Gene UL11 of Herpes Simplex Virus Type 1 Encodes a Virion Protein which Is Myristylated

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SUMMARY

We have investigated whether herpes simplex virus (HSV) contains structural polypeptides which are modified by myristic acid. We demonstrate that herpes simplex virions contain a family of myristylated proteins, Mr approximately 13000 to 16000. These were mapped, using HSV-1/HSV-2 intertypic recombinants, to 0-130 to 0-204 map units on the virus genome. Using anti-peptide sera, raised against the carboxy-terminus of the predicted UL11 gene product, we have established that the myristylated virion polypeptides are products of the viral gene UL11.

INTRODUCTION

Covalent modification with fatty acid is now a well established feature of several cellular and viral polypeptides (reviewed in Cross, 1987; Sefton & Buss, 1987). Two commonly described fatty acid modifications are palmitylation and myristylation. Palmitylation involves post-translational addition of the 16-carbon saturated fatty acid, palmitic acid, usually through a thio-ester linkage to an internal cysteine residue (Schmidt et al., 1979; Kaufman et al., 1984; Rose et al., 1984; Chen et al., 1985; Staufenbiel & Lazarides, 1986). This modification has been reported for a wide range of proteins, including several viral surface glycoproteins (Schmidt, 1982a), and may serve to increase the stability of membrane-protein interactions (reviewed in Schmidt, 1982b; Sefton & Buss, 1987). In contrast, myristylation occurs predominantly co-translationally (Wilcox et al., 1987) and involves addition of the 14-carbon saturated fatty acid, myristic acid, usually via an amide bond to an amino-terminal glycine residue (Carr et al., 1982; Henderson et al., 1983; Ozols et al., 1984; Schultz et al., 1985). Examples of myristylated cellular polypeptides are subunits of a number of guanine nucleotide-binding regulatory proteins (G-proteins) (Buss et al., 1987), the catalytic subunit of cAMP-dependent protein kinase (Carr et al., 1982), the membrane immunoglobulin heavy chain polypeptide (Pillai & Baltimore, 1987) and NADH-cytochrome b5 reductase (Ozols et al., 1984).

The precise function of the myristic acid moiety is not clear, but may not be simply that of a hydrophobic membrane anchor. Localization to the inner plasma membrane of the Rous sarcoma virus transforming protein pp60src and several retroviral gag proteins is dependent on myristylation (Cross et al., 1984; Rein et al., 1986; Rhee & Hunter, 1987). The apparent targeting of these proteins to the plasma membrane has led to the suggestion that specific cell membrane receptors for myristate may exist (Schultz & Oroszlan, 1983; Sefton & Buss, 1987). Not all myristylated polypeptides, however, may depend on the myristic acid moiety for membrane localization. The observation that myristylation should not be required for insertion of the membrane immunoglobulin heavy chain, or NADH-cytochrome b5 reductase into membranes, led Pillai & Baltimore (1987) and Ozols et al. (1984) to speculate that the modifying group may be involved in intermolecular protein–protein interactions. Furthermore, not all myristylated polypeptides are associated with membranes. The catalytic subunit of cAMP-dependent protein kinase is released from the membrane following activation by cAMP, which
led Carr et al. (1982) to suggest a role for the myristic acid moiety in the subunit interactions, or in the substrate specificity of this protein.

Structural polypeptides from several families of viruses, with either enveloped or non-enveloped morphology, have also been shown to be modified by myristic acid. Myristylation of retroviral gag proteins (Schultz & Oroszlan, 1983; Bradac & Hunter, 1986) appears to be essential for capsid assembly of Moloney murine leukaemia virus (Rein et al., 1986) and for the intracytoplasmic transport of preformed capsids of Mason–Pfeiffer monkey virus for budding and release (Rhee & Hunter, 1987). Myristylated proteins which have been suggested to play a role in virus assembly include proteins VP2 and VP6 of rotavirus (Clark & Desselberger, 1988), hepatitis B virus pre-S1 protein (Persing et al., 1987), virion proteins VP2 of both polovirus and simian virus 40 (Streuli & Griffin, 1987) and picornavirus protein VP4 (Chow et al., 1987; Paul et al., 1987).

In the work reported here we investigated whether herpes simplex virus (HSV), a large, enveloped, double-stranded DNA virus, also contains myristylated virion proteins. We show the presence of one such species and identify it as the product of viral gene UL11.

METHODS

Cells. BHK-21 clone 13 (C13) cells (Macpherson & Stoker, 1962) grown in Eagle's medium supplemented with 10% newborn calf serum were used throughout.

Virus. HSV-1 strain 17 syn* (Brown et al., 1973) and HSV-2 strain HG52 (Timbury, 1971) were the wild-type virus strains used in this study. The HSV-1/HSV-2 intertypic recombinants have been described previously (Marsden et al., 1978, 1982; Preston et al., 1978; Chartrand et al., 1981; Davison et al., 1981; Hope et al., 1982).

Preparation of infected cell extracts. Confluent cell monolayers in 50 mm Petri dishes were infected at 20 p.f.u./cell in Eagle's medium supplemented with 5% newborn calf serum. The virus was absorbed for 1 h at 37 °C and the infected cells were maintained in Eagle's medium containing one-fifth the normal concentration of methionine and 2% newborn calf serum for a further 3 h. [3H]Myristic acid (Amersham, specific activity 40 to 60 Ci/mmol), or [3H]palmitic acid (Amersham, specific activity 40 to 60 Ci/mmol), supplied in ethanol, were dried under a stream of nitrogen, redissolved in a small volume of ethanol and added to the cells at 125 to 250 µCi/ml; [35S]methionine (Amersham, specific activity > 1000 Ci/mmol) was added at 50 µCi/ml.

Infected cell polypeptides were harvested at 24 h post-infection by washing the cells twice with phosphate-buffered saline (PBS) and then adding 0.5 ml of extraction buffer containing 100 mM-Tris-HCl pH 8.0, 10% glycerol, 0.5% NP40, 0.5% sodium deoxycholate and 0.2 mM-PMSF (Zweig et al., 1980). Cell debris was pelleted by centrifugation at 11 000 g for 10 min in a Beckman microfuge. Aliquots of the supernatant were then boiled in denaturation buffer (50 mM-Tris-HCl pH 6.7, 2% SDS, 700 mM-2-mercaptoethanol, 10% glycerol and 1% bromophenol blue) and analysed by SDS–PAGE (Marsden et al., 1978).

Fluorography. SDS–PAGE gels were treated with En3Hance (New England Nuclear), dried and exposed against either Kodak X-Omat X-1 or X-6 film at −70 °C.

Purification of herpes simplex virions. BHK-21 C13 cells in 80 oz plastic roller bottles were infected at 5 or 0.003 p.f.u./cell and incubated at 37 °C for 24 h or 3 to 4 days, respectively. The infected cells were labelled at 3 h or 7 h post-infection, respectively, with [3H]myristic acid (125 µCi/ml) or [35S]methionine (25 µCi/ml) as described previously.

Infected cells were shaken into the medium and pelleted by centrifugation in a Fisons Coolspin centrifuge (2000 r.p.m., 10 min, 4 °C). Virions present in the supernatant were pelleted by centrifugation in a Sorvall GSA rotor (12000 r.p.m., 2 h, 4 °C), resuspended in a small volume of Eagle's medium without phenol red and layered on 12 ml continuous 5 to 15% Ficoll gradient, which was centrifuged in a Sorvall T41 rotor at 13000 r.p.m. for 2.5 h at 4 °C. The virion band was collected by side puncture, diluted and repelleted by centrifugation in a Sorvall T41 rotor at 21000 r.p.m. for 2 h at 4 °C.

Purified HSV virions were resuspended in denaturation buffer (for SDS–PAGE analysis), or in PBS (for electron microscopy).

Oligopeptide antisera. The peptide NH2-Tyr-Ala-Thr-Gln-Phe-Pro-Pro-Met-Ser-Asp-Ser-Glu-COOH represents the carboxy-terminal 12 amino acids of the predicted product of gene UL11 (McGeoch et al., 1986, 1988), with an additional amino-terminal tyrosine residue to facilitate coupling to bovine serum albumin (BSA) (Bassiri et al., 1979). Sheppard, 1983) using an LKB Biolyx peptide synthesizer. All chemicals were purchased from LKB Biocrom with the exception of dimethylformamide (Rathburn Chemicals), trifluoroacetic acid (Aldrich) and resin to which the first amino acid had been coupled via an acid-labile handle (Peptide and Protein Research).
Myristylated HSV structural proteins

Following synthesis the peptide was cleaved from the resin and side-chain protecting groups were removed with 95% trifluoroacetic acid in water. The M_r of the peptide determined by mass spectrometry (M-Scan) was identical to that predicted from the amino acid sequence.

Two New Zealand White rabbits were immunized intramuscularly with 100 µg of BSA-conjugated peptide emulsified in Freund's complete adjuvant for the first injection and in Freund's incomplete adjuvant for subsequent injections. The animals were bled 10 days after each injection and anti-BSA antibodies were removed by absorption with BSA immobilized on Sepharose 4B.

Immunoprecipitation. Fifty µl of antiserum was incubated at 37 °C for 30 min in the presence or absence of peptide. Infected cell extract (100 µl) or 25 µl of virion extract, diluted to 200 µl with extraction buffer, was then added and the samples were incubated overnight at 4 °C and then transferred to ice for a further 2 h in the presence of 60 µl of a 50% (v/v) suspension of Protein A-Sepharose. Immunoprecipitates were washed several times with extraction buffer and bound proteins then eluted by boiling in denaturation buffer and analysed by SDS-PAGE.

RESULTS

Myristylation of HSV-induced polypeptides

In order to investigate whether any HSV-induced polypeptides are myristylated, cells were labelled with [3H]myristic acid at various times after infection. Since it has previously been found that metabolic conversion of myristic acid to other fatty acids, especially palmitic acid, and to a much lesser extent to amino acids, occurs in vivo (Schmidt, 1984; Olson et al., 1985), infected cells were also labelled in separate experiments with [3H]palmitic acid. Preliminary analysis of labelled whole cell extracts by SDS-PAGE (results not shown) suggested that most of the myristic acid label is converted to palmitic acid. Four polypeptides appeared to be labelled exclusively with myristic acid: one with an M_r of approximately 26K and a cluster of three polypeptides of M_r approximately 13K to 16K. The latter three proteins were subsequently shown to be related to each other and were found to be components of the virus particle (see below). This report concerns only the 13K to 16K myristylated virion proteins.

Myristylation of HSV structural polypeptides

In order to determine whether any HSV-1 structural polypeptides are myristylated, HSV-1 was grown in the presence of either [35S]methionine, [3H]myristic acid or [3H]palmitic acid and purified virions were analysed by SDS-PAGE (Fig. 1). A group of polypeptides with an M_r of approximately 13K to 16K were labelled specifically by [3H]myristic acid (Fig. 1; compare lanes 3 and 4). The attached radiolabel was resistant to treatment with 1 M-hydroxylamine pH 9-5, or 2 M-NaOH, suggesting an amide rather than an ester linkage (Sefton et al., 1982; Magee & Courtneidge, 1985; Olson et al., 1985). In contrast, the label was lost from virion proteins labelled by both myristic acid and palmitic acid (results not shown). Thin layer chromatography of virions that were labelled with [3H]myristic acid and then hydrolysed (Chow et al., 1987) demonstrated comigration of about 70% of the released fatty acid with myristic acid and of most of the remainder with palmitic acid (results not shown). Taken together, these results indicate that the 13K to 16K polypeptides represent myristylated HSV-1 structural proteins. In contrast, virion proteins that labelled with both myristic acid and palmitic acid probably represent palmitylated polypeptides.

The identity of the palmitylated virion components was not investigated further. We note, however, that Johnson & Spear (1983) suggested that glycoprotein E (gE) may be palmitylated. Analysis of the predicted amino acid sequences of HSV-1 glycoproteins (McGeoch et al., 1985, 1988) for the presence of cysteine residues in or around the transmembrane region, the usual site of palmitic acid addition (Sefton & Buss, 1987), indicates that not only gE, but also gD, gG and gI could be palmitylated.

Mapping the myristylated virion proteins by SDS–PAGE

[3H]Myristic acid labelling of HSV-2 virions also revealed myristylated virion proteins that migrated with a slightly lower electrophoretic mobility than their presumed HSV-1 counterparts (Fig. 2a; also results not shown). Only the lowest M_r species can be seen in Fig. 2(a) but Fig. 2(c) shows more clearly the three myristylated HSV-2 virion proteins. The difference in
Fig. 1. Myristylation of HSV structural polypeptides. HSV-1 virions were purified from cells labelled with [³⁵S]methionine, [³H]palmitic acid or [³H]myristic acid (lanes 2, 3 and 4, respectively) and analysed on a 7.5 to 15% SDS-polyacrylamide gel, alongside an HSV-1-infected cell extract (E) labelled with [³⁵S]methionine (lane 1). Lane 5 represents a longer exposure of lane 2. A family of low Mr polypeptides acylated only in the presence of myristic acid are indicated (~1). Mr values are indicated on the left.

Electrophoretic mobility of the HSV-1 and HSV-2 myristylated polypeptides could also be distinguished in extracts from infected cells (Fig. 2a and b) and was utilized in mapping the HSV genes encoding the myristylated proteins. Cells were infected with either HSV-1 strain 17 syn+, HSV-2 strain HG52, or one of 17 HSV-1/HSV-2 intertypic recombinants and labelled with [³H]myristic acid. Infected cell polypeptides were then harvested and analysed by SDS-PAGE (Fig. 2b; also results not shown). The results summarized in Fig. 3 suggest that the gene(s) encoding the myristylated polypeptides are located between 0.130 and 0.204 map units on the HSV genome. None of the intertypic recombinants used synthesized the 13K species found in HSV-1 strain 17 syn+-infected cells; the reason for this is unknown. In order to confirm the map location obtained using infected cell extracts, recombinant virus RS5, which contains HSV-2 DNA sequences from 0.130 to 0.204 map units only, was grown in the presence of [³H]myristic acid and virions were purified. RS5 contains the HSV-2 myristylated virion species (Fig. 2c).

The region between map units 0.130 and 0.204 on the HSV-1 genome is expanded in Fig. 4 and contains all or part of the open reading frames UL8 to UL16. A consensus sequence
Fig. 2. Mapping the myristylated virion proteins. (a) HSV-1 and HSV-2 virions, labelled with [3H]myristic acid, were analysed on a 7.5 to 15% SDS–polyacrylamide gel (lanes 2 and 3, respectively). [35S]Methionine-labelled HSV-1 virions (lane 1) and [3H]myristic acid-labelled HSV-1- and HSV-2-infected cell extracts (lanes 5 and 4, respectively) are also shown. The myristylated virus polypeptides are indicated (HSV-1 \( \bullet \), HSV-2 \( \circ \)). Mr values are indicated on the left. (b) [3H]Myristic acid-labelled extracts from uninfected cells (lane 8), or cells infected with HSV-1 (lanes 4 and 7), HSV-2 (lanes 1 and 6) and intertypic recombinants RH6, RS5 and RE4 (lanes 2, 3 and 5, respectively), are shown. Only the relevant portion of the gel is presented. The myristylated polypeptides characteristic of strain 17 syn \( ^* \) (\( \bullet \)) or strain HG52 (\( \circ \)) are indicated. (c) HSV-1 virions labelled with [35S]methionine (lane 1) or [3H]myristic acid (lane 2) and RS5 virions labelled with [3H]myristic acid (lane 3), were analysed on a 7.5 to 15% SDS–polyacrylamide gel. The HSV-1 (\( \bullet \)) and RS5 (\( \circ \)) myristylated polypeptides are indicated. Mr values are indicated on the left.
Fig. 3. Mapping of the region of the HSV genome that encodes the myristylated virion polypeptides. The genome structures of, and myristylated polypeptides induced by, the 17 HSV-1/HSV-2 intertypic recombinants are shown. The genome arrangement of HSV DNA is shown at the top of the figure. Vertical dashed lines correspond to the ends of the long and short repeat sequences. Those sequences of the recombinant derived from the type 1 and type 2 parent are represented by a thick continuous line superimposed on the upper (HSV-1) and lower (HSV-2) of the two horizontal dashed lines. Crossover regions are indicated by one or two vertical lines between the thick continuous horizontal lines. The distance between two vertical lines indicates the remaining region of uncertainty for that crossover event. Where the uncertainty is small, the crossover appears as a single vertical line. The right of the figure shows for each recombinant whether it induces the HSV-1 (1) or the HSV-2 (2) myristylated protein species. The units on the bottom are expressed as a fraction of the genome length. Below is indicated the map location derived for DNA sequences encoding the myristylated proteins.

Fig. 4. Map location of the myristylated structural polypeptides. A schematic diagram of the HSV-1 genome (divided into 0 to 1-0 map units) is shown. U₅ and U₆ represent the long and short unique sequences, while TR₄/IR₄, TR₅/IR₅ represent the terminal and internal repeat sequences of the long and short regions, respectively. The genome is in the prototype orientation. The location of the region encoding the myristylated polypeptides is indicated (——) and expanded below to show the predicted open reading frames in this region (McGeoch et al., 1988). The solid bar represents the extreme mapped limits for sequences encoding the myristylated structural polypeptides.
Fig. 5. Immunoprecipitation of the UL11 gene products. Proteins from mock-infected or HSV-1-infected cell extracts labelled with [35S]methionine (lanes 3 and 4 to 6, respectively), or from HSV-1 virions labelled with [3H]myristic acid (lanes 8 and 9), were precipitated using preimmune serum (lane 4), or anti-UL11 serum alone (lane 3), or in the presence of 10 μg of the peptide against which the serum was raised (lanes 6 and 9), or an unrelated, control peptide (lanes 5 and 8) and analysed on 7.5 to 15% SDS–polyacrylamide gels. Bands which appear to be specifically precipitated are marked (●). Mock-infected and HSV-1-infected cell extracts labelled with [35S]methionine (lanes 1 and 2, respectively) and HSV-1 virions labelled with [3H]myristic acid or [35S]methionine (lanes 7 and 10, respectively) are shown. Mr values are indicated on the left.
Gly-X-X-Ser/Thr has previously been proposed to be present at the amino terminus of myristylated polypeptides (Chow et al., 1987; Towler et al., 1987). Only one of the identified open reading frames in HSV-1 strain 17 syn* (McGeoch et al., 1988), gene UL11, encodes this sequence immediately following the initiating methionine codon. Since gene UL11 lies within the limits defined by the intertypic recombinants (Fig. 4) and is predicted to encode a polypeptide of $M_r$ 10486, it is a good candidate for the gene encoding the myristylated virion proteins.

The equivalent gene products from HSV-2 (Draper et al., 1986), varicella-zoster virus (VZV) (Davison & Scott, 1986) and Epstein-Barr virus (EBV) (Baer et al., 1984) also contain the consensus sequence for myristylation at their amino termini. These are the products of genes 49 and BBLF1 for VZV and EBV, respectively (McGeoch et al., 1988).

**Gene UL11 encodes the myristylated structural polypeptides**

A synthetic oligopeptide representing the carboxy-terminal 12 amino acids of UL11 was synthesized and used to generate anti-peptide sera in two rabbits. Using $[^{35}S]$methionine-labelled infected cell extracts, immune serum (but not preimmune serum) precipitated a group of 13K to 16K polypeptides (Fig. 5). These polypeptides were not precipitated from mock-infected cell extracts. The presence of the relevant peptide, but not an unrelated control peptide, inhibited this reaction. The immune serum also precipitated the 13K to 16K myristylated polypeptides from $[^{3}H]$myristic acid-labelled virion extracts, the reaction again being inhibited by the relevant peptide only (Fig. 5, lanes 8 and 9). From these results it can be concluded that gene UL11 encodes the myristylated 13K to 16K virion polypeptides.

**DISCUSSION**

In this communication we have shown that herpes simplex virions contain a family of polypeptides ($M_r$ 13K to 16K) which are modified by myristylation. These polypeptides were mapped to 0·130 to 0·204 map units on the HSV genome and were shown, using anti-peptide sera, to be encoded by gene UL11. A number of important questions now remain to be addressed. Although elucidation of the function of the myristylated virion proteins may require the generation of suitable virus mutants, it will also be important to characterize the UL11 gene products with respect to their temporal regulation, post-translational modifications, intracellular location and their abundance and location within virus particles.

A 0·9 kb mRNA containing the UL11 open reading frame has previously been identified (Draper et al., 1986). This belongs to a family of five 3' coterminal mRNAs (Costa et al., 1983; Draper et al., 1986) and has been tentatively assigned to the beta-gamma class in terms of its kinetics of synthesis (Draper et al., 1986). No detailed study on the kinetics of synthesis of the myristylated UL11 polypeptides has been carried out, but preliminary pulse-labelling experiments suggest that these are synthesized as early (beta) or early-late (beta-gamma) gene products.

Three apparently related polypeptide species were detected in this study, all of which labelled with myristic acid and thus presumably contain the predicted amino-terminus of UL11. Furthermore, all three species were precipitated by the anti-peptide sera and were recognized by these sera in immunoblotting experiments (results not shown), suggesting that they also contain the intact, or almost intact, carboxy terminus of the UL11 product. It is possible that other post-translational modifications may be responsible for the apparent differences in $M_r$. Interestingly, none of the HSV-1/HSV-2 intertypic recombinants appear to synthesize the 13K species found with the wild-type HSV-1 strain 17 syn*. Absence of this polypeptide was not limited to the intertypic recombinants, as the HSV-1 temperature-sensitive mutant tsK, derived from strain 17 syn (Marsden et al., 1976), also synthesizes only the two higher $M_r$ species (results not shown).

Myristylated structural proteins of other viruses described to date appear to be relatively major components. The 0·9 kb transcript identified by Draper et al. (1986) appeared to be relatively abundant late in infection. However, the number of molecules of the myristylated UL11 products present per virion remains to be determined.
Myristylated HSV structural proteins

It will be interesting to establish the location of the myristylated proteins within the virus particle. Myristylated proteins are structural components of several non-enveloped viruses, suggesting that the UL11 products might not be located in the virus envelope. Herpes simplex virions consist of four main structural elements: the core, capsid, tegument and envelope. Only a limited number of proteins have been described in herpesvirus capsid structures (see, for example, Gibson & Roizman, 1972; Marsden et al., 1976; Heilman et al., 1979; Cohen et al., 1980). The major capsid proteins have Mr values of approximately 155K, 50K, 40K, 32K, 25K and 12K. The 13K to 16K myristylated species do not comigrate with the 12K capsid protein (results not shown) and no family of proteins of 13K to 16K have been described in HSV capsids. However, we cannot yet exclude the presence of these proteins in capsids, since, in our hands, labelling with myristic acid was relatively weak and yields of purified capsids were only 1 to 10% of those of virions. We are currently attempting to localize the UL11 products by immune electron microscopy.

What is the function of the myristylated herpes simplex virion proteins? Possession of a myristylated virion component is a feature of many virus families. Analysis of the effects of single point mutations affecting the myristylated glycine residue of gag proteins have shown that this modification is required for virion assembly in the case of two retroviruses (Rein et al., 1986; Rhee & Hunter, 1987). These facts, together with the observation that the amino termini of the predicted products of the EBV, VZV and HSV-2 genes equivalent to UL11 are also compatible with myristylation, might suggest an important role for the UL11 gene products. We are currently attempting to construct suitable virus mutants in order to elucidate the function of these myristylated virion proteins.

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