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N-Terminal Cleavage Fragment of Glycosylated Gag Is Incorporated into Murine Oncornavirus Particles

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Glycosylated Gag (Glycogag) is a transmembrane protein encoded by murine and feline oncornaviruses. While the protein is dispensible for virus replication, Glycogag-null mutants of a neurovirulent murine oncornavirus are slow to spread in vivo and exhibit a loss of pathogenicity. The function of this protein in the virus life cycle, however, is not understood. Glycogag is expressed at the plasma membrane of infected cells but has not been detected in virions. In the present study we have reexamined this issue and have found an N-terminal cleavage fragment of Glycogag which was pelleted by high-speed centrifugation and sedimented in sucrose density gradients at the same bouyant density as virus particles. Its association with virions was confirmed by velocity sedimentation through iodixanol, which effectively separated membrane microvesicles from virus particles. Furthermore, the apparent molecular weight of the virion-associated protein was different from that of the protein extracted from the plasma membrane, suggesting some level of specificity or selectivity of incorporation.

Glycosylated Gag (Glycogag) is an accessory protein of murine and feline oncornaviruses (4, 6, 12, 20). It is dispensable for virus replication (2, 7, 17, 21) but appears to be an important virulence determinant (2, 17). The protein is translated from an alternate initiation codon upstream and in frame with the initiation codon for pr65^{gag} (18), the precursor of the core proteins of the virus. The precursor of Glycogag, pr75^{gag}, consists of the sequence of pr65^{gag} but with a unique N terminus of 8 to 10 kDa (4, 5, 19). pr65^{gag} is synthesized as a cytosolic protein which is incorporated into virions and processed by the viral protease into the various components of the viral core. p75^{gag} is synthesized at the endoplasmic reticulum as a type II integral membrane protein (N_{cyto} C_{exo}); is N glycosylated to an 85-kDa protein, gp85^{gag}; and is expressed at the plasma membrane. The majority of the unique N-terminal sequence constitutes the cytoplasmic tail of the protein.

Our interest in Glycogag stems from evidence that it is an important determinant of neuroinvasiveness (17). The murine retrovirus CasFr^{KP} (abbreviated KP), a derivative of the wild mouse ecotropic virus CasBrE, is neuroinvasive after intraperitoneal inoculation of neonates and causes a paralytic disease associated with spongiform neurodegeneration (17). When the Kozak consensus sequence (11) surrounding the initiation codon of Glycogag was altered to knock out expression of the protein, the mutant virus, abbreviated KP^{gg-}, was no longer neuroinvasive (16, 17). Though Glycogag-null mutants still replicated, the kinetics of spread in vivo was measurably slower than that of wild-type virus (2, 17).

We have shown previously that the Glycogag protein of the virus KP is cleaved, presumably by a cellular protease, near the middle of the molecule. The C-terminal fragment, which contains much of the viral capsid (CA) and nucleocapsid sequences shared with pr65^{gag}, is secreted, whereas the N-terminal fragment, which contains the cytoplasmic tail, transmembrane domain, and sequences of the matrix protein and pp12^{gag}, remains associated with the cell as an integral membrane protein (8). It is this N-terminal fragment which is displayed at the plasma membrane (Fig. 1A).

Previous studies have concluded that Glycogag is not incorporated into virions (1, 4, 15). We have reexamined this issue using an antiserum specific for an octadecapeptide (designated 4210) located at the extreme N terminus of the cytoplasmic tail (8, 16). This antiserum, designated anti-Glycogag, is specific for Glycogag and reacts with both the precursor protein and the N-terminal cleavage fragment displayed at the plasma membrane (Fig. 1A). It does not react with pr65^{gag} or any of its cleavage products (8).

Our first approach was to ask whether Glycogag was pelleted by high-speed centrifugation using the Glycogag-null virus KP^{gg-} (17) as a negative control (Fig. 1B and C). Culture supernatants collected from *Mus dunni* fibroblasts infected with either KP or KP^{gg-} viruses were precentrifuged at 5,900 × *g* followed by filtration with 0.22-μm-pore-size Millex GS filters (Millipore) to remove cell debris. One milliliter of each filtered supernatant was removed and mixed with 0.25 ml of 5× sodium dodecyl sulfate (SDS) sample buffer (Fig. 1, Pre-spin). Twenty milliliters was centrifuged at 187,000 × *g* for 2 h to pellet the virions. One milliliter of the high-speed supernatant was also mixed with 0.25 ml of 5× SDS sample buffer (Fig. 1, HS Sup). The pellet was lysed with 1.0 ml of 1× SDS sample buffer and sonicated (Fig. 1, HS Pellet). All samples were boiled, and 20 μl of each was separated by SDS–9% polyacrylamide gel electrophoresis. The gel was electroblotted onto an Immobilon-P membrane (Millipore). The blot was incubated first with rabbit anti-CA, reactive with both the C-

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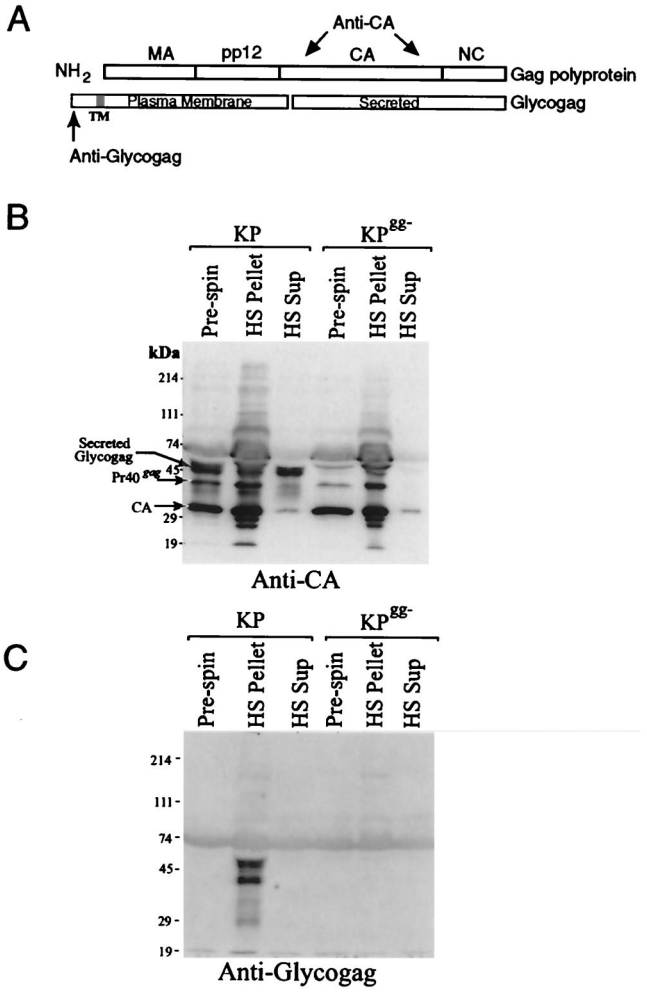

FIG. 1. Immunoblot analysis of Gag proteins in culture supernatants from KP- and KP^{gg-}-infected *M. dunni* fibroblasts. Supernatants from infected cells were subjected to ultracentrifugation as described in the text to pellet virus particles. Samples extracted with SDS sample buffer included the supernatant fluid before centrifugation (Pre-spin), the postcentrifugation high-speed pellet (HS Pellet), and the postcentrifugation supernatant (HS Sup). The blot was developed first with a rabbit antiserum to viral CA protein (B) which has been shown previously to react with both the CA protein and shared sequences in the secreted C-terminal fragment of Glycogag (8) (A). The CA protein band was prominent in the Prespin and HS Pellet of both KP and KP^{gg-}. A similar distribution was seen for pr40^{gag}, an intermediate cleavage product of the pr65^{gag} polyprotein. These two proteins therefore were pelleted. In contrast, the secreted fragment of Glycogag was seen in the Pre-spin of KP but not KP^{gg-} and was concentrated in the HS Sup. This result is consistent with this protein not being associated with pelleted virions. The anti-CA antibody was then eluted from the blot, and the blot was reprobed with an antiserum specific for Glycogag (C). This antibody reacts with the N-terminal transmembrane protein of Glycogag but does not react with any of the constituents of the viral core derived from the pr65^{gag} polyprotein (A). Two major bands are seen only in the HS Pellet of the KP virus, suggesting that these species may be associated with virions. Unlike the CA and pr40^{gag} bands in panel B, the Glycogag bands were not seen in the Pre-spin (see the text). MA, matrix protein; TM, transmembrane domain.

terminal secreted fragment of Glycogag and the CA protein of the viral core (Fig. 1B). Reactivity was revealed with horseradish peroxidase–anti-rabbit immunoglobulin G (IgG) (Biorad) followed by ECL chemiluminescent substrate (Amersham) (Fig. 1). Anti-CA detected a protein of ~45 kDa with minor species of 35 to 40 kDa in the HS Sup of KP- but not KP^{gg-}-infected cells (Fig. 1A). These bands represent secreted C-terminal proteolytic cleavage products of the gp85^{gag} precursor of Glycogag described previously (8). Anti-CA reacted with CA protein (p30) in the HS pellet of both viruses as well as a band of ~40 kDa which likely represents pr40^{gag}, an intermediate cleavage product of pr65^{gag} which contains both CA and nucleocapsid proteins (22). This experiment showed that the C-terminal fragment of Glycogag was likely not associated with virus particles.

After the anti-CA was stripped off the blot with 2% SDS at 70°C, the blot was reprobed with rabbit anti-Glycogag (Fig. 1C). Two major bands ranging from ~35 to 50 kDa were detected in the HS pellet of KP but not KP^{gg-}. These bands represent N-terminal cleavage fragments of Glycogag having different C termini. They appear not to be different glycoforms, since digestion with *N*-glycosidase F (PNGase; New England Biolabs) to remove N-linked sugars resulted in a shift down in the molecular weight of both bands (not shown). Their appearance in the HS pellet suggests that the N-terminal fragment of Glycogag may be associated with virions. It should also be noted that these bands were not observed in the prespin track even though this same material contained detectable CA protein (p30) (Fig. 1B). This suggests that if Glycogag was associated with virions, the relative quantity must be low.

To further examine the question of whether this protein was incorporated into virus particles, KP and KP^{gg-} viruses were subjected to equilibrium centrifugation through a 15 to 60% sucrose density gradients. The fractions were assayed by dot blotting and probed with anti-CA to identify virion cores and, after stripping, anti-Glycogag (Fig. 2). Glycogag cosedimented with KP virions at a density of 1.14 to 1.17 g/ml.

It is well-known that buoyant density alone is not sufficient to conclude that a protein is incorporated into virus particles (13). Microvesicles derived from the plasma membrane of infected cells can incorporate both cytosolic and membrane proteins and can sediment at densities comparable to that of retroviruses (3, 9). Thus, it was necessary to pursue this question using different techniques. As a first approach, we compared the electrophoretic mobilities of Glycogag species derived from the cell surface with that present in gradient-purified virion extracts. Proteins at the plasma membrane of KP- or KP^{gg-}-infected *M. dunni* cells were labeled with NHS*S*-*S*-biotin (Pierce Chemical) at 0°C as described previously (8). After lysis with 0.5% NP-40 in 0.01 M Tris-HCl–0.15 N NaCl–0.001 M EDTA (pH 7.2) (TNE), the lysates were divided equally. One half was boiled in SDS (Fig. 3, Total Cell Lysate). The other half was incubated with immobilized streptavidin (Ultralink; Pierce Chemical) to isolate the biotinylated proteins prior to boiling in SDS (Fig. 3, Cell Surface). The virion lysates (Fig. 3) were prepared using the peak fraction from viruses purified by sucrose density gradient centrifugation. Samples were separated by SDS–polyacrylamide gel electrophoresis, blotted as for Fig. 1, and probed with anti-Glycogag antiserum. The total cell lysate of KP-infected cells contained

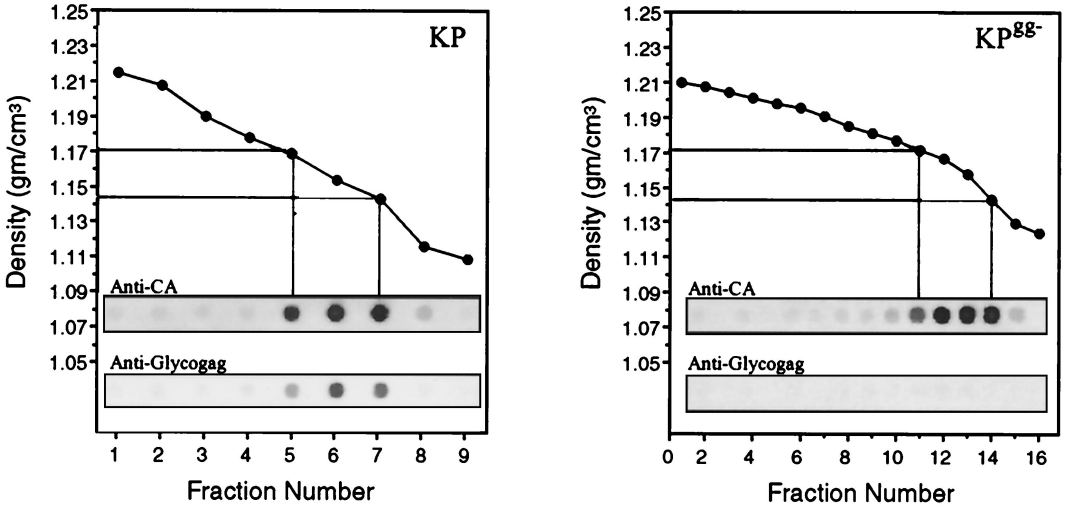

FIG. 2. Fractionation of culture supernatants from *M. dunni* cells infected with KP and KP^{gg-} by equilibrium centrifugation in 15 to 60% sucrose density gradients. Virus was first concentrated from 1 liter of supernatant by centrifugation for 18 h at $6.16 \times 10^3 \times g$. The pellets were suspended in TNE and layered onto preformed gradients. Gradients were centrifuged in an SW41 rotor (Beckman) at $1.88 \times 10^5 \times g$ for 1.5 h, and fractions were collected from the bottom. Specific gravities of the fractions were analyzed by refractive index measurement, and fractions were assayed by dot blot analysis using polyvinylidene difluoride membranes with either anti-CA to identify virus-containing fractions or anti-Glycogag. Both KP and KP^{gg-} virions had buoyant densities in the range of 1.14 to 1.17 g/cm^3, which is consistent with that of a typical murine oncornavirus. The Glycogag signals in the KP fractions appeared to cosediment with those of CA-containing virus particles.

two predominent species of Glycogag, one above and one below the 45-kDa marker (Fig. 3). As shown previously, the predominant species found at the cell surface consisted of an ~50-kDa band (8), which corresponded to the upper band of the total cell lysate. In contrast, the virion fraction contained a predominant species corresponding to the lower band of the total cell lysate. This lower band was also represented at low levels in the cell surface fraction. This difference suggests that the Glycogag sedimenting at 1.14 to 1.17 g/ml did not represent a random sampling of the plasma membrane protein but instead represented a subpopulation. This suggests, but certainly does not prove, that a portion of the Glycogag detected in the virion peak likely was associated with virions.

Two approaches to separating virions from plasma membrane microvesicles have been described. Ott et al. (14) have used a protease digestion technique followed by sedimentation through sucrose. This procedure has had great utility in identifying proteins which are inside virions and protected from the protease. However, the bulk of the N-terminal cleavage fragment of Glycogag is exposed externally and would be subject to protease digestion. Only the cytosolic tail and transmembrane domain (~88 amino acid residues in the case of the virus KP) should be protease resistant. We chose, therefore, an alternate technique which relies on velocity sedimentation through iodixanol (Optiprep) to separate virions from plasma membrane microvesicles (3, 10). Briefly, 8 ml of supernatant fluid from KP-infected *M. dunni* cells was precleared at $1,500 \times g$, filtered through a 0.22-μm-pore-size Millex GS filter, and centrifuged for 2 h at $100,000 \times g$ through a 20% sucrose cushion. The pellet was suspended in TNE, layered onto an 11-ml linear 6 to 18% iodixanol (Nycomed) gradient, and centrifuged for 1 h 20 min at $187,000 \times g$. Fractions (0.5 ml) were removed from the top of the gradient and analyzed by dot blotting. The blot was probed sequentially with three different antibodies to virus-

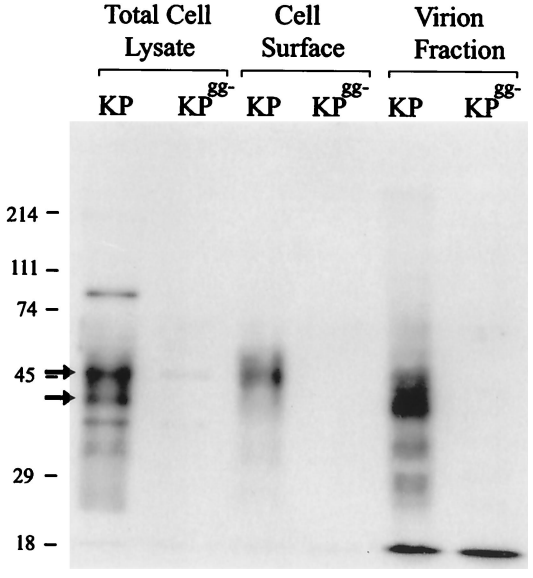

FIG. 3. Comparison of the electrophoretic mobility of Glycogag expressed at the cell surface versus that found in virions. Live *M. dunni* cells infected with either KP or KP^{gg-} were biotinylated to label protein at the plasma membrane as described previously (8). Cell lysates were analyzed directly (Total Cell Lysate), or the biotinylated proteins were isolated using immobilized streptavidin (Cell Surface). The virion fraction was derived from the peak fraction of a sucrose density gradient, pelleted by high-speed centrifugation prior to lysis in SDS sample buffer. The blot was probed with anti-Glycogag antiserum. Two major immunoreactive species (arrows), in addition to the gp85^{gag} precursor protein, are seen in the total cell lysate. The upper band appears to correspond with the major immunoreactive species in the cell surface sample, and the lower band corresponds to the major species in the virion fraction.

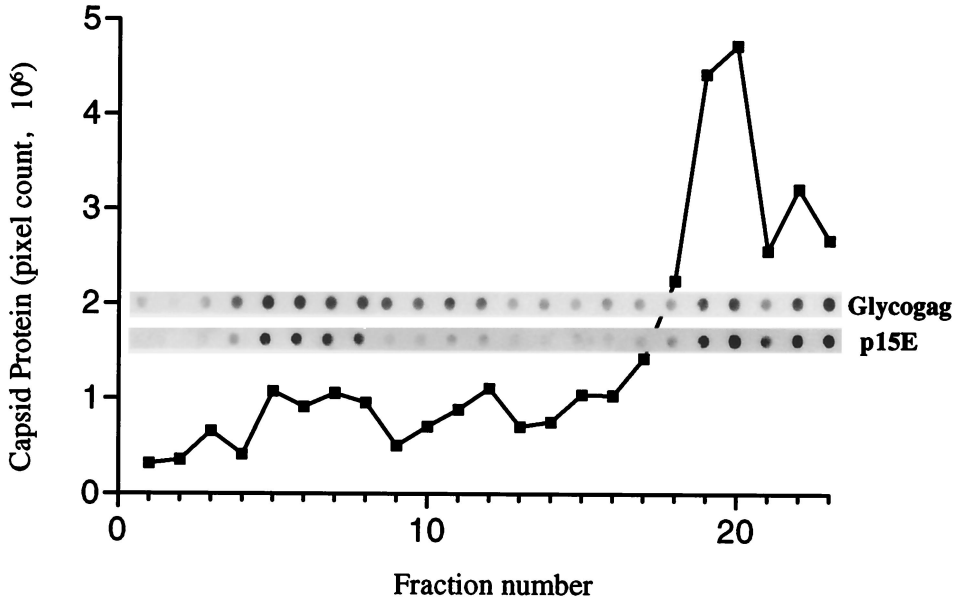

FIG. 4. Velocity sedimentation analysis of KP virus in a linear 6 to 18% iodixanol gradient. Fractions removed from the top of the gradient were subjected to dot blot analysis first with anti-CA protein, generating the graph. The vertical axis represents signal intensity measured by a PhosphorImager utilizing ImageQuant software (Molecular Dynamics). The blot was sequentially stripped and reprobed with anti-Glycogag and anti-transmembrane protein/p15E, shown as the strips of dots corresponding to the respective fractions. Under the conditions used (see the text), the peak of virus particles, represented by CA protein immunoreactivity, was located near the bottom of the gradient. Glycogag and p15E were found in two peaks, one coincident with the virion fractions and the other in the upper third of the gradient, likely representing plasma membrane microvesicles devoid of CA protein.

encoded proteins (CA, Glycogag, and transmembrane protein/p15E), each antibody being stripped off as described above before reprobing with the next antibody. Anti-CA protein was developed with an alkaline phosphatase-coupled anti-rabbit Ig and detected with a fluorescent substrate (AttoPhos; Promega) to allow quantification on a PhosphorImager (Molecular Dynamics). Anti-Glycogag and anti-p15E (kindly provided by (Garhard Hunsmann, Gottingen, Germany) were developed using horseradish peroxidase–anti-rabbit Ig followed by ECL (Amersham) substrate and were detected on film (Fig. 4). The peak of CA immunoreactivity was seen near the bottom of the gradient, marking the location of virus particles. There were two peaks of Glycogag immunoreactivity, one located in fractions 4 to 9, likely representing membrane microvesicles devoid of CA protein, and a second peak coincident with the virion peak near the bottom of the gradient. Interestingly, a similar distribution with two peaks was seen for p15E. Thus, Glycogag, like another transmembrane protein, p15E, was found in plasma membrane microvesicles but was also readily detectable in virion fractions, suggesting again that this protein is incorporated into virus particles.

This then raises the question of whether Glycogag is specifically concentrated in virus particle membranes, as might be expected for a viral structural protein. If one compares the relative distributions of p15E and Glycogag in the gradient (Fig. 4), it is apparent that p15E is partitioned predominantly, though admittedly not dramatically so, in the virion fractions. On the other hand, a larger fraction of Glycogag appeared to partition in the vesicular fractions. Though this could be simply a consequence of differences in the distribution of the two proteins in the plasma membrane, this difference could also indicate that Glycogag was not selectively concentrated in virions and that its presence in virions was a function of a passive rather than an active process. Indeed, there is now abundant evidence for passive incorporation of normal cellular membrane proteins into retroviral envelopes, indicating that the selective exclusion of cell membrane proteins during virus assembly is relatively weak (10). It should be reiterated, however, that the difference in apparent molecular weight between cell surface Glycogag and the Glycogag in virion fractions derived from equilibrium sedimentation experiments (Fig. 3) suggests some level of selectivity.

What role, if any, this virion-associated component could have in the virus life cycle is a matter of pure speculation at this point. It is of interest, in this regard, that Fan et al. (7) noted a small difference in the buoyant densities of Glygag-null and wild-type Moloney murine leukemia virus (1.185 versus 1.170 g/ml, respectively) and suggested that the protein may be involved in viral morphogenesis. This difference in buoyant densities was not observed in the current study. However, this could have been a consequences of technical differences and certainly warrants reexamination. It is also possible that Glycogag interacts with a coreceptor on host cells which could enhance viral infectivity and thus promote viral spread.

Although Glycogag is dispensable for virus replication, it is clear that its expression in infected cells has a strong influence on virus dissemination in vivo and that it plays a critical role in viral virulence. Understanding the nature of this effect should provide important clues to the selective forces driving expression of this protein (16) as well as insight into the virus-host interactions which ultimately lead to the induction of disease.

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