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Generation of a Water-Soluble Oligomeric Ectodomain of the Rous Sarcoma Virus Envelope Glycoprotein

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Sequences encoding the transmembrane domain of the Rous sarcoma virus envelope (Env) glycoprotein were deleted and replaced with sequences that signal addition of a glycosyl phosphatidylinositol (GPI) membrane anchor. Stable NIH 3T3 cell lines expressing either the wild-type transmembrane-anchored Env or the Env chimera with a GPI tail were established. The GPI-anchored envelope glycoprotein is expressed, oligomerized, and transported to the cell surface in a manner identical to that of its wild-type transmembrane-anchored counterpart. The GPI-linked protein is quantitatively removed from the cell surface by treatment with phosphatidylinositol phospholipase C. The phosphatidylinositol phospholipase C-released, water-soluble Env glycoprotein ectodomain retains the wild-type oligomeric structure and provides a useful tool for studying the subgroup-specific binding and fusion activities of a prototypic retroviral Env glycoprotein.

Enveloped animal viruses contain glycoproteins that mediate both binding and fusion between viral and cellular membranes. Studies with the influenza virus hemagglutinin (HA) have established a paradigm for viral glycoprotein-mediated membrane fusion (reviewed in references 27, 31, and 33). HA is produced as a trimeric nonfusogenic precursor that is cleaved to its mature fusogenic form during transport to the cell surface. This proteolytic event produces two subunits, HA1, which contains the receptor binding activity, and HA2, the membrane-anchored subunit, which possesses the fusion peptide. When subjected to low pH, HA undergoes conformational changes that are required for fusion activity; the fusion peptides are exposed (14, 16, 32), and the ectodomain acquires hydrophobic properties (27, 31, 33).

In contrast to influenza viruses, little is known about the mechanism by which retroviruses fuse with host cells. Similar to HA, retroviral envelope (Env) glycoproteins are translated as inactive precursor polypeptides that are proteolytically processed en route to the cell surface (11, 17, 19, 24). The resulting mature proteins are oligomers of heterodimers consisting of an outer surface (SU) glycoprotein which binds receptors on susceptible cells and a transmembrane (TM) glycoprotein which is the fusogen. Unlike influenza virus, most retroviruses fuse with host cells at neutral pH (13, 22, 23, 28). Binding of the Env glycoprotein to the host cell receptor or other cellular components may activate fusogenicity (reviewed in reference 29).

One of the most valuable reagents for studying influenza virus HA has been BHA, the water-soluble ectodomain produced by digesting HA with the protease bromelain (3). BHA contains all but nine amino acids of the ectodomain of HA and lacks the transmembrane domain and cytoplasmic tail. The water-soluble nature of BHA facilitated determination of its crystal structure (26, 34). Knowledge of the three-dimensional structure of HA, in combination with biochemical and biophysical studies of BHA, has been instrumental in elucidating the mechanism by which HA initiates membrane fusion (27, 31, 33) and in recent efforts at structure-based approaches to inhibit fusion (1). Understanding the mechanism by which retroviruses bind to and penetrate host cells would therefore be greatly aided by the development of an intact, water-soluble ectodomain fragment analogous to BHA.

We are interested in studying the fusion mechanism of the prototypic retrovirus, Rous sarcoma virus (RSV). Attempts to generate a stable soluble oligomeric RSV Env glycoprotein, either by limited proteolysis (4a) or by truncation (10), have not been successful. Env glycoproteins lacking the transmembrane domain sequences are secreted predominantly as monomers rather than as native trimers (10). Because formation of stable Env oligomers depends upon the transmembrane domain, anchoring the protein to the membrane by an alternative method might prove beneficial in maintaining the native form. Many extracellular proteins are attached to the plasma membrane by glycosyl phosphatidylinositol (GPI) linkages (for reviews, see references 6 and 12). The signal for addition of this moiety is encoded within a small peptide near the C terminus of the polypeptide chain (4, 25). During translocation into the endoplasmic reticulum, this peptide is cleaved and the GPI tail, which anchors the protein to the outer leaflet of the membrane, is added. GPI-linked proteins are subsequently glycosylated and transported to the cell surface. With the identification of the signal for GPI addition, several chimeric molecules containing GPI anchors in place of their normal transmembrane domains have been created. The recombinant proteins studied to date are appropriately expressed, oligomerized, and transported to the cell surface (5, 9, 21, 30). The phosphatidylinositol moiety of the GPI tail is cleaved by the enzyme phosphatidylinositol-specific phospholipase C (PI-PLC), releasing a soluble polypeptide. In the case of the major histocompatibility complex, PI-PLC releases heterodimers that bind and present ligands to T cells (30). Using a GPI anchor is an attractive approach for generating a water-soluble ectodomain fragment of a retroviral Env glycoprotein for several reasons: (i) the documented ability of chimeric constructs to be expressed and transported to the cell surface, (ii) the ease of removing the ectodomain with PI-PLC, (iii) the preservation of native oligomeric structure, and (iv) the retention of ectodomain functions.

We substituted the transmembrane and cytoplasmic tail

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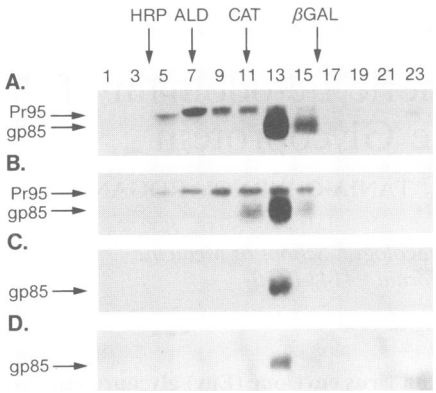

FIG. 1. Sucrose density gradient centrifugation of wt-Aenv and Aenv PI. Lysates of cells expressing wt-Aenv (A) or Aenv PI (B) were loaded onto 10 to 30% linear sucrose gradients in Tris-buffered saline containing 40 mM OG and were centrifuged in a SW41 rotor at 275,000 × *g* for 17 h at 4°C. Fractions were collected, precipitated with lentil lectin-agarose (Vector Labs), boiled and reduced, separated by SDS-polyacrylamide gel electrophoresis (PAGE [9% polyacrylamide]), and immunoblotted with anti-Env antiserum (gift of Martin Stoltzfus). The primary antibody was detected with a donkey anti-rabbit immunoglobulin G coupled to horseradish peroxidase (HRP) (Amersham) and was visualized by enhanced chemiluminescence (Amersham). Cells expressing wt-Aenv (C) or Aenv PI (D) were labelled with the membrane-impermeant reagent NHS-LC-biotin (Pierce) at a concentration of 2 mg/ml in phosphate-buffered saline plus 0.5 mM $MgCl_2$ (UCSF Tissue Culture Facility) for 45 min at 4°C and were then quenched with 100 mM glycine. The samples were then lysed and centrifuged as described above. Gradient fractions were collected, precipitated with avidin-agarose (Pierce), boiled and reduced, separated by SDS-PAGE (9% polyacrylamide), and detected as described above. The precursor, Pr95, and the mature terminally glycosylated SU glycoprotein, gp85, migrate as indicated. The TM glycoprotein comigrates in the same fractions as the SU glycoprotein, but only the SU glycoprotein is shown. Lane 1 contains the lightest fraction, and lane 23 contains the heaviest fraction. The internal S value standards (indicated by arrows) were β-galactosidase (Gal; 16S), catalase (CAT; 11.4S), alcohol dehydrogenase (ALD; 7.6S), and HRP (3.8S).

domains of the RSV Env glycoprotein with the 37 carboxy-terminal amino acids of decay-accelerating factor. This region constitutes a signal for GPI tail addition (4). The nucleotide sequences encoding the ectodomain of RSV (Schmidt-Ruppin A strain) envelope (up to and including amino acid 149 of gp37 [15]) were attached to the nucleotide sequence encoding the decay-accelerating factor-GPI signal (gift of D. Littman) by site-directed mutagenesis (20). This construct, Aenv PI, and the wild-type transmembrane version, wt-Aenv, were subcloned into the plasmid pCB6 under the control of the human cytomegalovirus promoter. The plasmids were transfected into NIH 3T3 cells, and stable cell lines were selected by growth in the presence of 500 μg of Geneticin per ml. Geneticin-resistant colonies were screened for increased Env expression upon overnight induction with sodium butyrate. Optimum butyrate concentrations for Env induction were found to be 25 mM for wt-Aenv cells and 5 mM for Aenv PI cells.

Cells expressing either wt-Aenv or Aenv PI were lysed in the nonionic detergent octylglucoside (OG), and subjected to sucrose velocity gradient centrifugation in the presence of OG. The wild-type transmembrane (Fig. 1A) and the GPI-anchored (Fig. 1B) forms migrated identically and localized in a discrete fraction as indicated by the SU subunit, gp85. The SU subunit, gp85, and the TM subunit, gp37, colocalized to the same

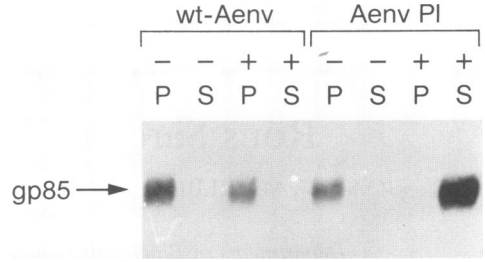

FIG. 2. PI-PLC releases Aenv PI but not wt-Aenv from the cell surface. Cells expressing either wt-Aenv or Aenv PI were labelled with NHS-LC-biotin as described in the legend to Fig. 1, and then the intact cells were treated with (+) or without (−) PI-PLC for 90 min at 37°C. The supernatants (S) or cell pellets (P) were harvested separately, the cell pellets were lysed as previously described, and then all samples were precipitated within avidin-agarose and processed for gel electrophoresis as described in the legend to Fig. 1 (panels C and D).

fractions (data not shown). The core glycosylated uncleaved precursor, Pr95, migrated in a heterogeneous fashion, presumably because of oligomerization intermediates (10). The calculated sedimentation coefficient for both wt-Aenv and Aenv PI is 12.8S, which is consistent with the interpretation that these glycoproteins migrate as oligomeric structures. These results agree with previous studies suggesting that the RSV Env glycoprotein oligomerizes to form a trimer (10).

To address whether the wt-Aenv and Aenv PI were expressed at the cell surface, cells expressing the glycoproteins were labelled with a membrane-impermeable biotinylation reagent. Cell lysates were prepared and subjected to sucrose velocity gradient centrifugation. Biotinylated proteins were then precipitated with avidin and analyzed for the presence of Env. For both wt-Aenv (Fig. 1C) and Aenv PI (Fig. 1D), only mature proteolytically processed glycoproteins reached the cell surface, because no Pr95 was detected. More importantly, cell surface wt-Aenv and Aenv PI sedimented identically to each other and to their counterparts in whole-cell lysates, indicating that only native oligomeric structures are present at the cell surface.

We next demonstrated that Aenv PI is indeed attached to the cell surface by a GPI linkage. Cells expressing either wt-Aenv or Aenv PI were biotinylated and then treated with recombinant PI-PLC (gift of P. Bjorkman [18]). The supernatants and cells were harvested separately and then immunoblotted to detect the presence of Env glycoproteins (Fig. 2). Treatment of cells that express GPI-anchored Aenv PI with PI-PLC released only mature fully processed Env glycoprotein into the supernatant. Env protein was not released from cells expressing the transmembrane form of the glycoproteins under any conditions nor was it released from Aenv PI-expressing cells in the absence of the enzyme. The PI-PLC treatment quantitatively and reproducibly removed the PI-linked Aenv glycoprotein from cells.

Membrane-anchored Aenv PI maintains the same oligomeric structure as wt-Aenv, as determined by its sedimentation properties (Fig. 1). To assess whether the PI-PLC-released Env glycoprotein maintains this oligomeric structure, its sedimentation properties in nonionic detergent were examined. Cells expressing Aenv PI were treated with PI-PLC, and the released material was subjected to sucrose gradient centrifugation in the presence of non-ionic detergent. The majority of the PI-PLC-released Aenv migrated identically to its membrane-bound counterpart (12.8S), indicating that it maintains its oligomeric structure (Fig. 3A).

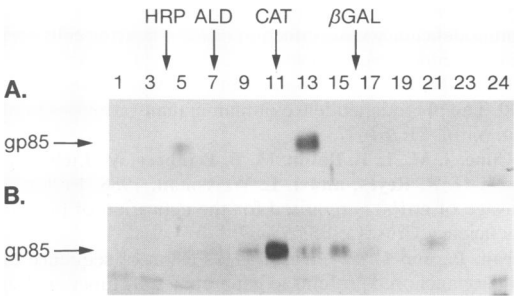

FIG. 3. Sucrose density gradient centrifugation of PI-PLC-released Aenv PI. Cells expressing Aenv PI were treated with PI-PLC. The supernatants were cleared of debris and then loaded onto 10 to 30% linear sucrose Tris-buffered saline gradients, with (A) or without (B) 40 mM OG, and then were centrifuged and processed as previously described. Again, the mature terminally glycosylated SU glycoprotein, gp85, migrates as indicated. Lane 1 contains the lightest fraction, and lane 24 contains the heaviest fraction. The internal S-value standards (indicated by arrows) are described in the legend to Fig. 1.

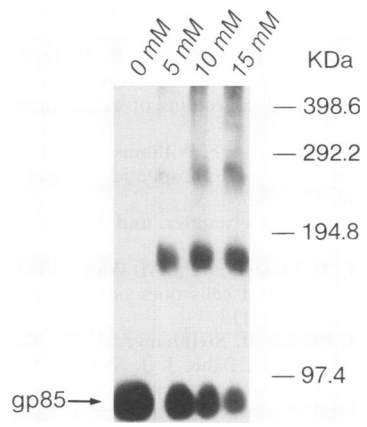

FIG. 4. Cross-linking of PI-PLC-released Aenv PI. Cells expressing Aenv PI were treated with PI-PLC and the supernatants were harvested and concentrated with a Centriprep 30 concentrator (Amicon). The samples were then treated with 0, 5, 10, or 15 mM difluorodinitrobenzidine (Sigma), a nonreducible cross-linking reagent, in phosphate-buffered saline for 45 min at 4°C and quenched with 100 mM glycine. The samples were then precipitated with ricin-agarose (Vector Labs), boiled, reduced, separated by SDS-PAGE (5% polyacrylamide), and detected as described in the legend to Fig. 1.

To determine whether the released protein would maintain its oligomeric state in aqueous solution, the PI-PLC-released Env glycoprotein was also subjected to sucrose gradient centrifugation in the absence of detergent. Under these conditions, the PI-PLC-released Aenv migrated with a sedimentation coefficient of 11.8S (Fig. 3B), corresponding to the peak fraction of Aenv protein (fraction 11). The sedimentation coefficient has shifted slightly from the sedimentation behavior in the presence of OG (12.8S) or Nonidet P-40 (12.2S [data not shown]). These small differences might be attributed to detergent binding to the oligomer. The PI-PLC-released Aenv appeared somewhat more heterogeneous when examined on aqueous gradients than when analyzed on the non-ionic detergent gradients.

To examine further the oligomeric state of the PI-PLC-released Env glycoprotein, we subjected it to chemical cross-linking with the reagent difluorodinitrobenzidine. With increasing concentrations of cross-linker, two additional, discrete bands appeared which migrated on reducing sodium dodecyl sulfate (SDS) gels with the apparent molecular masses predicted for dimers (170 kDa) and trimers (255 kDa) of gp85 (Fig. 4). Apparently, this procedure has not cross-linked the gp85 and gp37 subunits together, even though the PI-PLC-released purified material contains both subunits, as evidenced by silver stain analysis (data not shown). Although there is higher-molecular-weight antibody cross-reacting material, perhaps due to nonspecific aggregation, no discrete band migrated at the predicted molecular mass of tetramers (340 kDa). This result is most consistent with the previous suggestion that the wild-type Env oligomer is a trimer, because of its sedimentation properties (7, 10). Our cross-linking data for the PI-PLC-released Aenv PI and our sedimentation analysis of both wt-Aenv and Aenv PI agree with this prediction.

In conclusion, our results indicate that the chimeric Env glycoprotein, Aenv PI, is stably expressed in NIH 3T3 cells and is transported to the cell surface in its native oligomeric state. The protein is attached to the cell by a GPI anchor and can be quantitatively removed from the cell surface with the enzyme PI-PLC. The soluble ectodomain fragment is stable and maintains its oligomeric structure. Formation of stable oligomers depends on association with the lipid bilayer, and the GPI anchor appears to have mimicked the transmembrane domain in this regard. We have constructed an analogous GPI-linked form of the subgroup C RSV Env glycoprotein which behaves identically to the Aenv PI described here (12a). These PI-PLC-released soluble Env glycoproteins are valuable reagents for studying both subgroup-specific binding to susceptible and resistant host cells (2, 8) as well as the fusion mechanism of this prototypic retroviral Env glycoprotein.

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