, 1990). In W7MG1 cells grown without glucocorticoid, there are few if any MMTV glycoproteins containing endo H-resistant sugar chains. It is only after the addition of hormone that endo H-resistant oligosaccharides appear on MMTV glycoproteins, and then they are found only on the mature products, gp52 and gp33. This indicates that the primary hormone-regulated event in MMTV glycoprotein processing occurs at or before the acquisition of endo H resistance (Snider *et al.*, 1990). This finding allowed us to eliminate as candidates for the primary hormone-regulated step all the events occurring after the mannosidase II step, including all secondary glycosylation and sorting steps in the trans-Golgi, as well as transport to the cell surface.

It is our working hypothesis that the glucocorticoid hormone is acting through its receptor to regulate, either positively or negatively, the transcription of a cellular gene whose product either directly or indirectly controls a requisite step in the processing of the MMTV envelope precursor. To understand the detailed molecular mechanism of hormonal regulation in this system, we must first identify this requisite hormone-regulated step. Although our previous studies eliminated the distal part of the pathway

from consideration, there are still a large number of potential candidates for the primary hormone-regulated step. Therefore we have designed experimental strategies that will systematically test the remaining candidates to determine whether they satisfy the existing criteria for the hormone-regulated step in MMTV envelope protein processing. At present two criteria exist: 1) the hormone-regulated step must occur at or before the point where the MMTV envelope proteins become endo H resistant and 2) the hormone regulated step is an absolute prerequisite for the formation of the mature products, gp52 and gp33. Two of the most obvious candidates for this requisite hormone-regulated step are the two steps that have already been observed to be hormone dependent: the proteolytic cleavage step and the mannosidase II step, which renders the proteins endo H resistant. Because the order of these two events has not previously been determined for MMTV envelope proteins, both are still viable candidates for the hormone-regulated step.

The work presented here tested whether either proteolytic cleavage or formation of endo H-resistant carbohydrates represents the primary hormone-regulated event in the MMTV glycoprotein processing pathway. We addressed these questions by using pulse-chase analyses to identify processing intermediates that specify the order of these two events. In addition, we used the drug swainsonine to block the mannosidase II step and assess whether it was required for subsequent processing steps. Identification of either of these two steps as the primary regulated event would focus further experimentation on it; alternatively, elimination of these steps would focus attention on prior steps that occur in the endoplasmic reticulum or early Golgi cisternae.

Results

Identification of an MMTV glycoprotein processing intermediate

The 5 oligosaccharide chains on Pr74 are all endo H sensitive. The mature gp52 molecule has a mixture of endo H-resistant and -sensitive chains, and gp33 contains only resistant chains (Snider *et al.*, 1990). Thus conversion of Pr74 to the two mature products requires modification of the oligosaccharide chains to endo H-resistant forms and proteolytic processing. The oligosaccharide chain modifications entail an extensive series of core oligosaccharide trimming steps and subsequent secondary glycosylation reactions. Endo H resistance is ac-

Figure 1. Potential processing intermediates of MMTV Pr74^{env}. The intermediates are determined by the order of occurrence of the two observable processing events: proteolytic cleavage and final trimming of core oligosaccharide chains by mannosidase II. If proteolytic cleavage occurs first (A), then completely endo H-sensitive forms of gp52 and gp33 would be observed before subsequent processing to endo H-resistant forms by mannosidase II. Alternatively, should trimming of Pr74 by mannosidase II occur before proteolytic cleavage (B), then the intermediate species would be a modified form of Pr74 containing endo H-resistant oligosaccharide chains.

quired as a result of the final trimming step, which is catalyzed by mannosidase II. The order in which the mannosidase II and proteolytic steps occur dictates the type of processing intermediate that is produced. If proteolytic

cleavage of Pr74 occurred before its exposure to mannosidase II, then completely endo H-sensitive forms of gp52 and gp33 would be produced (Figure 1, Option A). Conversely, if the precursor were acted upon by mannosidase II before being proteolytically cleaved, then the intermediate species would be an endo H-resistant form of Pr74 (Figure 1, Option B). Because of differences in the status of oligosaccharide chain maturation, these intermediate species are expected to differ somewhat in molecular weight from the initial precursor and the fully mature products.

To detect these intermediates, we pulse-labeled dexamethasone-treated W7MG1 cells with [³⁵S]methionine for 20 min at 37°C; the cells were then chased in excess unlabeled methionine at 28°C. The lower chase temperature was used to reduce the rate of transport through the endoplasmic reticulum and Golgi, thus facilitating observation of the intermediates. Samples taken immediately after the pulse and at various intervals during the chase were immunoprecipitated and digested with endo H or endo F or mock digested (Figure 2). Endo F cleaves biantennary carbohydrate chains at the same site as endo H with relatively little specificity for the number of mannose residues (Plummer, 1984); it was used as a control to determine the migration pattern after all N-linked chains had been removed.

After the pulse the only observable species was Pr74 (Figure 2, lane 1); digestion of Pr74 with endo H or endo F produced the deglyco-

Figure 2. Pulse-chase analysis to determine MMTV envelope glycoprotein processing intermediates. Dexamethasone-treated W7MG1 cells were pulse-labeled with [³⁵S]methionine for 20 min at 37°C. Immediately after the pulse, an aliquot of cells was transferred to ice-cold chase medium containing excess unlabeled methionine. The remaining cells were chased at 28°C and aliquots removed at the indicated chase times. Each cell lysate was separated into three equal volumes and immunoprecipitated with combined antisera against gp52 and gp33. The immunoprecipitates were digested with endo H (lanes 2, 5, 8, 11, and 14) or endo F (3, 6, 9, 12, and 15) or mock digested (lanes 1, 4, 7, 10, and 13). For simplicity, only the mock digestion using endo H buffer is shown. Previous experiments indicated no difference in the mock-digestion patterns obtained with endo H or endo F buffers. The digested samples were run on a 12.5% SDS-polyacrylamide gel (acrylamide: bis-acrylamide 29.5%:0.5%) and visualized by autoradiography. The letters a-f indicate the endoglycosidase digestion products of Pr74, gp52, and gp33.

sylated species "a" (lanes 2 and 3). The mature products, gp52 and gp33, began to appear after 20 min of chase (lane 7) and were more readily seen after 40 and 100 min (lanes 10 and 13). Species "b" and "c" (lanes 8, 11, and 14) were endo H digestion products of gp52, containing two and one endo H-resistant carbohydrate chains, respectively (Snider *et al.*, 1990). Digestion with endo F removed all three N-linked chains, causing gp52 to migrate as a single deglycosylated species (band "d," lanes 9, 12, and 15). The other mature MMTV glycoprotein species, gp33, had two oligosaccharide chains that were both resistant to endo H (lanes 11 and 14); endo F digestion of gp33 yielded two bands with one and two chains removed, respectively (bands "e" and "f," lanes 12 and 15). Although endo F has relatively little substrate specificity with regard to number of mannose residues, it does not cleave bisected triantennary hybrid structures or triantennary and tetraantennary complex structures. At high concentrations, endo F will slowly cleave biantennary complex oligosaccharides (Elder and Alexander, 1982; Chilton *et al.*, 1988). Thus, band "e" is probably due to the endo F resistance of one oligosaccharide chain on some gp33 molecules.

We looked for the two alternative types of processing intermediates (see Figure 1) in the endo H digestion patterns near 74 kDa (for the endo H-resistant form of Pr74) and at the positions for bands d and f (for the completely endo H sensitive forms of gp52 and gp33, respectively). The only species observed that satisfied these criteria was an 80-kDa species (gp80), which was most easily observed in the endo H digestion pattern because it migrated only slightly more slowly than undigested Pr74. This species was barely (if at all) detectable at the end of the pulse (Figure 2, lane 2); it formed a distinct band by the 10-min chase point (lane 5), reached its maximum labeling intensity at 40 min (lane 11), and declined substantially in intensity by 100 min (lane 14). gp80, gp52, and gp33 were quantified from this autoradiogram by laser densitometry (Figure 3). As expected for a processing intermediate, gp80 appeared before the mature products and decreased as they increased. Essentially identical kinetics were seen in an additional pulse-chase experiment (not shown).

No species corresponding to fully endo H-sensitive gp52 or gp33 (at positions d and f, respectively) were observed in the endo H digestion patterns for any of the chase times (lanes 2, 5, 8, 11, and 14). The origin of the species migrating near band e in lanes 8 and 11 is

Figure 3. Kinetics of appearance of processing intermediate and mature products from pulse-chase analysis. Quantification of gp80 (●), gp52 (▲), and gp33 (■) from the autoradiogram in Figure 2 was performed by laser densitometry. gp80 levels were determined from the endo H-digested lanes (2, 5, 8, 11, and 14), as gp80 is obscured by Pr74 in undigested lanes. gp52 was quantified from the endo F-digested lanes (3, 6, 9, 12, and 15) and gp33 from the mock-digested lanes (1, 4, 7, 10, and 13) because these lanes provided the sharpest single bands. The maximum value for each species was taken to be 100%, and the lower values were expressed as a percentage of this value. A densitometer value of zero indicates that no peak was detected.

uncertain. However, on several counts, the species does not meet our criteria for an MMTV glycoprotein processing intermediate. Its migration rate is not perfectly matched with species e. In addition, even if it were the same as species e, we previously established that species e contains one undigested oligosaccharide chain (Snider *et al.*, 1990); this is not consistent with a completely endo H-sensitive species. Furthermore, there is no corresponding gp52 species with three endo H-sensitive chains, i.e., one that migrates at position d after endo H digestion. Thus, we were unable, after repeated attempts, to detect any completely endo H-sensitive gp52 and gp33. This result adds weight to the other data suggesting that gp80 is the processing intermediate and that proteolytic cleavage therefore occurs after Pr74 is acted upon by mannosidase II in the cis/medial Golgi.

To show that gp80 is an MMTV protein, we performed a series of control experiments with nonimmune serum and with uninfected cells. The pulse-chase experiment was repeated, and the 40-min chase point was selected because it yielded the maximum amount of gp80. gp80 was precipitated from extracts of glucocorticoid-treated W7MG1 cells by combined antisera against gp52 and gp33 (Figure 4, lanes 1 and

Figure 4. Verification of gp80 as an MMTV protein. Hormone-induced W7MG1 cells (lanes 1–4), uninduced W7MG1 cells (lanes 7 and 8), and uninduced WEHI7.2 cells (lanes 5 and 6) were pulse-labeled with [35 S]methionine for 20 min at 37°C and then chased in excess unlabeled methionine for 40 min at 28°C. The cells were lysed and immunoprecipitated using combined antisera against gp52 and gp33 (lanes 1, 2, and 5–8) or nonimmune goat serum (lanes 3 and 4). The immunoprecipitates were mock digested (odd lanes) or digested with endo H (even lanes), resolved on an SDS–polyacrylamide gel and visualized by autoradiography. Band “a” denotes the endo H digestion product of Pr74.

2), but not by normal goat serum (lanes 3 and 4). The sera against gp52 and gp33 did not precipitate any gp80 or other proteins from WEHI7.2 cells (lanes 5 and 6), the uninfected parental cells from which the W7MG1 line was derived. Under these labeling conditions, no gp80 was detected in W7MG1 cells grown without glucocorticoid (lanes 7 and 8). As usual, these experiments involved two successive immunoprecipitations for each sample; the first immunoprecipitate pellet is dissociated by heating in sodium dodecyl sulfate (SDS) before the second immunoprecipitation is performed. Therefore, gp80 cannot be a cellular protein that coprecipitates with the viral proteins by remaining associated with them.

Glucocorticoid regulates production of the processing intermediate

To determine rigorously whether the efficiency of production of gp80 was glucocorticoid dependent, we performed parallel pulse-chase experiments with induced and uninduced W7MG1 cells (Figure 5, A and B, respectively). Glucocorticoid, in addition to its effect on MMTV protein processing, also stimulates transcription from the MMTV promoter and thereby causes a four- to fivefold increase in the level of MMTV RNA and the rate of synthesis of Pr74

in W7MG1 cells (Danielsen *et al.*, 1983; Rabin-dran *et al.*, 1987). To assess the hormonal effects on protein processing, investigators must account for the increased rate of synthesis of Pr74. However, rather than attempting to balance accurately the amounts of MMTV proteins loaded from all the samples in this complex experiment, we loaded and exposed both gels in a manner that allowed accurate quantification by densitometry of the ratio between gp80 (measured at its peak intensity during the chase) and Pr74 (measured at the end of the pulse). This ratio served as a measure of the efficiency of converting Pr74 to gp80 in hormone-treated and -untreated W7MG1 cells. All samples were digested with endo H to augment the separation between Pr74 and gp80. During exposure of the autoradiogram, the length of time required to detect gp80 resulted in overexposure of Pr74. To allow densitometry of both species on the same autoradiogram, we loaded 20% and 75% of the sample taken immediately after the pulse (lanes 1 and 2, respectively) and 100% of all samples taken during the chase (lanes 3–6).

In hormone-treated cells (Figure 5A) the ratio of gp80 at its peak intensity (40-min chase point, lane 5) to Pr74 at the end of the pulse (lanes 1 and 2) was 0.18 (using lane 1) and 0.19 (using lane 2); these ratios included appropriate adjustments for the differences in sample loading

Figure 5. Parallel pulse-chase analyses for gp80 in dexamethasone-treated and untreated cells. Hormone-induced (A) and uninduced (B) W7MG1 cells were pulse-labeled as in Figure 2 and chased at 28°C for 0 min (lanes 1 and 2), 10 min (lane 3), 20 min (lane 4), 40 min (lane 5), and 90 min (lane 6). Cell lysates were prepared, immunoprecipitated with combined antisera against gp52 and gp33, and digested with endo H before electrophoresis. To allow densitometry of gp80 and Pr74 on the same autoradiogram, 20% and 75% of the sample taken immediately after the pulse was loaded on the gel (lanes 1 and 2, respectively). Lanes 3–6 contain 100% of samples taken during the chase. Major MMTV species are identified. The arrow indicates a band that we do not consider to be gp80 (see text). Pr74 runs as a doublet. The lower band is fully deglycosylated. The upper band contains one undigested oligosaccharide chain and is the product of an incomplete digestion.

volumes. When samples from uninduced cells were loaded in the same manner (Figure 5B), no gp80 was clearly discernible at any point during the chase period. We do not believe the band indicated by the arrow to be gp80, because it is very sharp rather than diffuse, migrated slightly faster than gp80, and appeared immediately at the end of the pulse (lane 2). Note that the same species was also observed in hormone-treated cells at the end of the pulse (Figure 5A, lane 2) and migrated slightly faster than gp80. Even if we assumed that the species in the untreated cells were gp80, the ratio of its intensity at the 40-min chase point (Figure 5B, lane 5) to Pr74 at the end of the pulse was 0.02, using either lane 1 or 2 to quantify Pr74. Therefore, by this measure the efficiency of converting Pr74 to gp80 was at least nine times higher in glucocorticoid-treated cells than in untreated cells.

Inhibition of mannosidase II by swainsonine

Pulse-chase analysis provided evidence that proteolytic cleavage of the MMTV envelope polypeptide precursor occurs after the mannosidase II step. To determine whether proteolytic processing depended on prior conversion of Pr74 oligosaccharide chains to endo H-resistant forms, we used the drug swainsonine to inhibit mannosidase II activity (Tulsiani *et al.*, 1982) in dexamethasone-treated cells. If pro-

teolytic cleavage were dependent on the prior action of mannosidase II, then no cleaved products would be observed.

Hormone-induced cells were treated with swainsonine before as well as during a 2-h metabolic labeling. A cell homogenate was prepared, the viral glycoproteins were precipitated with specific antisera, and immunoprecipitates were digested with endo H or endo F or mock-digested before gel electrophoresis (Figure 6). The amount of labeled gp52 and gp33 indicated that the efficiency of proteolytic processing was not reduced in swainsonine-treated cells (Figure 6, lanes 5–8), compared with control cells (lanes 1–4). Swainsonine did efficiently inhibit formation of endo H-resistant carbohydrate chains, because most gp52 and gp33 molecules produced in the presence of the drug were endo H sensitive; i.e., they had similar mobilities after digestion with endo H (lane 6) or endo F (lane 8). Swainsonine also had a significant effect on the mobility of undigested gp33 (lanes 5 and 7, labeled gp33*) and on that of undigested gp52 (labeled gp52*), presumably resulting from the inability of endo H-sensitive carbohydrate chains to undergo full terminal glycosylation in the trans-Golgi. In contrast, all gp52 and gp33 molecules produced in the absence of swainsonine had one or two endo H-resistant oligosaccharide chains (compare lanes 2 and 4). The fact that proteolytic processing of the MMTV glycoprotein precursor still occurred efficiently

Figure 6. The effect of mannosidase II inhibition on proteolytic cleavage of Pr74. Dexamethasone-induced W7MG1 cells were treated with 2 μ g/ml swainsonine for 1 h before as well as during the 2-h metabolic labeling (lanes 5–8). Control cells were also induced with hormone but were not treated with the inhibitor (lanes 1–4). Swainsonine had no effect on [35 S]methionine incorporation into total protein. Cell lysates were prepared and immunoprecipitated as in Figure 2. The immunoprecipitates were digested with endo H or endo F or mock digested, as indicated. The digested samples were subjected to gel electrophoresis and visualized as in Figure 2. The major MMTV species are identified, and endoglycosidase digestion products a–f are described in the text. The asterisks indicate species whose mobilities were altered by the effects of swainsonine on the cells.

when mannosidase II activity was inhibited demonstrated that endo H-resistant chains (and thus the mannosidase II step) are not prerequisite for proteolytic processing.

Discussion

Our previous studies defined two criteria for the primary hormone-regulated event in MMTV envelope protein processing in W7MG1 cells: 1) it must occur at or before the point where MMTV envelope proteins become endo H resistant and 2) it is an absolute prerequisite for the production of gp52 and gp33 (Rabindran *et al.*, 1987; Snider *et al.*, 1990). Two observable steps in the pathway were found to depend either directly or indirectly on the action of the hormone; these two steps were the proteolytic cleavage and the mannosidase II step, which confers resistance to endo H. Therefore, in the present study we directly tested whether either of these two visibly hormone-dependent events was the requisite hormone-regulated step in this pathway.

We began by using pulse-chase analyses to determine the relative order of the proteolytic cleavage step and the acquisition of endo H resistance. We detected an 80-kDa glycoprotein that satisfied the criteria for a processing intermediate between Pr74 and the mature prod-

ucts, gp52 and gp33. Identification of this intermediate indicates that proteolytic cleavage occurs after Pr74 acquires endo H-resistant oligosaccharide chains. The identification of gp80 as an intermediate was based on several findings: gp80 contained endo H-resistant oligosaccharide chains, appeared and disappeared with appropriate kinetics, and was detected only in glucocorticoid treated cells. We have previously shown that gp80 is a galactose-rich species (Snider *et al.*, 1990) and has thus undergone secondary glycosylation in the trans-Golgi before its proteolytic cleavage. Equally important was the failure to detect any of the other theoretically possible processing intermediates, namely the completely endo H-sensitive forms of gp52 and gp33 (see Figure 1). Although the evidence presented does not definitively prove that gp80 is the processing intermediate, the absence of any other viable candidates adds considerable weight to this conclusion. The identification of this intermediate demonstrates that proteolytic processing of MMTV envelope proteins occurs after their oligosaccharide chains become endo H resistant and acquire at least some terminal glycosylation. Therefore, the proteolytic cleavage step does not meet the criteria for the requisite hormone-regulated step in MMTV envelope protein processing.

The proteolytic cleavage of cellular and viral glycoproteins in the Golgi system invariably occurs at peptide bonds that immediately follow two basic amino acids (Strauss *et al.*, 1986). Thus, proteinases of similar specificity are apparently responsible for processing of all these glycoproteins. The site and timing of proteolytic processing, as well as the identity of the responsible proteinases, are still subjects of considerable debate. In the most definitive study to date, immunoelectron microscopy with monoclonal antibodies specific for the processed or unprocessed proteins placed the cleavage of proinsulin in maturing post-Golgi secretory granules (Orsi *et al.*, 1987). However, examination of a variety of other glycoproteins by less rigorous criteria has led to conclusions that some are proteolytically processed before becoming endo H resistant, whereas others are cleaved after acquiring endo H resistance. Studies of the envelope proteins of various retroviruses illustrate the dichotomy of conclusions that exists in the literature. On one hand, identification of specific processing intermediates demonstrated that the envelope proteins of avian reticuloendotheliosis virus (Tsai and Oroszlan, 1988) and MMTV (this report) are proteolytically processed in the trans-Golgi or beyond. In contrast, other studies concluded that the envelope proteins of murine leukemia virus (Machida and Kabat, 1982), Rous sarcoma virus (Wills *et al.*, 1984), and human immunodeficiency virus (Stein and Engleman, 1990) were proteolytically cleaved in early or mid-Golgi compartments. We note that these latter three studies used less complete kinetic analyses that did not involve identification of processing intermediates. These authors' conclusions were based primarily on the lack of endo H-resistant oligosaccharide chains in the initial envelope precursor protein and the presence of endo H-resistant chains in the mature proteolytically processed products. Our study and that of Tsai and Oroszlan (1988) demonstrated that these criteria, without identification of specific processing intermediates, are not sufficient to determine whether proteolytic cleavage occurs before or after the protein becomes endo H resistant. Thus, we suggest that further studies of processing intermediates are required to resolve this issue for other retroviral envelope proteins.

After eliminating proteolytic cleavage from consideration, we determined whether trimming by mannosidase II was a required step in the processing pathway. Mannosidase II catalyzes the final core oligosaccharide trimming step

(Man₅GlcNAc₂ to Man₃GlcNAc₂), which renders the oligosaccharide chains resistant to endo H (Kornfeld and Kornfeld, 1985). When mannosidase II was inhibited by swainsonine, the MMTV envelope proteins produced lacked endo H-resistant oligosaccharide chains but were still proteolytically processed at the normal efficiency. Thus, the mannosidase II step is not a prerequisite for subsequent proteolytic processing. Because the primary hormone-regulated event is absolutely required for the complete processing of precursor to products and because the swainsonine experiment demonstrated that mannosidase II is not a required step in the processing sequence, we conclude that mannosidase II is not the requisite hormone-regulated step.

From the summation of this and our previous results, we conclude that the primary hormone-regulated event for MMTV glycoprotein processing in W7MG1 cells must precede both the mannosidase II and proteolytic processing steps. Therefore, hormone regulates the ability of Pr74 to interact with these two enzymes and does not directly regulate the level of enzyme activity. We conclude that the requisite hormone-regulated event occurs in the endoplasmic reticulum or possibly the early Golgi.

This finding is in contrast to the studies of Haffar *et al.* (1988) involving the ionophore monensin. These investigators concluded that the primary hormone-regulated step for MMTV glycoprotein processing in rat hepatoma cells occurs after the acquisition of endo H-resistant oligosaccharide chains but at or before the site of galactose addition. The dramatic contrast between our conclusion and that of Haffar *et al.* (1988) could be due to differences in the processing pathways in the two different cell lines (lymphoma vs. hepatoma, and mouse vs. rat) or to the use of monensin in one study but not in the other. There are precedents in the literature for both of these eventualities. In some cases, a single protein precursor has been shown to be processed differently in different cell lines (Pratt *et al.*, 1988; Smith and Funder, 1988). Also, monensin is known to exert pleiotropic inhibitory effects on cells, and the exact effects on protein processing and sorting can vary from cell line to cell line and even from protein to protein within the same cell line (Tartakoff and Vassalli, 1979; Tartakoff, 1983; Strous and Lodish, 1980; Alonso and Companys, 1981). In the rat hepatoma cells, monensin prevented proteolytic processing of the MMTV glycoproteins but allowed Pr74 to be converted to an endo H-resistant species, gp70, which was subse-

quently galactosylated and transported to the cell surface (Haffar *et al.*, 1988). In contrast, in our mouse lymphoma cell system, monensin did not prevent proteolytic cleavage of the MMTV glycoproteins, their galactosylation, or their transport to the cell surface, but did inhibit addition of terminal sialic acid residues to them (our unpublished results). Further studies will be required to resolve these differences.

We have previously eliminated from consideration all steps in the trans-Golgi and beyond (Snider *et al.*, 1990); in this report we eliminated proteolytic processing and mannosidase II activity as the requisite hormone-regulated step in this pathway. We can now focus attention on the early events in glycoprotein processing and sorting, which occur in the endoplasmic reticulum and early Golgi cisternae (Figure 7). The endoplasmic reticulum is the site of several required events in protein sorting, including protein subunit folding and (for multimeric proteins) oligomerization. Newly synthesized glycoprotein species appear to interact with resident endoplasmic reticulum species as they accomplish these two steps, and mutant proteins that cannot fold properly remain associated with these resident endoplasmic reticulum proteins (Pelham, 1989; Doms *et al.*, 1987). Many viral glycoproteins, including VSV G protein and RSV envelope glycoprotein, form trimers in the endoplasmic reticulum; this oligomerization appears to be required for transport to the Golgi (Einfeld and Hunter, 1988; Hurtley and Helenius, 1989). The transport of a number of heterooligomeric cellular proteins—such as the T cell receptor, MHC proteins, and immunoglobulin molecules—to the Golgi also requires prior completion of the oligomerization process in the endoplasmic reticulum. In each of these cases, a different accessory protein has been implicated in the oligomerization and/or transport steps (Hendershot and Kearney, 1988; Hurtley and Helenius, 1989). We propose that a similar mechanism involving an accessory protein is operating in our system, and that the hormonally regulated association of this accessory protein with MMTV Pr74 in the endoplasmic reticulum or early Golgi is responsible for the hormone-dependent processing/sorting of the MMTV glycoproteins.

One obvious possibility consistent with the above mechanisms is that glucocorticoid might regulate the exit of Pr74 from the endoplasmic reticulum to the Golgi. However, thus far our published and preliminary data does not support this hypothesis. First, Pr74 can be metabolically labeled with [³H]galactose, both in hormone-

Figure 7. Schematic representation of protein transport and processing steps for Pr74 through the endoplasmic reticulum and Golgi complex. The steps occurring below the horizontal line have been eliminated as candidates for the requisite hormone-regulated step in the processing pathway of MMTV envelope glycoproteins. Still under investigation are the steps above the horizontal line (see Discussion).

treated and untreated W7MG1 cells (Snider *et al.*, 1990). Second, we have detected Pr74 on the surface of hormone-treated and untreated cells by binding antibodies to intact metabolically labeled cells, washing away excess antibodies, and then lysing the cells and adding fixed *Staphylococcus aureus* to complete the immunoprecipitation (our unpublished results). Both of these results indicate that Pr74 is not restricted to the endoplasmic reticulum, either in hormone-treated or untreated cells. The preliminary implication is that the hormone-regulated event controls the ability of Pr74 to be processed to mature products, but not its exit from the endoplasmic reticulum. We are currently conducting immunofluorescence and subcellular fractionation studies to examine further the cellular localization of Pr74.

Materials and methods

Materials

W7MG1, an MMTV-infected subclone of the mouse T-lymphosarcoma cell line WEHI7, was grown as described pre-

vously (Danielsen *et al.*, 1983). Swainsonine was acquired from Boehringer-Mannheim Biochemicals (Indianapolis, IN). All other materials were as described previously (Snider *et al.*, 1990).

Metabolic labeling and analysis of MMTV glycoproteins

Cells used for metabolic labeling experiments were either untreated (control) or grown in the presence of 1 μ M dexamethasone for 6–8 h before labeling. Dexamethasone was included in all subsequent incubations of hormone-treated cells. Labeling with [³⁵S]methionine was performed as described previously (Snider *et al.*, 1990), except that 10⁷ cells were used for each sample.

Immunoprecipitation of MMTV envelope proteins, digestion of immunoprecipitates with endoglycosidases, and analysis of proteins by SDS–polyacrylamide gel electrophoresis and autoradiography were performed as described previously (Snider *et al.*, 1990). Autoradiograms were scanned using an LKB Ultrascan XL (Pharmacia LKB Biotechnology, Piscataway, N.J.).

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