Lipoproteins of varicella-zoster virus

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Human fibroblast cells infected with varicella-zoster virus (VZV) showed a slight increase in lipoprotein synthesis, with the production of two major viral lipoproteins, as detected by radioimmunoprecipitation (RIP). Three bands of Mr 73000, 90000 and 97000 were identified as forms of the VZV gpl glycoprotein. All three incorporated both palmitic and myristic acid, and were shown by thin-layer chromatography to contain myristic, palmitic and stearic acids. A very strong band corresponding to 7000 Mr, which may represent the product of VZV gene 49, was detected after RIP and in VZV-infected cells, and was shown to contain almost entirely myristic acid. Several minor bands were also detected. The possible functions of the lipoproteins are discussed.

Varicella-zoster virus (VZV) is the causative agent of both chickenpox (varicella), resulting from primary infection, and shingles (zoster), resulting from reactivation of latent virus. The genome of the virus has recently been sequenced (Davison & Scott, 1986), but many of the mechanisms of virus pathogenesis are still poorly understood. VZV is known to possess many functions which require interaction with membranes (Heath, 1987). With other viruses such functions are known to involve viral lipoproteins (Schmidt, 1983; Schultz & Oroszlan, 1984), which also appear to be important in many stages of viral pathogenesis. With the human herpesviruses, lipoproteins have been identified only for herpes simplex virus type 1 (HSV-1), of which the gE glycoprotein is known to incorporate palmitic acid (Johnson & Spear, 1983), and the UL11 gene to encode a myristylated protein with an apparent Mr of 13000 to 16000 (MacLean et al., 1989). Up to 13 lipoproteins (of which up to nine also appear to be glycosylated) were observed in early work with virions of equine herpesvirus type 1 by chemical staining (Abodeely et al., 1971) or radiolabelling with [3H]choline (Perdue et al., 1974). Here we report an analysis of lipoprotein production in cells infected with VZV.

Human MRC-5 fibroblast cells (Flow Laboratories) were infected with a clinical isolate (H-551) of VZV. Trypsin-dispersed VZV-infected cell monolayers were used at an uninfected : infected cell ratio of 4 : 1; sterile medium was used for mock infection. Cells were incubated at 37 °C in Eagle's MEM plus 5 % foetal calf serum. Radiolabelling was done with D-[6-3H]glucosamine hydrochloride (Amersham) at 100 µCi per 25 cm² monolayer from 24 to 72 h after infection, or with L-[35S]methionine (Amersham) at 75 µCi per 25 cm² monolayer from 66 to 72 h after infection. After radiolabelling, monolayers were washed with Dulbecco's phosphate-buffered saline A (PBS) and either lysed in SDS sample buffer containing 2-mercaptoethanol for separation by SDS-PAGE (Laemmli, 1970), or prepared for immune precipitation by sonication in PBS plus 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium azide, 1 mM PMSF, 1 mM p-hydroxymercuribenzoate. For SDS-PAGE 10-5% acrylamide/0.32% bisacrylamide gels were used. For resolution of low Mr proteins (less than 10000 Mr), gels also contained 50% (w/v) sucrose, and were run using 20 mA of constant power (Chambers & Samson, 1982). After electrophoresis, gels were infiltrated for fluorography using 2,5-diphenyloxazole (Bonner & Laskey, 1974).

Overall, we observed a slight increase in the level of lipoprotein biosynthesis in VZV-infected cells relative to mock-infected cells, whereas the total amount of protein present in infected cells was not significantly altered (data not shown). Only two lipoproteins were observed that were clearly specific to VZV-infected cells, at 7000 and 5000 Mr, (Fig. 1). These were apparent from both myristic acid and palmitic acid radiolabelling, but appeared to incorporate primarily myristic acid and palmitic acid, respectively. It was clear that, as expected (Magee & Courtneidge, 1985; Olson et al., 1985), the two fatty acids radiolabellled different populations of proteins in both uninfected and VZV-infected cells.

For radioimmunoprecipitation (RIP), cell lysates were clarified by centrifugation at 85000 g for 1 h, and
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Fig. 1. Fluorogram of 3H-fatty acid-labelled cell lysates and immunoprecipitates using pooled post-zoster sera (10.5% acrylamide/50% sucrose gel). Lanes 1 to 4 cell lysates; lanes 5 to 8, radioimmunoprecipitates. Lanes 1, 3, 5 and 7 represent mock infections; lanes 2, 4, 6 and 8, VZV infections. Lanes 1, 2, 5 and 6, labelling with [3H]myristic acid; lanes 3, 4, 7 and 8, labelling with [3H]palmitic acid. Bands indicated are referred to in the text.

Preabsorbed for 1 h with *Staphylococcus aureus* cells (Calbiochem) at 37 °C prior to RIP with an equal mixture of four highly reactive convalescent sera from patients with zoster (complement fixation titres 1024 to >2048), or with monoclonal antibody (MAb) 3B3 against VZV gpI glycoprotein (kindly supplied by Professor C. Grose). After reaction for 2 h at 37 °C, immune complexes were collected on *S. aureus* cells for 1 h at 37 °C and separated from non-reactive proteins by four washes in PBS plus 0.1% Tween-20. Immune complexes were solubilized in SDS sample buffer containing 2-mercaptoethanol prior to electrophoresis and fluorographic infiltration. From RIP of a VZV-infected cell lysate, three major virus-specific bands were observed with myristic acid radiolabelling (Fig. 1, lanes 5 and 6), at Mₐ 7000 and a doublet at 90000/97000 (gpI). Also observed was a minor band at Mₐ 73000 (ppgI), a complex of five minor bands at Mₐ 35000 to 42000. Two bands at Mₐ 16000 and 28000 which were present with uninfected cell material were more intense with virus-infected cells. With palmitic acid (Fig. 1, lanes 7 and 8), the bands at Mₐ 73000 (ppgI) and 90000/97000 (gpI) were clearly more intense than with myristic acid. Minor bands at Mₐ 38000, 40000 and 42000 were observed, but the Mₐ 5000 band observed in lysates of infected cells (Fig. 1, lane 4) was only very weakly apparent after RIP.

After RIP with a MAb against gpI, the nonglycosylated form (pgpI) was apparent at Mₐ 73000 (Fig. 2, lane 8), whereas the glycosylated forms were observed at Mₐ 90000/97000 (Fig. 2, lane 6). When fatty acid-radiolabelled antigen was radioimmunoprecipitated with MAb, after prolonged exposure faint bands were apparent at Mₐ 90000/97000 with palmitic acid (Fig. 2, lane 4), as well as extremely faint bands at Mₐ 90000/97000 with myristic acid, and at Mₐ 73000 with palmitic acid. These latter bands were visible on the original fluorogram, but were too faint to reproduce in the figure. Their presence was confirmed by densitometry using a Joyce-Loebl Chromoscan 3 integrating densitometer.

It is known that the 14-carbon myristic acid radiolabel can be incorporated as (16-carbon) palmitate after a biochemical conversion, and that the palmitic acid radiolabel can be similarly incorporated as myristate (Buss & Sefton, 1985; Hedo *et al.*, 1987), although the former (a chain elongation) seems to be more common (Olson *et al.*, 1985; Schmidt, 1984). Therefore it was necessary to determine the nature of the fatty acids bound to gpI. In order to do this, the fatty acids present were analysed directly (by reverse-phase thin-layer chromatography, RP-TLC) or indirectly (by assaying hydroxylamine-resistant fatty acid incorporation).

Fig. 2. Fluorogram of radiolabelled immune precipitates using a MAb to VZV gpI (10.5% acrylamide gel). Lanes 1, 3, 5 and 7 represent mock infections; lanes 2, 4, 6 and 8, VZV-infections. Lanes 1 and 2, labelling with [3H]myristic acid; lanes 3 and 4, labelling with [3H]palmitic acid; lanes 5 and 6, labelling with [3H]glucosamine; lanes 7 and 8, labelling with [35S]methionine. Bands indicated are referred to in the text.
For the direct assay, sections of dried polyacrylamide gel containing the lipoprotein of interest were cut, rehydrated, and hydrolysed in vacuo for 16 h in 6 M-hydrochloric acid at 110 °C. After hydrolysis, fatty acids were extracted three times with n-hexane, and analysed by RP-TLC on Merck RP-18 plates using acetonitrile : acetic acid (1:1) (Schmidt et al., 1989). Unlabelled fatty acid standards visualized with 2,5-diphenylhexatriene (Sigma) were used to locate the corresponding regions of the sample lanes, which were then assayed by liquid scintillation counting (Table 1a). This method was also used to confirm the purity of fatty acid radiolabels. It was found that approximately half of the myristic acid radiolabel appeared to be incorporated in an unaltered form, while the remainder underwent chain elongation to palmitic and (18-carbon) stearic acids. With palmitic acid, elongation to stearic acid was observed, but no significant conversion to myristate was detected, a result which is in accord with those of Olson et al. (1985) and Schmidt (1984). However, it was clear from these data that gpI appeared to contain both myristic and palmitic acids, and also to contain significant levels of stearic acid. It has been reported that the latter is incorporated into a similar range of proteins as that of palmitic acid (Marinetti & Cattieu, 1982). By contrast, the 7000 Mr band, which incorporated primarily myristic acid, was shown by this method to contain 87.5% of the myristic acid radiolabel in the original form, while the remainder being palmitic acid.

For the indirect assay, polyacrylamide gels containing electrophoresed radioimmunoprecipitates were incubated for 20 h in 1 M-hydroxylamine pH 7-0 prior to fluorography. The resultant image was compared with that from a control gel which was similarly incubated in 1 M-Tris–HCl buffer pH 7-0, and the total intensity of individual bands on the two fluorograms was quantified by densitometry. Comparison of the two was used to determine the level of hydroxylamine-resistant incorporation (Table 1b). The thiester bonds which link palmitic and stearic acids (Schmidt, 1984; Schmidt et al., 1979) to the polypeptide are cleaved more readily by hydroxylamine than are the amide bonds which link the majority of myristic acid residues to the polypeptide, resulting in the preferential removal of palmitic acid, although some myristic acid is removed after exhaustive digestion (Olson et al., 1985). This method provides a useful control for the direct method. It was found (Table 1b) that hydroxylamine resulted in the removal of 72.4% of the myristic acid radiolabel, and 93.5% of the palmitic acid radiolabel. These results are in good agreement with those obtained by direct analysis of the fatty acid content of gpI, and provide further evidence of the presence of both myristic and palmitic acids in VZV gpI. The presence of fatty acids in the Mr 73000 band (Fig. 1, lanes 6 and 8) suggests that fatty acylation occurs before substantial glycosylation, as reported also for palmitylation of the HSV-1 gE glycoprotein (Johnson & Spear, 1983) and other lipoglycoproteins (Dunphy et al., 1981; Magee & Courtneidge, 1985; Schmidt & Schlesinger, 1980).

From densitometric quantification of the myristic and palmitic acid radiolabelled immunoprecipitates, together with the results obtained by direct fatty acid analysis, it is clear that gpI contains higher levels of palmitic than myristic acid. With other lipoproteins, palmitic acid is known to be attached to multiple sites, primarily cysteine residues, along the polypeptide chain (Kaufman et al., 1984; Magee et al., 1984), whereas myristic acid is usually present only on N-terminal glycine residues (Kamps et al., 1985; Pellman et al., 1985; Towler et al., 1987). Our observations are in accordance with this. There is some evidence that myristylation may occur at other sites (Hedo et al., 1987), possibly at lysine residues (Wold, 1986), but this does not appear to be a common occurrence.

It is clear that gpI is a lipoglycoprotein, as is the homologous (Davison & Scott, 1986) gE glycoprotein of HSV-1 (Johnson & Spear, 1983). From the published sequence of the VZV genome (Davison & Scott, 1986), it is apparent that the predicted product of the VZV gpI gene has the N-terminal glycine residue normally required for myristylation. However, the other residues present do not conform to the optimal configuration for myristylation, notably in the lack of a serine or threonine residue four residues from the N-terminal glycine (Chow et al., 1987; Paul et al., 1987; Towler et al., 1987). Also, it appears that the N-terminal region of the gpI transcript is a signal sequence, and thus removed cotranslationally. It is not yet clear how this fits with the observed myristylation of gpI. The bands identified as gpI represented the major radioimmunoprecipitated lipoprotein (and apparently the only major lipoglycoprotein) with palmitic acid radiolabelling. It is apparent from the genomic sequence (Davison & Scott, 1986) that the gpI
gene encodes two cysteine residues in the transmembrane region, which are likely to represent palmitylation sites.

For myristic acid, the most intense band by far was at $M_r$ 7000. This does not correspond to any well characterized VZV protein, or to any known VZV gene product with an N-terminal glycine residue (Davison & Scott, 1986). However, gene 49 encodes a protein of 8907 $M_r$ with such a residue. This gene also encodes the other residues in this region which are optimal for myristylation, and is the VZV homologue of the UL11 gene of HSV-1, which encodes a polypeptide of $M_r$ 10486 (McGeoch et al., 1988). This is myristylated, and migrates on polyacrylamide gels as a group of bands corresponding to 13000 to 16000 $M_r$ (MacLean et al., 1989). Therefore it would seem probable that the VZV myristoprotein of $M_r$ 7000 represents the product of gene 49. The 5000 $M_r$ palmitated lipoprotein is also smaller than the product of any known VZV gene. The poor detection of this band by immunoprecipitation may be due to poor solubility in immunoprecipitation buffer, or to low immunoreactivity. If the latter is the case, this could suggest that the 5000 $M_r$ band is not viral in origin, but rather a cellular protein overexpressed after VZV infection, as has been suggested for the 24000 $M_r$ lipoprotein observed late in infection with the Newcastle disease paramyxovirus (Veit et al., 1989). The high relative intensity of the bands representing $M_r$ 5000 and 7000 suggests that they are not breakdown products of a larger lipoprotein, although an extremely rapid breakdown of larger precursors cannot be ruled out. The well characterized maturation pathway of gp1 (Montalvo et al., 1985; Okuno et al., 1983) does not allow for the removal of such large polypeptides. Therefore it appears likely that these two lipoproteins are not merely by-products and, by analogy with the importance of lipoproteins for other viruses, may play significant roles in VZV infection. The nature and function of these lipoproteins are currently under investigation.

We have shown that VZV gp1 is a lipoglycoprotein, that VZV produces other lipoproteins, and that these incorporate both myristic and palmitic acids. Palmitylated lipoproteins appear to be exclusively membrane-associated (Olson & Spizz, 1986; Olson et al., 1985; Schmidt, 1983; Sefton et al., 1982), whereas myristylated lipoproteins have been observed in both membrane-associated and cytoplasmic forms (Buss et al., 1985; Carr et al., 1982; Olson & Spizz, 1986; Olson et al., 1985; Schultz & Oroszlan, 1984). Myristylation is often associated with glycoproteins (Schmidt, 1984), being involved with structural and functional membrane interactions (Schmidt, 1983), whereas many possible roles have been suggested for myristylation, including that of mediating transient membrane associations (Schultz & Oroszlan, 1984). Among the other human herpesviruses, cytomegalovirus is known to induce membrane permeability changes (Rugolo et al., 1986), which may involve viral myristoproteins (Cross et al., 1984; Kamps et al., 1985; Van der Valk et al., 1987). HSV is known to alter lipid metabolism in infected cells (Ben-Porat & Kaplan, 1971; Compton & Courtney, 1985), whereas VZV alters cellular lipid metabolism in a strain-dependent manner (Jerkofsky & De Siervo, 1986). VZV lipoproteins may be involved in such changes. VZV gp1 is well characterized, but the 5000 and 7000 $M_r$ lipoproteins reported herein have not been reported previously.

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