A Novel Intermediate in Processing of Murine Leukemia Virus Envelope Glycoproteins

PROTEOLYTIC CLEAVAGE IN THE LATE GOLGI REGION*

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The intracellular processing of the murine leukemia virus envelope glycoprotein precursor Pr85 to the mature products gp70 and p15e was analyzed in the mouse T-lymphoma cell line W7MG1. Kinetic (pulse-chase) analysis of synthesis and processing, coupled with endoglycosidase (endo H) and neuraminidase digestions revealed the existence of a novel high molecular weight processing intermediate, gp95, containing endo H-resistant terminally glycosylated oligosaccharide chains. In contrast to previously published conclusions, our data indicate that proteolytic cleavage of the envelope precursor occurs after the acquisition of endo H-resistant chains and terminal glycosylation and thus after the mannosidase II step. In the same W7MG1 cell line, the type and order of murine leukemia virus envelope protein processing events was identical to that for the mouse mammary tumor virus envelope protein. Interestingly, complete mouse mammary tumor virus envelope protein processing requires the addition of glucocorticoid hormone, whereas murine leukemia virus envelope protein processing occurs constitutively in these W7MG1 cells. We propose that all retroviral envelope proteins share a common processing pathway in which proteolytic processing is a late event that follows acquisition of endo H resistance and terminal glycosylation.

Proteins destined for secretion, the plasma membrane, the Golgi, the endoplasmic reticulum (ER),† or the lysosomes enter the cellular transport pathway via co-translational translocation into the ER (Lingappa, 1989; Pfeffer and Rothman, 1987; Walter and Lingappa, 1986). During their passage into the ER, the signal peptide is removed and core oligosaccharides are added (Rothman and Lodish, 1977; Kornfeld and Kornfeld, 1985). After folding and/or assembly into a conformationally competent form, the protein is transported to the Golgi (Pelham, 1989; Hurtley and Helenius, 1989). Numerous modifications of the protein occur during transport and targeting to its final destination. Such protein modifications include but are not restricted to carbohydrate removal and addition, proteolytic cleavage, fatty acid addition, and disulfide bond formation (Kornfeld and Kornfeld, 1985; Roth, 1987; Schmidt and Schlesinger, 1979; Freedman, 1984).

In the case of retroviral envelope proteins, extensive carbohydrate chain modifications and proteolytic cleavage at a single site generate the two mature envelope protein species that remain associated with each other either covalently or noncovalently (Kornfeld and Kornfeld, 1985; Schlesinger and Schlesinger, 1987). Interestingly there are differences of opinion within the literature regarding the specific cellular location of envelope protein cleavage. For example, some investigators argued that proteolytic cleavage of the murine leukemia virus (MuLV) envelope protein precursor Pr85 to the mature viral products gp70 and p15e (Karshin et al., 1977) is an early event in the processing pathway, probably occurring in the ER or cis-Golgi (Machida and Kabat, 1982; Fitting and Kabat, 1982; Polonoff et al., 1982; Ulmer and Palade, 1991). Wills et al. (1984) determined that Rous sarcoma virus envelope protein cleavage occurs in an ill-defined region within the Golgi apparatus between the addition of palmitic acid and fucose. Others looking at reticuloendotheliosis virus and mouse mammary tumor virus (MMTV) envelope proteins have found that cleavage occurs later in the transport pathway, most likely in the trans-Golgi or beyond (Tsai and Oroszlan, 1988; Corey and Stallcup, 1990). In addition, examination of human immunodeficiency virus (HIV) envelope protein processing in CD4+ cells by two different investigators has resulted in two different conclusions. Willey et al. (1988) concluded that cleavage was a late event in the cell transport pathway, while Stein and Engleman (1990) suggested that cleavage occurs early in the transport pathway prior to the acquisition of endo H-resistant oligosaccharide chains.

In view of the dichotomy of conclusions regarding the timing of retroviral envelope protein processing, we decided to re-examine this question using MuLV. We believe it unlikely that the location of proteolytic cleavage within a cell would be a function of the specific retroviral envelope protein for several reasons. First, all retroviral envelope proteins appear to utilize the constitutive secretory pathway within the cell, probably reflecting their common viral function. Second, all viral envelope proteins examined thus far which are cleaved in the constitutive secretory pathway share the same highly conserved Arg-X-(Lys/Arg)-Arg sequence in which cleavage occurs after the final Arg residue (Strauss et al., 1986; McCune et al., 1988; Dickson and Peters, 1983; Perez and Hunter, 1987). Finally, careful examination of the published work on the timing and cellular location of retroviral...
envelope protein processing suggested to us that some of the conclusions were based upon insufficient evidence (see "Discussion"). In an effort to address this issue, we examined the order of proteolytic processing and carbohydrate maturation of the envelope protein from endogenous MuLV proviruses in the mouse T-lymphoma cell line W7MG1. Specifically we asked where and when proteolytic cleavage occurs during MuLV envelope protein transit through the ER/Golgi system. Based on ours and other data, we propose that there is a common pathway for the processing of all retroviral envelope proteins.

It has previously been reported that complete MMTV envelope protein processing in two MMTV-infected cell lines, W7MG1 and rat hepatoma M1.54, requires glucocorticoid hormone (Rabindran et al., 1987; Rabindran and Stallcup, 1987; Firestone et al., 1982). In the W7MG1 cell line, both MuLV and MMTV envelope proteins are expressed and presumably utilize the same cellular transport pathway due to their similar roles in virus assembly. This situation provided an opportunity to address the question of whether all glycoproteins in the ER/Golgi system of W7MG1 cells require glucocorticoid hormone for their processing and maturation, or whether this hormone-dependent processing is highly specific for the MuLV envelope protein.

**EXPERIMENTAL PROCEDURES**

Materials—W7MG1, an MMTV-infected subclone of the mouse T-lymphosarcoma cell line WEHI7.2, was grown as described previously (Danielson et al., 1983). Endoglycosidases H and F and neuraminidase were purchased from Boehringer Mannheim. Goat antiserum against Rauscher murine leukemia virus envelope protein gp70 was obtained from the National Institutes of Health Virus Cancer Program (Bethesda, MD). Antiserum against p15e was kindly provided by Dr. Bruce Chesebro, Rocky Mountain Laboratories (Hamilton, MT). All other materials were as described previously (Snider et al., 1990).

**Analysis of MuLV Envelope Protein Synthesis and Processing—**

[**[35S]**]Methionine was purchased from Du Pont-New England Nuclear (Wilmington, DE), and the labeling was performed as described previously (Snider et al., 1990). For endo H, endo F, and neuraminidase analyses, 10⁶ cells were labeled with 1 mCi of [**[35S]**]methionine. Cells used for metabolic labeling were grown in the absence or presence of 1 μM dexamethasone for 6–8 h before labeling. Immunoprecipitation of MuLV envelope proteins, digestion of immunoprecipitates with endo H, endo F, or neuraminidase, and subsequent analysis of proteins by SDS-polyacrylamide gel electrophoresis and autoradiography were performed as described previously (Snider et al., 1990). For the isolation and digestion of gp95 with endo F, 10⁶ W7MG1 cells were pulse labeled with 2 mCi of [**[35S]**]methionine for 55 min, washed, and chased for 45 min. Cell extracts were immunoprecipitated and envelope proteins were fractionated by SDS-PAGE (9%). The gel slice containing gp95 was excised and soaked in 0.1% SDS for 16–20 h. The eluted gp95 was diluted in immunoprecipitation buffer and immunoprecipitated. Gp95 was subsequently mock digested or endo F digested and analyzed by SDS-PAGE (9%) as referenced above.

**RESULTS**

**Identification of MuLV Envelope Species—**Many murine cells including the T-lymphoma W7MG1 cell line produce MuLV envelope protein from endogenous proviruses.² Immunoprecipitation studies were used to identify the envelope protein species. W7MG1 cells were pulse labeled with [**[35S]**]methionine for 2 h, washed, and resuspended in medium containing excess nonradioactive methionine for a 1-h chase period. Proteins were immunoprecipitated from cell extracts with antiserum against MuLV envelope protein gp70 or p15e. Immunoprecipitated proteins were then digested with either of two endoglycosidases and analyzed by SDS-PAGE and autoradiography. Digestion with endo H removes only N-linked carbohydrate chains containing 5 or more mannose residues (Robbins et al., 1977). Digestion with endo F removes almost all N-linked carbohydrate residues (except triantennary and tetrantennary complex oligosaccharides) by cleaving at the same site as endo H (Plummer et al., 1984). Endo F digestion serves as a control to define the migratory position of a protein on a gel if all N-linked sugars are removed. Those glycoproteins that are present transiently or reside in the ER tend to be totally endo H sensitive. After glycoproteins are transported from the endoplasmic reticulum and reach the medial Golgi region, all or most of the carbohydrate chains become resistant to digestion by endo H. This is a direct consequence of the cellular mannosidase II enzyme, located in the medial Golgi, removing 2 of the 5 mannose residues and leaving three mannose sugars in the core oligosaccharide (Kornfeld and Kornfeld, 1985; Roth, 1987).

Antiserum against p15e (Fig. 1, panel A) immunoprecipitated the MuLV envelope precursor protein Pr85 and one of the cleaved products p15e (lane 1). Gp70 was not immunoprecipitated by this antibody. Note that when the immunoprecipitates were digested with either endo F or endo H (lanes 2 and 3, respectively), there was no mobility shift of p15e, indicating that p15e lacks N-linked sugars. However, the mobility of Pr85 increased to the same extent after digestion with either endo H or endo F, indicating that all N-linked chains on Pr85 are totally endo H sensitive. As expected, this result suggests that Pr85 is located proximal to the site where endo H-resistant chains are acquired, in the ER or early Golgi compartments. Immunoprecipitation of the same samples with antiserum against gp70 (Fig. 1, panel B) yielded Pr85 again, as expected (lane 4). A second faster migrating band, absent in panel A, was observed when immunoprecipitating with antiserum against gp70 (panel B, lane 4). Since this species was immunoprecipitated with antibody specific for gp70 but not p15e, it is identified as the gp70 envelope species. Endo H digestion of gp70 (gp70*, lane 6) increased its mobility slightly such that it approximately comigrated with endo H-digested Pr85 (Pr85*). However, endo F digestion of gp70 (lane 5) resulted in the appearance of three discrete bands with greatly increased mobility, denoted as a, b, and c. Thus, the N-linked carbohydrate chains on gp70 consist of a mixture of both endo H-sensitive and resistant types of oligosaccharides. The multiple products (a,b,c) from endo F digestion

² R. M. Bedgood and M. R. Stallcup, unpublished observations.
indicate that a few of the mature carbohydrate chains are resistant to endo F. The identity of the various envelope species was further confirmed by kinetic analysis of synthesis and processing (see below).

Identification of an MuLV Envelope Protein Processing Intermediate Gp95—A kinetic (pulse-chase) analysis was performed to determine the time course of MuLV envelope protein processing, and more importantly, to identify any potential processing intermediates. W7MG1 cells were labeled for 30 min with [35S]methionine, washed, and incubated in chase medium for 0–120 min. After immunoprecipitation with polyclonal antiserum against gp70, MuLV envelope proteins were analyzed by SDS-PAGE and autoradiography (Fig. 2). At the 0-min chase time point, precursor Pr85 was the first envelope species detected, along with a second faster migrating band that is likely either the result of initiation at an internal AUG site on the envelope message or degradation of Pr85 during the sample preparations. At the 15-min chase point a high molecular weight band designated gp95 first appeared and reached a maximum level at 30–45 min of chase time. Gp95 decreased at the 60-min chase and was gone by the 90-min chase point. Gp70 first appeared at the 30-min chase point, at which time the level of gp95 had already reached its maximum; gp70 increased to a maximum level at 90–120 min of chase time. Formation of the novel species gp95 clearly occurred prior to that of the cleaved product gp70, but only after synthesis of Pr85. In addition, the kinetic pattern of gp95 appearance, accumulation, and disappearance was closely linked to the appearance and accumulation of gp70. This pattern is consistent with gp95 functioning as an envelope processing intermediate that is cleaved to form gp70 and p15e.

Carbohydrate Analysis of MuLV Envelope Proteins—The fact that formation of gp95 and the appearance of gp70 are tightly coupled kinetically suggests a precursor-product relationship and provides a means of determining where proteolytic cleavage occurs within the cell. Pr85 N-linked oligosaccharides are all endo H-sensitive and thus Pr85 is localized to the ER and early Golgi regions. Mature gp70 contains endo H-resistant oligosaccharides and is thus localized beyond the site of mannosidase II, i.e. beyond the medial Golgi. In order to determine where the processing intermediate gp95 exists within the cellular transport pathway relative to mannosidase II, we performed a pulse-chase experiment and coupled it to carbohydrate analysis by digestion with endo H and endo F. W7MG1 cells were pulse labeled and subsequently chased as previously described. Immunoprecipitated envelope proteins were digested with endo H or endo F, or were mock digested, and analyzed by SDS-PAGE. At the 0-min chase time point Pr85 and the truncated precursor species were the only envelope species present (Fig. 3, lanes 1–3). At the 40-min chase time point envelope species gp86 reached a maximum (lane 7). Endo H digestion of gp95 (gp95*, lane 8) resulted in a slight increase in mobility relative to undigested gp95 (compare lanes 7 and 8). However, endo F digestion of gp95 caused the disappearance of gp95 such that it now presumably migrates with Pr85* (lane 9). Indeed, when gp95 was isolated from a polyacrylamide gel and digested with endo F, it comigrated with endo F-digested Pr85 (data not shown). This is consistent with the interpretation that Pr85 and gp95 differ only in the degree of processing of their carbohydrate chains; removing all N-linked carbohydrate by digestion with endo F resulted in the conversion of gp95 and Pr85 to a single band Pr85*. Since endo H digestion resulted in only a slight increase in the mobility of gp95 (gp95*) and endo F digestion resulted in a more dramatic mobility shift (Pr85*), most but not all of the oligosaccharides on gp95 are endo H resistant. This evidence indicates that gp95 has been modified by mannosidase II and exists only at or beyond the medial Golgi. Finally, at the 90-min chase point gp95 disappeared and gp70 accumulated as expected (lane 10); gp70 yielded the same endo H and endo F digestion products as in Fig. 1 (gp70* in Fig. 3, lane 11; a, b, c in lane 12).

Proteins that are localized to, or transiently reside in, the trans-Golgi characteristically contain terminal sugars, such as sialic acid, on their oligosaccharide chains. To determine if gp95 contains sialic acid, we digested the envelope immunoprecipitates with the sialic acid cleaving enzyme neuraminidase. W7MG1 cells were pulse labeled for 35 min with [35S]methionine, washed, and chased for 40 min as previously described. Immunoprecipitated envelope proteins (Fig. 4) were split into three equal samples: undigested (lane 1), neuraminidase-digested (lane 2), and mock digested (lane 3) proteins were fractionated by SDS-PAGE (9% gel). Undigested envelope species are indicated on the left of the panel, neuraminidase-digested products are indicated on the right side of the panel.
The existence of an endo H-resistant uncleaved processing intermediate, gp95, strongly indicates that cleavage of the envelope protein occurs after the acquisition of endo H-resistant chains. If cleavage were to occur prior to the acquisition of endo H-resistant chains, we would expect to see a completely endo H-sensitive form of gp70 as a processing intermediate; upon endo H digestion, this species should migrate with band c. Our extensive pulse-chase analysis of the MuLV envelope protein, coupled with endo H and endo F digestions, failed to identify any completely endo H-sensitive form of gp70. The absence of a totally endo H-sensitive gp70 intermediate argues against cleavage prior to the acquisition of endo H-resistant chains. Moreover, the fact that gp95, like gp70, contains sialic acid residues suggests that gp95 is terminally glycosylated and present in the trans-Golgi, consistent with cleavage late in the transport pathway.

**MuLV Envelope Processing Occurs Constitutively**—Our results indicate that the sequence of proteolytic processing and carbohydrate maturation for the MuLV (this report) and MMTV (Corey and Stallcup, 1990) envelope glycoproteins are identical in W7MG1 cells. Both involve the synthesis of an endo H-sensitive primary precursor which is first converted to an endo H-resistant processing intermediate prior to the formation of cleaved products. Thus, the processing intermediate is proteolytically cleaved to products at or after the site of mannosidase II activity.

It has been demonstrated that in W7MG1 cells, formation of the endo H-resistant MMTV envelope processing intermediate and subsequent cleavage to products require the action of glucocorticoid hormone (Snider et al., 1990; Corey and Stallcup, 1990). In contrast, processing of MuLV envelope proteins occurred in virus-infected fibroblast cells without any apparent requirement for hormone (Ikuta and Luftig, 1986). Therefore, we asked whether the hormonal regulation of MMTV envelope processing is specific for the MMTV envelope protein or is a peculiar property of the W7MG1 T-lymphoma cell line and applies to other glycoproteins produced in these cells. Fig. 5 illustrates a pulse-chase analysis of MuLV envelope processing in W7MG1 cells in the presence and absence of the synthetic glucocorticoid hormone dexamethasone. The same kinetics and efficiency of processing from Pr85 to gp95 to gp70 were observed in the presence (even numbered lanes) or absence (odd numbered lanes) of hormone. Thus in the same cell line two retroviral envelope proteins with apparently identical transport and processing pathways undergo differential processing regulation by glucocorticoid hormone. Specifically, complete MMTV envelope processing is hormone dependent while MuLV envelope processing is hormone independent (constitutive).

**FIG. 5. MuLV envelope protein processing occurs constitutively.** Cells were grown with (even lanes) or without (odd lanes) 1 μM dexamethasone for 6 h, pulse labeled for 30 min, and chased for the indicated times. MuLV envelope proteins were immunoprecipitated with antiserum against gp70 and analyzed by SDS-PAGE.

**DISCUSSION**

We have undertaken the effort to resolve the question of where and when cleavage of the MuLV envelope protein occurs within the ER/Golgi transport pathway of mouse T-lymphoma W7MG1 cells. Our studies have shown that processing of the MuLV envelope protein occurs through a previously unidentified high molecular weight intermediate gp95. Gp95 appears only after the synthesis of Pr85 but prior to the formation of gp70. The additional observation that disappearance of gp95 correlates well with the accumulation of gp70 is kinetically consistent with gp95 functioning as the immediate precursor to proteolytic cleavage and formation of gp70/p15e. Endo H and Endo F digestion patterns of the various MuLV envelope species were utilized to localize their position in the processing pathway relative to mannosidase II and determine the size of the polypeptide backbone of each glycoprotein species. We found that both the processing intermediate gp95 and the mature product gp70 possessed primarily endo H-resistant oligosaccharide chains, and thus both must exist after the mannosidase II site in the medial Golgi. In addition, gp95 like gp70 contains sialic acid indicative of terminal glycosylation and localization within the trans-Golgi. Thus, kinetic data as well as carbohydrate analysis of the envelope species are consistent with cleavage occurring after the formation of an endo H-resistant processing intermediate gp95.

Previous studies of MuLV envelope protein processing (Fitting and Kabat, 1982; Polonoff et al., 1982; Machida and Kabat, 1982) and HIV envelope protein processing (Stein and Engleman, 1990) concluded that proteolytic cleavage of the envelope proteins occurs early in the transport pathway, prior to the mannosidase II site. However, these conclusions were based on less rigorous criteria in which cleavage was assumed to occur early in the pathway because the precursors were endo H-sensitive and the cleaved products were endo H-resistant. Notably, these studies failed to consider the identification of a processing intermediate as a relevant criterion for establishing the order and localization of envelope processing. For example, for MuLV, if cleavage of the envelope protein occurred prior to the acquisition of endo H-resistant chains as the previous reports have suggested, then a totally endo H-sensitive form of gp70 must exist as a processing intermediate prior to the conversion, by mannosidase II, to endo H-resistant chains. After careful studies, we have been unable to detect any form of gp70 that is totally endo H sensitive. Ulmer and Palade (1991) also concluded that proteolytic cleavage of the MuLV envelope protein occurs prior to the acquisition of endo H-resistant chains based on their finding that gp70 contains endo H-sensitive chains. However, their work failed to address the possibility that gp70 also contains endo H-resistant chains; no endo F digestion of the envelope species was performed. In contrast, our results demonstrated that gp70 contains a mixture of endo H-resistant and endo H-sensitive oligosaccharide chains. Moreover, the relative magnitude of the mobility shift caused by endo F versus endo H digestion indicates that most, but not all of the seven N-linked carbohydrate chains on gp70 are endo H resistant. The failure of some oligosaccharide chains to become endo H resistant is likely due to inaccessibility of some chains on Pr85 to mannosidase II. Ulmer and Palade (1991) also designated a putative processing intermediate (gp78) by the methodology of increasing labeling times to follow the processing of the precursor. Using this methodology, one cannot profile accurately the kinetics of gp78 accumulation and turnover because the amount of MuLV envelope protein labeled was increasing from time point to time point; this also makes it difficult to determine when gp78 first appears. In our pulse-chase studies, where the total level of labeled MuLV envelope protein was relatively constant throughout the
study, we found that gp78 appeared simultaneously with (rather than subsequent to) the full size precursor Pr85. Thus, the characterization of gp78 as a processing intermediate is unsubstantiated. To our knowledge, no totally endo-H-sensitive form of any cleaved retroviral envelope protein has been shown to exist.

The bulk of the evidence now appears to support processing through an endo H-resistant terminally glycosylated intermediate and proteolytic cleavage late in the ER/Golgi pathway for most, if not all proteins that require such processing (except for cleavage by signal peptidase). Our finding of a late cleavage event for MuLV envelope proteins is consistent with previous conclusions regarding the MMTV (Corey and Stallcup, 1990), HIV (Willey et al., 1988), and reticuloendotheliosis virus (Tsai and Orozlan, 1988) retroviral envelope proteins as well as many nonviral proteins. This is not surprising since it appears that both cellular and viral proteins that require intracellular proteolytic processing and reach the cell surface by the constitutive vesicular transport system should share the same highly conserved cleavage recognition sequence Arg-X-(Lys/Arg)-Arg (Strenes et al., 1986; Dickson and Peters, 1983; Ebina et al., 1985; Ullrich et al., 1986; Bentley et al., 1986). In addition, cellular proteins secreted via the regulated secretory pathway, such as proopiomelanocortin, proenkephalin, and proinsulin, contain a variation of this conserved cleavage/recognition sequence lacking the -4 arginine residue and consisting of a basic-basic dipeptide, usually (Lys/Arg)-Arg (Smith and Funder, 1988; Thomas et al., 1986; Docherty and Steiner, 1982). It has been demonstrated by immunoelectron microscopy with monoclonal antibodies that proinsulin cleavage occurs in post-Golgi secretory granules (Orci et al., 1987) by a calcium and acidic pH-dependent endopeptidase (Davidson et al., 1988). It has also been found that NH4Cl inhibits cleavage of the HIV envelope protein in CD4+ cells, probably due to the neutralization of the acidic pH required for proper protease function or vesicular transport in the late Golgi (Willey et al., 1988). All of this data is consistent with a common family of endoproteases localized to the trans-Golgi, in the case of constitutively transported proteins, or in post-Golgi secretory vesicles, in the case of proteins secreted via the regulated pathway. Candidate endoprotease genes such as FUR, PC1, PC2, and PC3 have been isolated from various mammalian cell lines and show extensive homology to the yeast KEX2 gene (van den Steen, 1990; Smeekens and Peters, 1983; Ebina et al., 1985; Ullrich et al., 1986; Bentley et al., 1986). In addition, transfected KEX2 or FUR into a processing defective mammalian cell line, BSC-40, which possesses only the constitutive secretory pathway can induce cleavage of proopiomelanocortin and proβ-nerve growth factor, respectively, after the dibasic Lys-Arg signal. In fact, our recent data indicates that the MuLV envelope precursor Pr74 is restricted to the ER in these cells in the absence of glucocorticoid (Corey and Stallcup, 1992). We are currently investigating the specific sequences of the MMTV envelope protein precursor that prevent its transport from ER to Golgi in the absence of hormone.

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