Topography of Murine Leukemia Virus Envelope Proteins: Characterization of Transmembrane Components

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Trypsinization of intact Moloney murine leukemia virus resulted in cleavage of p15(E) and Pr15(E) at a site near the middle of the molecule, producing a 9,000-dalton amino-terminal fragment which contains the disulfide linkage site to gp70 and which carries p15(E) epitopes *b* and *c*, but not epitope *a*. After solubilization of the viral membrane, trypsinization occurred at a second site within 1,000 daltons of the carboxy end of p15(E). This site is not exposed in intact virions, indicating that p15(E) and Pr15(E) are transmembrane proteins.

Murine leukemia viruses (MuLVs) contain two major envelope proteins, gp70 and p15(E). These proteins are produced by cleavage of a common precursor (2, 8, 12, 16) and form disulfide-linked complexes with each other (9, 11). Many observations indicate that gp70 is only loosely attached to the viral membrane, whereas p15(E) appears to be an integral membrane protein. Intracellularly, Pr15(E), a precursor form of p15(E), is found which is usually processed to p15(E) at a late step in the budding process (3); however, low levels of intact Pr15(E) are often incorporated into virions. Studies with monoclonal antibodies have indicated that p15(E) contains at least three distinct antigenic epitopes, *a*, *b*, and *c*. Site *a* is specific for ecotropic virus, site *b* is present in ecotropic and xenotropic MuLVs, and site *c* is conserved as well for feline leukemia virus (6). Recently, the amino acid sequences of these proteins have been deduced from the DNA sequence of the *env* gene (13). This sequence indicates that Moloney Pr15(E) is 196 amino acids long, whereas mature p15(E) contains 180 residues. Hydrophobic regions are present near each end of p15(E): a 42-residue stretch occupies positions 2 through 43, and a 30-residue stretch occupies positions 135 through 164. Both of these regions are long enough to span the membrane, and thus several possible models for the association of these proteins with the membrane can be constructed. In this paper, we present evidence of the orientations of p15(E) and Pr15(E) in viral membranes and of the localization of several p15(E) epitopes to amino-terminal regions of the molecule.

Treatment of freshly purified intact virions with high concentrations of trypsin resulted in the complete degradation of gp70 and in a large diminution in the levels of p15(E) and Pr15(E)

(Fig. 1). Fragments of gp70 and an approximately 9,000-dalton (9K) fragment of p15(E), recognized specifically by monoclonal antibody 42/114 (lanes C) but not by antibody 9-E8 (lanes B), were formed. (9-E8 recognizes epitope *a*; 42/114 has a reactivity pattern similar to that of 19-F8 in an analysis against a broad panel of virus isolates, and thus we tentatively assigned the specificity of this antibody to epitope *c*.) The concentrations of the internal proteins p30 and p12 remained unchanged, confirming the integrity of the viral envelope. These results confirmed a previous report that the *env* proteins gp70, Pr15(E), and p15(E) are all exposed at the surface of intact virions (7) and further suggested that the accessibility of gp70 to trypsin is greater than that of p15(E).

The relative sensitivity of p15(E) to trypsinization in intact versus solubilized virus was compared (Fig. 2A). The titration indicated that the susceptibility of gp70 to cleavage is greater after lysis, whereas the extent and kinetics of cleavage of p15(E) are comparable for intact and lysed virions. From other studies, we know that the major gp70 fragments obtained are derived from the carboxy terminus of the molecule, and there appears to be a relationship between the reduction in size of the gp70 fragments formed and the cleavage of p15(E), suggesting that degradation of the carboxyl domain of gp70 may increase the accessibility of p15(E) to proteolysis. After treatment with the highest trypsin concentration, a slight shift in the mobility of p15(E) was detected for the solubilized protein but not for the intact virus (lanes 3). This size shift is more clearly visualized in Fig. 2B and corresponds to a molecular weight change of 500 to 1,000. Analysis of the predicted amino acid sequence of Moloney p15(E) indicated that the

FIG. 1. Effect of trypsinization of intact Moloney MuLV. Freshly purified [35 S]methionine-labeled virions (200,000 cpm per sample) were incubated with trypsin (1.25 mg/ml) for 30 at 37°C. The trypsin was inactivated with Trasylol (1,000 U/ml; Bayer), and the virions were solubilized with 0.5% Nonidet P-40. Both treated and nontrypsinized (control) samples were then solubilized with radioimmunoprecipitation buffer (0.01 M Tris buffer [pH 7.4] 0.5% Nonidet P-40, 0.5 M NaCl) and immunoprecipitated with the following antisera: lanes A, hyperimmune goat α Rauscher gp70 serum; B, monoclonal α p15(E) antibody 9-E8 (6); C, monoclonal α p15(E) antibody 42/114; D, rabbit α R serum, which specifically recognizes Pr15(E) but not p15(E) (15); and E, normal goat serum. Immunoprecipitates were solubilized by being boiled with 1% sodium dodecyl sulfate—1% mercaptoethanol and analyzed on a 12% polyacrylamide gel by the method of Laemmli (4). Lanes v represent the complete virus samples. Analysis of these immunoprecipitates under nonreducing conditions indicated that the coprecipitation of p15(E) by the α gp70 serum (lanes A) was due in part to the presence of disulfide-linked complexes and in part to the noncovalent association of these proteins.

conversion to this 14K fragment must involve cleavage at the carboxy terminus of p15(E), as there are basic amino acid residues 8 and 10 residues from the carboxy end (assumed to be residue 180 [13]), whereas the first trypsin site at the amino end of the molecule occurs at position 48 (see Fig. 5A). The accessibility of this site only after solubilization of the viral particles strongly suggests that this region of the protein is located inside the viral membrane. This carboxy-terminal region immediately follows the hydrophobic sequence at positions 135 to 164, thus implicating that region as the transmembrane segment of the protein.

This trypsin site near the carboxy terminus of p15(E) provided us with a specific marker for

that end of the protein. The fact that an identically sized 9K fragment was formed upon trypsinization of both intact and solubilized virions (Fig. 2B) indicated that this fragment could not be derived from the carboxy end of the molecule. Consideration of the fragment size and of the predicted distribution of trypsin sites on p15(E) (see Fig. 5A), led us to conclude that the 9K band corresponds to the amino-terminal domain of the molecule.

We have previously shown that p15(E) is disulfide bonded to a site in the carboxy-terminal domain of gp70 (10). To determine whether the cysteine(s) of p15(E) involved in this linkage is located in the 9K fragment, we examined the association of this band with gp70 fragments from virus samples in which disulfide bonds between gp70 and p15(E) had been stabilized by treatment with *N*-ethylmaleimide (NEM) (11). The 9K band was efficiently coprecipitated by α gp70 serum from NEM-treated virus, but not from virus for which the NEM treatment was omitted, indicating that it is disulfide linked to gp70 (Fig. 3). Consistent with this conclusion, the bulk of the coprecipitated 9K band was not seen upon analysis of this sample under nonreducing conditions (data not shown). It thus appears that coprecipitation of the 9K fragment by α gp70 sera is dependent upon disulfide bond formation with gp70, and unlike the situation for intact p15(E) (Fig. 1A), noncovalent associations do not play a major role. Further confirmation of the disulfide linkage of the 9K band to gp70 was provided by analysis of the reactivities of four monoclonal α p15(E) antibodies towards 125 I-labeled p15(E)-gp70 disulfide-linked fragments from AKR MuLV (Fig. 4). Trypsinization generated a 32K carboxy-terminal fragment of gp70, which remained disulfide linked to p15(E) and to the p15(E) 9K fragment also formed, resulting in resolution under nonreducing conditions of a 47K band and a 41K band, present in roughly equivalent amounts. In agreement with our previous results, both the 47K complex and the 41K complex were recognized by 42/114, whereas 9-E8 recognized only the 47K band. Antibodies 19VIII-E8 and 19-F8, directed against p15(E) epitopes *b* and *c* (6), also recognized the 41K band, indicating that these epitopes are also located in the 9K amino-terminal p15(E) fragment. These results are in agreement with the observations by Stone and Nowinski (14) that antibodies against epitopes *b* and *c* exhibit binding competition with each other but not with antibodies against epitope *a*.

In addition to the 9K band recognized by 42/114, a slightly larger fragment was found in variable amounts in immunoprecipitates obtained with antibodies 42/114 and 9-E8 (Fig. 2 and 3). This band, corresponding to a fragment

FIG. 2. Comparison of trypsin sensitivity of gp70 and p15(E) in intact and lysed virions. Freshly purified [35 S]methionine-labeled Moloney MuLV, either intact or after solubilization, was treated with trypsin. After neutralization of trypsin with Trasylol, the samples were diluted with radioimmunoprecipitation buffer, immunoprecipitated with 9-E8, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (A) The following trypsin concentrations were used: lanes 1, 62.5 μ g/ml; 2, 250 μ g/ml; and 3, 1 mg/ml. (B) A trypsin concentration of 1 mg/ml was used, and samples were immunoprecipitated with the following antisera: lanes 1, hyperimmune goat α Rauscher gp70; 2, monoclonal α p15(E) antibody 9-E8; 3, monoclonal α p15(E) antibody 42/114; and 4, normal goat serum.

FIG. 3. Coprecipitation of the p15(E) 9K fragment by α gp70 serum. [35 S]methionine-labeled Moloney MuLV was treated with NEM (0.1%, wt/vol) for 15 h, solubilized with 0.5% Nonidet NP-40, and treated with trypsin (1.25 mg/ml). Control samples for which the trypsin or NEM treatments were omitted were also analyzed. After immunoprecipitation, samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% gels. Antisera used: lanes 1, goat α Rauscher gp70; 2, 9-E8; 3, 42/114; and 4, normal goat serum.

of approximately 10K, was identical in size for both intact and solubilized virions and was formed in greater yield for the solubilized virions (Fig. 2B). That the size of this band was not decreased after solubilization indicates that it cannot be a carboxy-terminal p15(E) fragment.

FIG. 4. Coprecipitation of gp70 fragments with α p15(E) monoclonal antibodies. [125 I]-labeled AKR MuLV (10^6 cpm) was solubilized, treated with trypsin (250 μ g/ml), and then immunoprecipitated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels both before and after reduction with mercaptoethanol. Antibodies used: lanes 1, 42/114; 2, 9-E8; 3, 19-F8; and 4, 19VIII-E8.

It might represent a larger amino-terminal fragment, which may be a precursor to the 9K band. If so, this would indicate that epitope *a* recognized by antibody 9-E8 is located near the carboxy terminus of the 10K fragment. An alternate possibility is that the 10K fragment may represent a gp70 fragment which is strongly associated with p15(E) and is thus coprecipitated by α p15(E) antibodies. Clarification of the structure and origin of this fragment will require analysis of these components by peptide mapping.

The conclusions derived from this study are summarized in diagrammatic form in Fig. 5B. Both p15(E) and Pr15(E) are integral membrane proteins, with a carboxy-terminal hydrophobic

sequence traversing the membrane (a similar model has been proposed by Lenz et al., based on the deduced amino acid sequence of the Akv *env* gene [5]). The transmembrane region is drawn as a helix, because a consideration of energetics has indicated that this is the most stable form for a polypeptide in the apolar portion of membrane bilayers (1). A linear map, drawn to scale, of the distribution of charged residues on p15(E) indicates the presence of many potential trypsin sites in the central portion of the p15(E) protein which could account for the formation of the observed fragments (Fig. 5A). At least one trypsin-sensitive site is exposed on the surface of intact virions, although cleavage at this site required high trypsin

FIG. 5. Diagrammatic representation of structural features and membrane orientation of Pr15(E). (A) Linear map indicating location of charged residues of Pr15(E) (3). Every 10th residue is marked off. Positively charged residues (lysines and arginines) are represented by a dot above the line; negatively charged residues (aspartic and glutamic acids) are represented by a dot below the line. The carboxy termini of p15(E) and Pr15(E) are indicated by arrows. (B) Diagram illustrating membrane orientation of Pr15(E). Single arrowheads represent trypsin sites involved in formation of 9K and 14K fragments; the double arrowhead represents the natural cleavage site utilized in processing Pr15(E) to p15(E). The carboxy-terminal nonpolar region of p15(E) is presented traversing the membrane in helical conformation. The heavy lines represent the nonpolar regions at the amino terminus of p15(E) and carboxy terminus of gp70, which may participate in the noncovalent association of these two molecules.

concentrations, suggesting that accessibility to the protease is somewhat blocked, presumably owing to the proximity of gp70. The deduced sequence of p15(E) indicates that there are three cysteines in the amino-terminal half of Pr15(E) at positions 86, 93, and 94 (13), one or more of which must be involved in this disulfide linkage. An additional trypsin site is located in the cytoplasmic region of p15(E); this site is accessible only after solubilization of the viral membrane. The DNA sequence of the *env* gene indicates the presence of another hydrophobic region at the amino terminus of p15(E), and one near the carboxy terminus of gp70 (13). A model in which these regions also traverse the bilayer is inconsistent with the known peripheral association of gp70 with the membrane. These regions may be involved in noncovalent associations between the two proteins.

The cytoplasmic location of the carboxy-terminal domain of Pr15(E) suggests the possibility that these sequences may be recognized by the viral *gag* proteins and thereby provide the association between *gag* and *env* proteins necessary for assembly of infectious virions. If this is the case, then the reported hypervariability at the extreme carboxy terminus of Pr15(E) starting at residue 191 (5), would indicate that this region is not critical for such interactions but, rather, that the conserved residues 165–190 of the cytoplasmic domain may be involved.

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