Characterization of the vaccinia virus L1R myristylprotein as a component of the intracellular virion envelope

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In many cases, virus-encoded acylproteins appear to localize to specific cellular and viral membranes and to be directly involved with the processes of virus morphogenesis and/or egress from the infected cell. It was therefore of interest to determine whether the major vaccinia virus (VV) myristylprotein, L1R, is specifically associated with one or more of the membranes enveloping various infectious forms of VV virions. To this end, single-membraned intracellular virions (INV) and extracellular enveloped virions (EEV), which are surrounded by at least two distinct membranes, were purified from VV-infected cell lysates. The location of the VV L1R protein was determined by using a monospecific anti-L1R serum to detect the L1R protein by immunoblot in INV- and EEV-containing fractions, by examining the proteinase K sensitivity of the L1R protein in intact INV and EEV particles, and by immunoelectron microscopy. The data obtained clearly indicate that although the L1R protein is a constituent of both the INV and EEV particles, it is exclusively found in the inner INV-specific membrane. These results are discussed with regard to the potential role of the VV L1R protein in the primary intracellular envelopment of infectious VV particles.

Our understanding of the complex events leading to the assembly and envelopment of many mammalian viruses is rudimentary, at best. For vaccinia virus (VV), the prototype member of the poxvirus family, most of what we know about the maturation and egress of the virus from infected cells is based on electron microscopic studies. Using the transmission electron microscope, the first defined viral structures which become visible after infection are spicule-coated membranes which enclose a granular matrix. Further differentiation leads to the characteristic electron-dense core structures which can be found throughout the cytosol. These particles, which are infectious, are designated intracellular ‘naked’ virions (INV); a misleading term as the INV are enclosed within at least one lipid-containing structure.

Another form of infectious VV virion exists which differs from INV both in terms of its relative buoyant density in isopycnic cesium chloride gradients and the mode by which it exits from the infected cell. These latter particles are thought to obtain two additional membranes by becoming enwrapped by a double layer of the Golgi trans cisternae. One of these membranes is lost by fusion with the plasma membrane in a process which can be thought of as reverse phagocytosis, resulting in release of the virus from the interior of the infected cell. Consequently, after release, the extracellular VV particles have an additional lipoprotein envelope and are called extracellular ‘enveloped’ virions (EEV). The process of EEV formation and release from the cell surface requires both an intact cytoskeleton, as evidenced by experiments using the drug cytochalasin which inhibits microfilament formation (Payne & Kristensson, 1982a), and the glycosylation of viral proteins (Payne & Kristensson, 1982b). Furthermore, there appear to be at least 10 EEV-specific proteins, nine of which are glycosylated, which may influence EEV egress. In particular, the p37K protein [the product of the F13L open reading frame (ORF)], which is the most abundant species present in the EEV outer envelope (Hiller & Weber, 1985), has been implicated as having a potential role in virion envelopment and release from the infected cell (Blasco & Moss, 1991; Payne & Kristensson, 1979). In addition to INV and EEV, purified virions from lysates of VV-infected cells prepared by sucrose gradient sedimentation may contain two more types of particles. These are the intracellular enveloped virions (IEV), and the cell-associated virions (CEV), for which a role in virus dissemination in tissue culture has been demonstrated (Blasco & Moss, 1991, 1992).

Given the bifurcated nature of VV development, it becomes of interest to determine the molecular signals for differentiation, as well as the targeting mechanisms employed to ensure that virus proteins are directed to the correct subcellular compartment for incorporation into these different types of particles. In recent years, it has become evident that modification of proteins by the
covalent attachment of various lipid moieties, collectively referred to as acylation, can play a central role in determining the localization and functional activity of the modified protein (Grand, 1989). Although a specific function for many of the proteins modified by the addition of lipids remains to be elucidated, it appears that for many viral proteins the attachment of a fatty acid moiety may aid in interactions with other molecules and ultimately orchestrate the assembly of progeny virions. This has been established definitively for picornaviruses, for which analysis of the crystal structure data confirmed a direct and critical interaction between the myristylated VP4 protein and VP3 within the virus capsid (Chow et al., 1987). Similarly, the myristylated VP2 proteins of simian virus 40 and other polyomaviruses appear to have a role in virus particle scaffolding as evidenced most recently by a mutant virus expressing a non-myristylated form of VP2 which revealed a decrease in both viral infectivity and virus assembly (Sahli et al., 1993). Other acylated viral proteins which may have a function in viral morphogenesis include the Sindbis and vesicular stomatitis virus glycoproteins, the influenza virus haemagglutinin and the preS1 protein of hepatitis B virus, which is thought to influence nucleocapsid assembly and/or infectivity (Persing et al., 1987).

We and others have previously demonstrated that VV encodes a large number of acylated proteins, including the EEV-specific p37 protein which is palmitylated (Child & Hruby, 1992; Franke et al., 1989; Hiller & Weber, 1985). Another VV acylprotein of particular interest is the major myristylated protein, product of the VV L1R ORF (previously termed M25 protein), which is expressed at late times during the infectious cycle and is cotranslationally modified by the addition of myristic acid (tetradecanoate, C14:0) to the penultimate NH2-terminal glycine residue in a hydroxylamine-resistant fashion (Franke et al., 1989, 1990). The L1R protein is a component of sucrose gradient-purified virions and is partitioned in the detergent-soluble fraction upon treatment of whole virions with NP40, thus suggesting an association with a virion membrane (Franke et al., 1989; Ravanello et al., 1993). In our previous studies we observed that upon treatment of whole purified virions with a protease, such as proteinase K, immunoreactivity to the L1R protein was lost when using a monospecific antiserum raised to this polypeptide (Ravanello et al., 1993). This finding raises the possibility that the L1R molecule may be targeted and localized to one or more of the membranes enveloping mature virus particles. In this report, we have extended our investigation regarding the virion localization of the L1R protein by using immunodetection techniques and proteinase sensitivity assays to determine whether the L1R protein is a component of caesium chloride-purified, mature INV and EEV particles, or is restricted to one or the other virion form. Furthermore, immunogold electron microscopy techniques were employed in order to examine where the L1R protein is localized within the vaccinia virion.

Confluent monolayers of RK13 (rabbit kidney) cells were infected at a multiplicity of 5 infectious particles/cell with the IHD-J strain of VV. After 24 h of incubation at 37 °C, INV and EEV forms of VV were purified as follows. The infected cells were scraped from the plate, transferred to a conical tube and centrifuged at 1000 g for 5 min at 4 °C to obtain a primary pellet and supernatant fraction. EEV was pelleted from the supernatant in a Beckman SW28.1 rotor tube at 35000 g for 30 min at 4 °C. This preparation was further purified on a discontinuous caesium chloride gradient (100000 g for 17 h at 15 °C) that was prepared by prelayering 1:30 g/ml (1:3 ml), 1:25 g/ml (1:8 ml) and 1:20 g/ml (1:8 ml) caesium chloride in a Beckman SW50.1 ultracentrifuge rotor tube. INV was purified from lysed harvested cell pellets (the primary pellet described above) by centrifugation on a 36% (w/v) sucrose cushion in a Beckman SW41 rotor tube at 40000 g for 80 min at 4 °C. The resulting virus pellet was further purified on a discontinuous caesium chloride gradient to obtain INV. After centrifugation, the bands corresponding to the EEV (density 1.22 g/ml) or the INV (density 1.26 g/ml) forms of VV were removed through the side of the tubes using a needle and syringe. The volume of each fraction was adjusted to 1.5 ml with distilled H2O and the fractions were centrifuged at 6500 g for 30 min at 4 °C. The supernatant was removed by aspiration and the pellet resuspended in 100 μl distilled H2O.

The INV and the EEV forms of VV were examined for the presence of the L1R protein by immunoblot analysis using a monospecific antiserum for this polypeptide. A small amount (15 μg) of each purified virus preparation was analysed by discontinuous SDS–PAGE followed by immunoblotting (Fig. 1). After immunoblotting the membranes were incubated in 3% gelatin before treatment with specific antisera. The membranes were then incubated in goat anti-rabbit IgG conjugated with alkaline phosphatase followed by the chromogenic substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indoylphosphate in carbonate buffer.

Using a monospecific, polyclonal antiserum raised to a histidine-tagged L1R protein, anti-his:L1R (Ravanello et al., 1993), approximately equal amounts of the L1R polypeptide were observed in both the INV and the EEV forms of VV (Fig. 1a, lanes 1 and 2). As controls to ensure the purity and identity of the INV and EEV preparations, replicate immunoblots were probed with a polyclonal antiserum directed against either p37 or 25K (VP8, the product of the L4R gene). The p37 protein should be found only in the EEV fraction whereas the
25K core protein should be present in both INV and EEV. This analysis easily detected p37 protein in the EEV fraction (Fig. 1b, lane 2). The 25K protein is proteolytically cleaved from its precursor form (p25K) and accounts for approximately 7% of the total virus by dry weight. An antiserum raised to a TrpE fusion protein (Lee & Hruby, 1993) recognizes both the precursor (p25K) and the product (25K) of the cleavage reaction. The levels of protein p25K/25K detected in the INV and EEV samples were roughly equivalent (Fig. 1c, lanes 1 and 2) indicating the presence of mature virions and that the lanes were loaded with approximately equal numbers of virions. Together these results demonstrate that the L1R protein is a *bona fide* component of mature vaccinia virions and that it is found in both INV and EEV.

The carboxy terminus of the L1R protein is predicted to possess a high degree of hydrophobicity (Goebel et al., 1990), consistent with the hypothesis that this portion of the molecule serves to anchor the L1R protein into the membrane surrounding VV virions. However, in view of the fact that the L1R protein is present in both INV and EEV, it was of interest to determine whether the protein was restricted to the INV-specific membrane or was also present in the outer EEV membrane. To approach this question, protease digestion experiments were undertaken using proteinase K, an endo- and exoprotease, which has previously been used for studies to characterize membrane protein attachment (Marimoto et al., 1983). Equivalent amounts (20 μg) of INV or EEV were incubated in the presence of proteinase K (50 μg/ml) for 60 min on ice. At the completion of the incubation period, the protease inhibitor PMSF was added to a final concentration of 5 mM. The digested extracts were subjected to discontinuous SDS-PAGE. The resolved protein bands were transferred to nitrocellulose membranes which were then probed by immunoblotting using the three antisera described above. Immunoreactivity to L1R protein was lost completely in the INV fractions treated with proteinase K, (Fig. 2, anti-L1R, lane 1), whereas the amount of L1R detected in the EEV fraction was unaffected by treatment with the proteinase (lane 2). In contrast, the p37 protein appeared to be completely removed from the EEV fraction by incubation with proteinase K (anti-p37). There was no detectable change in immunoreactivity for the p25K/25K core protein in either the INV or EEV samples (anti-p25K/25K, lanes 1 and 2). These data strongly suggest that whereas the L1R protein is a component of both the INV and EEV forms of VV, it is only in direct physical and biochemical association with the primary membrane enveloping the INV.

As an alternative means to investigate the location of the L1R protein within the VV virion, immunoelectron microscopy was carried out on thin sections of purified EEV. The EEV fraction was pelleted by centrifugation in a microcentrifuge rotor at 6500 g for 10 min. Fixing, embedding and slicing of the blocks with a diamond knife was carried out by personnel at the Oregon State University Electron Microscopy Laboratory. The slices prepared for immunocytochemistry were placed on uncoated Pelco nickel grids, 200 mesh. These grids were then subjected to immunogold labelling (Erickson et al., 1987) using a 1:1000-fold dilution of the primary antibody and a 1:20 dilution of the goat anti-rabbit IgG 10 nm gold conjugate. Samples were stained with uranyl acetate and lead citrate (Russell & Rohrmann, 1990; Venable & Coggeshall, 1965). The gold particles and virions were visualized on a Philips EM 300 electron microscope. Using p25K/25K antiserum as the primary antibody strict localization of the gold particles to the virion core was demonstrated (data not shown; VanSlyke...
Fig. 3. Localization of viral proteins in EEV particles by immuno-electron microscopy. Thin sections of purified EEV particles were incubated with antisera specific for either the L1R or p37 proteins. Bound antibody was visualized using colloidal gold (10 nm particles) conjugated to goat anti-rabbit immunoglobulin serum and visualized by transmission electron microscopy. (a) Anti-p37 serum. (b) Anti-L1R serum. Gold labelling of EEV particles was not observed when preimmune serum was employed as the primary serum (data not shown). The bar markers represent 100 nm.

& Hruby, 1994). Since the 25K protein is a core protein, this result was as expected and confirmed our ability to localize specifically viral proteins by this procedure. Treatment of the thin sections with the anti-p37 antibody resulted in gold particles being deposited in the vicinity of the distal envelope membrane (Fig. 3a). This is consistent with the p37 protein being a major surface determinant for the extracellular form of VV. In contrast, when the anti-L1R serum was used as the primary antibody, gold particles were seen throughout the internal regions of the virion with a majority being found between the inner and outer membranes (Fig. 3b). Furthermore, gold particles bound to the L1R-specific antibody were entirely limited to the mature vaccinia virions, whereas some of the gold particles bound to anti-p37 antibody were found associated with other membranes (Fig. 3a, arrow). The identity of these structures may correspond to membranes of either viral or Golgi origin that had been disrupted during the preparation of the samples. In any case, the results of the immuno-electron microscopy confirm and support the conclusion that the VV L1R protein is an integral component of the INV membrane.

The results presented here have demonstrated that the VV L1R protein is a constituent of both INV and EEV particles, but that it is associated exclusively with the primary membrane surrounding the virion core. Given the rather specific localization of the VV L1R protein, it will be of interest to elucidate the salient protein targeting mechanisms that are being employed to guide it to this site. It is likely that this will involve a display of primary amino acid sequence, secondary and tertiary structure of the protein and the presence of a hydrophobic modification, myristic acid. We have demonstrated previously that a fusion protein, in which the first 12 amino acids of the L1R protein were abutted to the entire bacterial chloramphenicol acetyltransferase (CAT) protein, was myristylated and targeted to the vaccinia virion purified from the cytoplasm of infected cells (Ravanello et al., 1993). However, in contrast to the results reported here, the L1R–CAT fusion protein was resistant to proteinase K treatment (Ravanello et al., 1993). The proteinase K sensitivity of L1R protein in INV particles (Fig. 2) indicates that an additional membrane anchoring domain might aid in exposing the majority of the mass of the L1R protein (ectodomain) to the outside surface of the virion. The putative hydrophobic membrane-spanning region found near the carboxy terminus together with the amino-terminal myristic acid moiety of this protein could provide such a function. This would be consistent with the analysis of other viral membrane proteins (Doms et al., 1993).

Since expression of the VV L1R protein is apparently essential for replication (Franke et al., 1989, 1990) and it
appears to be associated with the nascent virion envelope, it is reasonable to postulate that the L1R protein may play a central role during virion morphogenesis. If the long-held assumption that INV particles acquire their membranes de novo is true (Dales & Mosbach, 1968; Dales & Pogo, 1981), it is conceivable that L1R may help recruit lipids to the virosome and thus lead to the condensation event that marks the initiation of virion morphogenesis. If this is the case, the myristic acid moiety may serve a function in holding the lipid components and L1R protein in a stable conformational state until the membrane is formed around the previrion. Alternatively, in the event that the INV particle acquires its membrane by intracellular budding through the intermediate compartment (Sodeik et al., 1993), then it is possible that the L1R protein may function to direct the assembling virion to this site, much like the matrix protein of Mason-Pfizer monkey virus (Rhee & Hunter, 1990).

Although the L1R protein appears to be restricted to the primary membrane surrounding the VV core, this does not preclude it also playing a role in EEV formation. For example, it is possible that L1R and p37 proteins may interact with each other by virtue of the altered hydrophobicity conferred on them by their respective acyl moieties. Essential oligomeric interactions between viral membrane proteins have previously been demonstrated in a number of cases, including the influenza virus haemagglutinin (Wilson et al., 1981). Such a cooperative interaction between the L1R and p37 proteins may ultimately aid in virus egress from the cell.

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References


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