Novel Glycosylation Pathways of Retroviral Envelope Proteins Identified with Avian Reticuloendotheliosis Virus

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Previously, we identified two mature glycoproteins, gp90, the surface glycoprotein, and gp20, the transmembrane protein, from avian reticuloendotheliosis virus and an avian reticuloendotheliosis virus *env* gene-encoded intracellular polyprotein gPr77^{env}, but the precise relationship of gPr77^{env} to the mature envelope proteins was not determined (W.-P. Tsai, T. D. Copeland, and S. Oroszlan, *Virology* 155:567-583, 1986). In the present study, using metabolic labeling of viral proteins with [³⁵S]cysteine, radioimmunoprecipitation, and carbohydrate structure analysis, we have identified a higher-molecular-weight endo-H-resistant *env* gene-encoded polyprotein designated gPr115^{env} in addition to the endo-H-sensitive gPr77^{env}. It appears that gPr77^{env} is the primary polyprotein precursor, modified with mannosyloligosaccharides that are processed into sialic-acid-rich extraordinarily large complex-type carbohydrates (up to 17 kilodaltons for each N-linked site) on the gp90 domain but not on the gPr22 domain. In this process, gPr77^{env} is converted into the apparently endo-H-resistant secondary polyprotein, gPr115^{env}, which is rapidly processed into gp90 and gPr22. The proteolytic processing which occurs only after the appearance of an endo-H resistant precursor is now clearly demonstrated for a retrovirus. Some important aspects of carbohydrate structure, including the site-specific glycosylation, as well as the intracellular location and nature of the potential enzyme involved in the proteolytic cleavage of gPr115^{env} are discussed.

The *env* gene of type C retroviruses codes for two principal mature viral envelope components. They are gp85 and gp37 in Rous sarcoma virus (RSV), the prototype avian type C virus (7, 15, 20), and gp70 and p15(E) in Moloney murine leukemia virus (Moloney MuLV), a prototype of mammalian type C viruses (7, 32). The major glycoproteins gp85 and gp70 are present as surface knobs on the viral envelope, and gp37 and p15(E) are transmembrane proteins which are the spikes to which gp85 and gp70 attach, by either disulfide bonds or weak noncovalent bonds or both (14, 37, 46, 47). The two envelope proteins for each virus are derived from single glycosylated precursors called gPr95^{env} for RSV and gPr85^{env} for MuLVs. These precursors are initially cotranslationally glycosylated in the rough endoplasmic reticulum and further processed by proteolysis and transported to the plasma membrane, where the cleavage products are incorporated into budding viruses. The precursors were found to be endo-glycosidase-H sensitive, containing high-mannose-type sugars (9, 18, 50, 55, 68, 69). In contrast, RSV gp85 and MuLV gp70 were shown to be endo-H resistant and to contain fucose or sialic acids or both (9, 23, 29, 39, 41, 42, 68, 70). However it remains obscure whether intracellular cleavage of the precursors occurs prior to, immediately after, or during the final stages of glycosylation.

Avian reticuloendotheliosis viruses (REVs) are a group of serologically related type C retroviruses (38, 53) that were isolated from various bird species (71). The replication-competent reticuloendotheliosis-associated helper virus (REV-A) was isolated from REV strain T, the prototype virus, which also possesses a replication-defective component (27). Previous studies on nucleotide sequence, protein sequence, and antigenicity show that REV-A is highly re-

lated to mammalian type C viruses (1, 3, 11, 30, 44, 45, 52, 58, 64, 65, 67). Previously, we identified two glycoproteins, gp90, the surface glycoprotein, and gp20, the transmembrane protein, from the virus and a related intracellular precursor polyprotein gPr77^{env} (previously called gPr75-79^{env}) (65). From the nucleotide sequence (67) and N- and C-terminal protein sequences (65), we estimated the molecular weight of the gp90 peptide backbone to be ~39,500 (356 amino acid residues). In a preliminary study, endo- β -acetylglucosaminidase F was shown to reduce the size of gp90 by 40 to 50 kilodaltons (kDa), indicating that gp90 is highly glycosylated. Thus we speculated that gPr77^{env} may be processed into the transmembrane protein gPr22/gp20 and an immature form of gp90 which is further glycosylated into the full-sized surface glycoprotein gp90 (65). However, the existence of an unidentified larger precursor which would serve as the proximal intermediate to gPr22 and gp90 could not be excluded. In the present study, using metabolic labeling of viral proteins with [³⁵S]cysteine, radioimmunoprecipitation, and carbohydrate structure analysis, we identified, in addition to gPr77^{env}, a higher-molecular-weight *env* precursor designated gPr115^{env} and showed it to be modified with sialic acid-rich complex-type carbohydrates (endo-H resistant). We also showed that gPr115^{env} is the proximal precursor to gp90 and gPr22. The glycosylation pathway and the proteolytic processing of an endo-H-resistant precursor, as described in this report for REV-A, have yet to be identified in other retroviral systems.

MATERIALS AND METHODS

Cell lines and virus. REV/cBMC, a continuous line of transformed chicken bone marrow cells that produces REV-A (27), was used in this study.

Antisera. The antisera directed against purified REV-A gp20, p30, or the synthetic peptide corresponding to the

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C-terminal 13 residues of gp90 have been described previously (64, 65).

Radioimmunoprecipitation of cell cultures and viruses. REV/cBMC, grown actively in RPMI 1640 supplemented with heat-inactivated 10% fetal bovine serum, 1% L-glutamine, and penicillin-streptomycin, was labeled with [35 S]cysteine (40 μ Ci/ml) in cysteine-free RPMI 1640 plus 10% dialyzed fetal bovine serum. After labeling, the cells were harvested by centrifugation at 500 to 1,000 rpm for 5 min by using an IEC HN-S centrifuge (Damon/IEC Div.). The virus particles were obtained by pelleting from clarified culture medium by using ultracentrifugation at 25,000 to 50,000 rpm for 90 min in a Beckman L3-50 ultracentrifuge in nitrocellulose tubes which were cushioned at the bottom with 20% sucrose in TNE buffer (10 mM Tris hydrochloride [pH 7.0], 0.1 M NaCl, 0.001 M EDTA).

Radioimmunoprecipitation. After being labeled with [35 S]cysteine, cells were washed with cold phosphate-buffered saline and then disrupted with either lysing buffer (0.02 M Tris hydrochloride [pH 7.5], 0.05 M NaCl, 0.5% sodium deoxycholate, and 0.5% Nonidet P-40 plus approtinin [10 to 20 μ g/ml] and 1 mM phenylmethylsulfonyl fluoride) or with RIPA buffer (0.02 M Tris hydrochloride [pH 7.5], 2 mM EDTA, 0.15 M NaCl, 0.25% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and 10 to 20 μ g of approtinin per ml). Cell lysates were then clarified at about 15,000 rpm for 10 to 30 min in a Sorvall RL-5 centrifuge or an Eppendorf microcentrifuge 5415. The pelleted viruses were solubilized with lysing buffer. To perform immunoprecipitations, samples of cell lysates or viruses were incubated with antisera in the presence of Sepharose-protein A at 4°C overnight. The immunoprecipitates were then washed two or three times with RIPA buffer followed by two washes with Tris buffer (10 mM Tris hydrochloride, pH 7.8) with protease inhibitors. The washed precipitates were ready for autoradiography.

SDS-PAGE and autoradiography. The washed immunoprecipitates described above were first subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by using a 7.5 to 18% gradient of acrylamide (34). The gels were then dried, and radioactive bands were located by scintillation autoradiography (36).

Analysis of carbohydrate structures of the glycoproteins by glycosidases. Glycoprotein samples that were isolated by immunoprecipitation as described above were further washed with cold distilled water. At this point they were used in assays or stored at -20°C for future uses. Endoglycosidase D (endo-D), endoglycosidase H (endo-H), and endoglycosidase F (endo-F) were purchased from Boehringer Mannheim Biochemicals, and neuraminidase (type X from *Clostridium perfringens*) was purchased from Sigma Chemical Co. The enzymes were used as recommended by the manufacturers, but they were usually incubated for a longer time to ensure complete digestions and to monitor possible proteolytic activities. Aliquots of Sepharose-protein A-bound substrates prepared from a large scale of precipitation were used for assays in all experiments. For digestion with enzymes, 30 μ l of an appropriate buffer (see below) was added to a sample of Sepharose-protein A-bound substrate contained in a 0.5 ml-microfuge tube; after being boiled for 2 min, the sample was incubated with enzymes in a shaking water bath at 37°C. The amounts of enzymes used for the assays were 0.1 U for endo-F, 0.001 U for endo-H, 0.001 U for endo-D, and 0.01 U for neuraminidase. Endo-F or endo-H was assayed with endo-F buffer containing sodium phosphate buffer (100 mM, pH 6.0), EDTA (50 mM), Noni-

FIG. 1. Detection of REV-A *env* gene-encoded precursor polyproteins in REV/cBMC by radioimmunoprecipitation. Cells were labeled for 1 h, and cell lysates and solubilized virus were prepared as described in Materials and Methods. Samples were then precipitated (2×10^6 cpm of cell lysate or 0.2×10^6 cpm of solubilized virus for each immunoprecipitation) with either antibody to gp90 peptide or gp20 in the presence of Sepharose-protein A at 4°C overnight. The immunoprecipitates were washed and subjected to SDS-PAGE of a 7.5 to 18% gradient. The gels were dried, and bands were visualized by autoradiography. Lanes: 1, cell lysates; 2, virus; S, molecular weight markers in thousands [K].

det P-40 (1%), 2-mercaptoethanol (1%), and SDS (0.1%), and the incubation time was 2 h to overnight. Endo-D was assayed with endo-D buffer containing sodium phosphate buffer (100 mM, pH 7.5), and the incubation time was the same as that for endo-F or endo-H. Neuraminidase was assayed with Neu buffer containing sodium acetate (50 mM, pH 5.5), NaCl (100 mM), Triton X-100 (0.5%), and calcium acetate (1 mM) with or without NaN_3 (0.4%). The incubation time was 16 to 24 h. For the negative control without enzymes, the samples were incubated in comparable buffers. After incubation, the samples were added with 20 μ l of 4 $\times$ electrophoresis loading buffer without SDS (34) and 7 μ l of 10% SDS to a final concentration of about 1%. The samples were then boiled and ready for SDS-PAGE followed by autoradiography.

RESULTS

Identification of gPr115^{env} precursor polyprotein. To identify the REV-A *env* gene-encoded precursor in REV-A-infected chicken bone marrow cells (REV/cBMC), we labeled cells with [35 S]cysteine for 1 h. The lysates of labeled cells as well as the virus pelleted from the medium were then analyzed by immunoprecipitation by using antisera to purified REV-A transmembrane protein gp20 and surface glycoprotein gp90 synthetic peptide. The results are shown in Fig. 1. From the cell lysate (lanes 1), gPr115^{env} and gPr77^{env} were detected by both antisera, indicating that both of these molecules contain gp90 and gp20 antigenic domains. The antibody to gp90 peptide (left panel) detected gp90 in both the cell lysate (lane 1) and the virus (lane 2). This suggested that mature viral gp90 is processed intracellularly from its precursor. Antibody to gp20 (right panel), on the other hand, detected gPr22 from the cell lysate (lane 1) and gp20 from the virus (lane 2). This confirms that gPr22 is a proximal intra-

FIG. 2. Analysis of the metabolic relationship among REV-A *env* gene-encoded proteins by pulse-chase experiments followed by immunoprecipitation and autoradiography. REV/cBMC and REV-A viruses were grown, labeled, and harvested as described in the legend to Fig. 1. The cells were labeled for 5 and 10 min for the kinetic study and for 15 min in the presence of tunicamycin (10 μ g/ml) (preceded by an additional 15 min treatment before labeling). The cells that were labeled for 10 min were then chased for different periods of time in complete medium. The cells were then lysed in 1 ml of RIPA buffer, and viruses were lysed in 50 μ l. Cell lysates were precipitated with either anti-gp90 or gp20, and solubilized viruses were precipitated with a combination of these two antibodies. For cell lysates, samples with 10^6 cpm were used for each immunoprecipitation. For viruses, samples of 45 μ l (containing 10×10^3 to 70×10^3 cpm, except for that for the 10-min pulse which had higher counts) were used. (A) Cell lysates precipitated with anti-gp20. (B) Cell lysates precipitated with anti-gp90. (C) Viruses precipitated with a mixture of anti-gp20 and anti-gp90. Lanes: 1 and 1v, pulsed for 5 min; 2 to 6 and 2v to 6v, pulsed for 10 min and not chased (lanes 2 and 2v) or chased for 5 min, 20 min, 50 min, and 2 h (lanes 3 to 6 and 3v to 6v, respectively); 7 and 7v, pulsed in the presence of tunicamycin; S, molecular weight markers (in thousands [K]).

cellular cleavage product of *env* precursor and serves as an immediate precursor to the mature gp20 as we described previously (65). A band of 90 kDa in the anti-gp20 precipitate (lanes 1 and 2) represents coprecipitation of gp90, since the gp20 protein that was used for the preparation of antibody was highly purified and antibody was shown to detect only gp20 in the virion (65).

In the previous study (65) in which we pulse-labeled cells with [35 S]methionine for 20 min followed by a chase for various periods of time, gPr115^{env} was not noticed. There are two reasons for the failure to detect this molecule. First, the predicted sequence for the *env* precursor polypeptide contains more cysteine residues (a total of 23) than methionine residues (a total of 9). Second, gPr115^{env} was shown to be transient in the pulse-chase experiment using [35 S]cysteine. In addition, the use of antisera to both gp90 and gp20 in the present study (only anti-gp20 was used previously) may have facilitated the ready detection of gPr115^{env}.

Metabolic relationship among the *env*-gene products. To understand the metabolic relationship of gPr115^{env} to gPr77^{env} and their end products a time course study using pulse-chase was performed. REV/cBMC was labeled with [35 S]cysteine, chased, and lysed. The cell lysates were further processed by immunoprecipitation and SDS-PAGE followed by autoradiography. The virus pelleted from the medium was treated similarly. The results are shown in Fig. 2. The lysates were reacted with anti-gp20 (panel A) and anti-gp90 (panel B) separately, whereas the virus samples were reacted with a mixture of gp20 and gp90 antisera (panel C). From the data in Fig. 2, gPr77^{env}, which was synthesized

rapidly after a 5- to 10-min pulse (lanes 1 and 2 in panels A and B) and maintained at the same level after a 20-min chase following the 10-min pulse (lanes 3 and 4 in panels A and B), appears to be the immediate precursor to gPr115^{env}. Without accumulating to a substantial level (compared with gPr77^{env}), gPr115^{env} was rapidly processed into intracellular gp90 and gPr22 (lanes 4, 5, and 6 in panels A and B). The data further show that the intracellular gp90 and gPr22 peak at the 50-min chase concomitant with the first appearance of gp90 and gp20 in the virion (lane 5 in panels A and B and lane 5v in panel C). At the end of the chase (2 h) (lane 6 in panels A and B, and lane 6v in panel C), as the intracellular supply of gPr22 and gp90 decreased, the amount of proteins detected in the released virion reached its maximum level.

Analysis of the nature of glycosylation of the proteins. The experimentally determined molecular sizes of gPr115^{env} (115 kDa) and gp90 (90 kDa) are substantially larger than those predicted from the DNA sequences (546 amino acids and four Asn-X-Ser/Thr sites for gPr115^{env} and 356 amino acids and three Asn-X-Ser/Thr sites for gp90) (67). To determine the extent of glycosylation, we treated REV/cBMC cells with tunicamycin before and during labeling with [35 S]cysteine. The results of immunoprecipitation are shown in Fig. 2, lane 7 in panels A and B and lane 7v in panel C. From the cell lysates, only a single protein of 58 kDa was detected by antibodies to gp20 (lane A7) or gp90 (lane B7). No proteins were detected in the extracellular pellet (lane C7v). The data indicate that the REV-A *env* gene precursor is glycosylated at the N-linked sites, since tunicamycin is known to inhibit N-linked glycosylation (16).

To analyze the nature of glycosylation of these proteins,

FIG. 3. Analysis of carbohydrate structure of REV-A *env* gene-encoded glycoproteins by glycosidases. REV-A proteins isolated from viruses and cell lysates with or without treatment with glycosidases are shown, respectively, in panel A and panel B (except for lanes 6 in which proteins isolated from the virion were used). *env* gene-encoded proteins were used in lanes 1 to 6 of panel A and in lanes 1 to 10 of panel B; P58 isolated from tunicamycin-treated cells was used in lane B4. *gag*-encoded Pr60^{gag} was used in lanes 9 and 10 of panel B. Enzymes used for the treatment of the proteins are indicated above the panel. (—), No glycosidase; D, endo-D; H, endo-H; F, endo-F; N, neuraminidase; S, molecular weight marker (in thousands [K]). REV/cBMC and REV-A viruses were grown and harvested as described in the legend to Fig. 1. The cells were labeled for 1 h with [³⁵S]cysteine (20 μ Ci/ml) in cysteine-free medium. The viruses were produced by the cells labeled with [³⁵S]cysteine (60 μ Ci/ml) for 20 h, during which the density of the cells was 1×10^7 to 0.5×10^7 cells per ml in the first 4.5 h and 0.5×10^6 cells per ml by dilution at the beginning of the remaining period. To obtain tunicamycin-induced proteins, the cells were treated as described in the legend to Fig. 2. The *env* gene-encoded proteins were isolated by immunoprecipitation, with a mixture of antibodies to gp90 peptide and gp20 in the presence of Sepharose-protein A, from either cell lysates or viruses prepared as described in the legend to Fig. 1. The precipitates from viruses were shown to contain gp90 and gp20, with more counts in gp90 than in gp20, a pattern similar to that shown in Fig. 1, lane 2. Precipitates from cell lysates contained gPr77^{env}, gPr115^{env}, gp90, and gPr22, with more gPr77^{env} than other components and with gPr22, as the smallest amount. P58 was isolated from the cells treated with tunicamycin by using the same antibodies described above. Pr60^{gag} was isolated by using antibody to REV-A p30. P58 and Pr66^{gag} were used to serve as controls to monitor the possible proteolytic activities present in the glycosidases. The details of the assays are described in Materials and Methods.

we used a panel of glycosidases. The proteins were metabolically labeled with [³⁵S]cysteine, immunoprecipitated, and then digested with various enzymes. The results are shown in autoradiographs (Fig. 3). Panel A shows proteins isolated from viruses, and panel B shows proteins from cell lysates. The apparent molecular weights of the end products generated by glycosidases were estimated and are summa-

TABLE 1. Effect of glycosidases on REV-A *env* gene-encoded glycoproteins^a

Glycoprotein and source	Predicted mol wt of peptide chain	Observed molecule size (kDa) after treatment with:			
		Endo-H	Endo-D	Endo-F	Neuraminidase
Virus					
gp90	39,160	ND ^b	ND	47	77
gp20	17,000 ^c	17	ND	17	ND
Cells					
gPr77 ^{env}	60,060	62	62	62	ND
gPr115 ^{env}	60,060	ND	ND	62	92.5
gp90	39,160	ND	ND	47	77
gPr22	20,900	19	ND	19	ND

^a A summary of the results shown in Fig. 3 is given. The observed molecule size of the proteins was calculated from the predicted sequences on the basis of 110 daltons per amino acid residue.

^b ND, No detectable size shift.

^c Estimated value. No determined C-terminal sequence of gp20 is available.

rized in Table 1. Compared with the control without glycosidases (lane B3), digestion with endo-F of immune precipitates containing gPr77^{env}, gPr115^{env}, and gp90 was shown to produce a 62-kDa molecular species (Table 1 and lane B5) and a 47-kDa molecular species, derived from gPr77^{env}/gPr115^{env} and from gp90, respectively, as discussed below. This result is consistent with those from the tunicamycin experiment, suggesting that gPr115^{env} is highly glycosylated (carbohydrates account for almost half of its molecular mass). Only gPr77^{env} was sensitive to endo-H and yielded a 62-kDa species (lane B2 and Table 2), similar in size to that obtained with endo-F. In contrast, gPr115^{env} was shown to be resistant to endo-H. High-mannose-type and complex-type carbohydrates were shown to be endo-H sensitive and endo-H resistant, respectively (61, 62). Therefore, it appears that gPr77^{env} is the only precursor that is a high-mannose type carbohydrate, whereas gPr115^{env} is a complex-type carbohydrate. These results, together with the kinetic studies (Fig. 2), clearly indicate that gPr77^{env} is processed into gPr115^{env} by conversion of the high-mannose moieties into complex-type oligosaccharides. Both gPr22 and gp20 were reduced in size by ~3 kDa after digestion with endo-H (Table 1 and Fig. 3A and B, lanes 2) as compared with the controls without the glycosidase (lanes A1 and B3), indicating that they contain high-mannose-type oligosaccharide as found for gp20 of Mason-Pfizer monkey virus (10). On the other hand, gp90, like gPr115^{env}, was shown to be unaffected

TABLE 2. Comparison of estimated size of N-linked carbohydrate chains of REV-A and other retroviral *env* proteins

Virus	Protein	Size (kDa) of N-linked chain in carbohydrate type:		
		High mannose	Complex	Sialoligosaccharide
REV-A ^a	gPr77	4.8		
	gPr115		17.0	5.5
	gp90		14.3	4.3
	gPr22	3.0		
MuLV ^b	gp20	3.0		
	gPr85	2.4		
	gp70		3.0–3.5	0.3–0.6
RSV ^c	gPr95	2.2		
	gp85		3.0–3.5	1.0
	gp37		6.6–7.6	

^a The values for REV-A are calculated from the data in Table 1. The *env* gene sequence has four potential N-linked sites, three on the gp90 domain and one on the gp20/gPr22 domain. The N-linked carbohydrate-free *env* polyprotein (P58) identified in tunicamycin-treated cells is 58 kDa.

^b The values are taken or calculated from data from references 17, 50, 56, and 70.

^c The values are taken or calculated from data from references 8, 9, and 31.

by endo-H treatment (lane 2 in panels A and B). This indicates that complex-type carbohydrates are present in gp90. Endo-F reduced gp90 to a carbohydrate-free polypeptide of 47 kDa (lanes A4 and Table 2), suggesting an average mass of about 15 kDa per complex-type carbohydrate moiety (three Asn-X-Ser/Thr sites are present in the deduced sequence). The large size of this complex-type carbohydrate seemed to be unique among retroviruses, and we decided to investigate it further.

Neuraminidase was used to test for sialic acids in REV-A glycoproteins. The results show that the complex-type gPr115^{env} and gp90, but not the high-mannose-type gPr77^{env} or gPr22/gp20 (Fig. 3, lane A6 compared with lane A5 without the enzyme; and lane B8 compared with lane B7 without the enzyme), are sensitive to this enzyme, indicating that the complex oligosaccharides are probably terminally sialylated. Taken together, these results could suggest that gp90 and its polyprotein precursor gPr115^{env} contain sialylated biantennary hybrid and/or biantennary complex oligosaccharides that are sensitive to endo-F (61). However, the presence of tri- or tetraantennary complex-type carbohydrates cannot be completely excluded. Such structures were identified in the major oligosaccharides of Friend MuLV envelope proteins (24). It remains to be seen if O-linked sugars (6, 28, 35, 48) are also present on REV-A *env* proteins. We note that the product of gPr115^{env} obtained by endo-F digestion (Fig. 3, lane B5) was ~4 kDa larger than that induced by tunicamycin treatment (lane B4) and the endo-F digestion product of gp90 is 47 kDa (lane A4 and Table 1), about 8 kDa larger than the size of the predicted gp90 peptide backbone.

The high-mannose-type oligosaccharides, which are sensitive to endo-H, are known to have various structures (62), one of which is sensitive to endo-D and possesses an N-linked Man₅GlcNAc₂ component with an unsubstituted α -mannosyl residue that links to core trisaccharide by an α 1→3 linkage (40, 60). Endo-D was used to check whether this specific heptasaccharide structure exists in the REV-A endo-H-sensitive proteins. Glycoproteins immunoprecipitated from the cell lysate and treated with the enzyme (Fig. 3, lane B1) were shown to give rise to a new band of 62 kDa apparently derived from gPr77^{env} and similar in size to but less intense than that detected after endo-H treatment (lane

B2 and Table 1). The enzyme-free control (lane B3) did not show this band. In contrast, gPr22 (lane B1) and gp20 (lane A3) appear to be resistant to endo-D, since the bands of either 19 or 17 kDa, which were detected after digestion with endo-H, were not detected after treatment with endo-D. The combined data indicate that a portion of gPr77^{env} molecules was modified with this specific heptasaccharide on the gp90 domain but not on the gPr22 domain. In view of the findings that the endo-D-resistant form of vesicular stomatitis virus G protein is converted to the endo-D-sensitive form before becoming endo-H resistant (4, 16), it is likely that gPr77^{env} molecules are similarly processed and that the endo-D-sensitive species is the proximal intermediate to the complex-type gPr115^{env}.

DISCUSSION

From the present analysis of the carbohydrate types of REV-A glycoproteins, the primary precursor, gPr77^{env}, appears to be modified with endo-H-sensitive high-mannose-type oligosaccharides on both the gp90 and gPr22 domains. By additional processing of the mannosyloligosaccharides on the gp90 domain into endo-H-resistant, sialic acid-rich complex carbohydrates, gPr77^{env} was converted into the secondary precursor, gPr115^{env}, which was rapidly cleaved into the endo-H-resistant gp90 and the endo-H-sensitive gPr22 intracellularly. gPr22 together with gp90 was incorporated into the virion and further processed into gp20, which was also endo-H sensitive.

We have also demonstrated that biosynthesis of an endo-H-resistant complex-type precursor precedes the proteolytic cleavage of the precursor in the REV-A *env* protein synthesis. This metabolic pathway has not been clearly demonstrated in other retroviruses. In a study (43) of Moloney MuLV-infected cells, a fucosylated 92-kDa protein was detected in addition to the unfucosylated smaller precursor, but this slow-migrating band did not incorporate [³H]leucine and its metabolic relationship to the mature products was not demonstrated. In other studies of MuLVs and RSV (9, 42, 50, 68), the precursors were found to be devoid of fucose or sialic acids or both, contrasting with the mature cleavage products, which were shown to be endo-H resistant and to contain fucose or sialic acids or both. From the data of the present study, it is evident that the intracellular cleavage of the REV-A *env* precursor polyprotein occurs after the final stages of glycosylation, generating unusually large structural units of carbohydrates. The novel glycosylation pathway demonstrated here in REV-A may not be unique; gPr115^{env} may represent a possible missing link in the metabolic pathways of other retroviruses.

REV-A gPr115^{env} was shown to be modified with complex-type carbohydrate on the gp90 domain and with high-mannose-type carbohydrate on the gPr22 domain, demonstrating site-specific variations in carbohydrate type within the molecule. The mechanism that dictates the site-specific modification is not clear. However, in view of the fact that only the gp90 domain on gPr77^{env} but not the gPr22 domain was sensitive to endo-D, the structure of the endo-D-sensitive heptasaccharide may be essential for further glycosylations that give rise to the complex-type carbohydrate on the gp90 domain within gPr115^{env}. The distribution of the two different carbohydrate types on the gPr115^{env} molecule falls into a pattern, based on a survey of about 50 proteins, in which the complex-type oligosaccharides are located closer to the N terminus of the protein and the high-mannose-type oligosaccharides are located closer to the C

terminus (49). Furthermore, the different sensitivities to endo-D of mannoside chains on the two different domains within gPr77^{env} indicate site-specific variations in trimming of glucose and mannose residues on parent tetradecasaccharide chains. Conceivably, the site-specific trimming is directed by the folding of gPr77^{env} molecules during translation that allows only the carbohydrate chains on the gp90 domain but not that on the gPr22 domain to be accessible to the action of glucosidases or mannosidases or both in the glycosylation pathway. The limited number of the glycosylation sites on gPr77^{env}/gPr115^{env} may make REV-A a unique system in which to explore the parameters that direct the site-specific variation in glycosylation.

The present study establishes the intracellular cleavage pathway for REV-A *env* proteins. A relevant question that has been discerned can now be addressed. What is the nature of the cleavage enzyme, and where is it localized? It has been found that in some cell types sialyltransferase is associated with the distal Golgi or even with more distal tubular structures appearing ready to exit to the plasma membrane (25, 57). The addition of sialic acid to gPr115^{env} may also take place in such subcellular structures. Thus, the cleavage of the gPr115^{env} into gp90 and gPr22 is likely to occur at the distal Golgi structures or en route to the plasma membrane. The *env* precursor of RSV is perhaps also cleaved at a similar cellular location (12). A cellular protease is speculated to catalyze the cleavage at the carboxyl end of the last dibasic residue of the gp90 C-terminal tetrapeptide (Arg-His-Lys-Arg) (65). A pair of basic residues commonly occurs at the cleavage site of many hormone and neuropeptide precursors (59). Recently, the KEX2 protease that was found to be associated with the Golgi membrane of yeast cells was shown to specifically cleave such sequences of yeast prepro- α -factor (21) and human proalbumin (2). These findings support our arguments for a potential cellular protease for the REV-A gPr115^{env} cleavage and the localization of this enzyme. The consensus tetrapeptide (Arg-X-Arg/Lys-Arg) that has three basic residues has been found to be conserved in viral glycoproteins of all retroviruses (13, 65) and some other enveloped viruses (22, 51, 54). Thus, the tetrapeptide appears to be the specific recognition site for the cellular enzyme responsible for the cleavage of the viral *env* precursors.

The apparent large size of the complex-type carbohydrate identified on REV-A *env* proteins is of interest. To explore its potential significance, REV-A N-linked carbohydrate chains and their sialic acid contents were compared with those of other retroviral *env* proteins (Table 2). It is evident that gPr115^{env} and gp90 are equipped with extraordinarily large carbohydrates, up to 17 kDa for each N-linked chain, unlike the complex-type chains found in MuLV and RSV that are small. The sialylated N-linked oligosaccharide is known to consist of a general structure of (sialic acid-Gal-GlcNAc)₂₋₄-Man₃-(GlcNAc)₂-, a core pentasaccharide with two to four sialylactosamine outer branches (5, 33). It is highly likely that the biantennary complex oligosaccharides, one of the two possible types that equip gPr115^{env}/gp90, may assume a structure with a similar scheme. In the REV-A complex carbohydrate, the structure corresponding to the pentasaccharide core is likely derived from the endo-D-sensitive heptasaccharide by further removal of two outer mannose residues. Considering the large size of sialoligosaccharides, the complex-type structure is likely to be sialylated with unusual polysialic acid chains. Polysialosyl glycopeptides have only recently been described on neural cells (19, 33, 63). The size of at least 4.2 kDa for each N-linked chain

on the gp90 domain, as estimated from the size of the complete complex-type chain less that of the polysialic acid chain and that of the mannoside chain on gPr77^{env}, appears too large for single lactosamine units in the biantennary complex chain on gPr115^{env}. gPr115^{env} may contain variations in its lactosamine, with substitutions on *N*-acetylglucosamine residues; or instead of single units, it may contain repeating lactosamine units that have been identified in the minor fraction of oligosaccharides isolated from the Friend MuLV gp71-p15E/p12E complex (24) and in some hematopoietic cells (33).

In summary, we have identified an endo-H resistant REV-A *env* gene-encoded polypeptide, gPr115^{env}, in addition to endo-H-sensitive gPr77^{env}, which we previously described. It is concluded that gPr77^{env} is the primary polypeptide precursor, modified with mannosyloligosaccharides on both the gp90 and gPr22 domains. By a mode of site-specific glycosylation that processes mannosyloligosaccharides on the gp90 domain but not on the gPr22 domain into sialic-acid-rich complex-type carbohydrates, gPr77^{env} is converted into the endo-H-resistant secondary polypeptide gPr115^{env}, which is the immediate precursor to gp90 and gPr22. gPr22, which is endo-H sensitive, is incorporated together with gp90 into the virion and gives rise to endo-H-sensitive gp20. The proteolytic processing which occurs only after the appearance of an endo-H-resistant precursor is now clearly demonstrated for a retrovirus. It has been proposed that glycosylation inhibitors, such as inhibitors of trimming enzymes (26, 66), may control retroviral diseases. REV-A may provide a convenient system in which to define the precise parameters of oligosaccharide processing and to study inhibitors of envelope maturation.

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