**Myristylation of Rotavirus Proteins**

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**SUMMARY**

After labelling with [3H]myristic acid during replication the structural proteins VP6 and VP2 of purified rotavirus particles were found to be myristylated in an amide bond. [3H]Palmitic acid was converted to myristic acid before bonding to the viral proteins. Possible functions of rotavirus protein myristylation are discussed.

Post-translational modification of cellular and viral proteins is a frequently encountered event which localizes, stabilizes and trims them for various functions. Acylation with fatty acids has been described for immunoglobulins in B lymphocytes (Pillai & Baltimore, 1987) and for regulatory G proteins in tumour cells (Buss et al., 1987) and has been found in proteins of influenza and Semliki Forest viruses (Schmidt, 1984), of various retroviruses (Buss & Sefton, 1985; Rhee & Hunter, 1987) and more recently in proteins of the envelope-free simian virus 40 (SV40) and polyoma virus (Streuli & Griffin, 1987) and of various members of the Picornaviridae family (Chow et al., 1987; Paul et al., 1987). Myristic acid (n-tetradecanoic acid) and other fatty acids were found in an ester linkage in the viral glycoproteins E1 and E2 of Semliki Forest virus and the HA2 subunit of fowl plague influenza virus; there was metabolic conversion of smaller fatty acid precursors to longer fatty acids before esterification (Schmidt, 1984). The lipid bound to the transforming protein of Rous sarcoma virus, p60src, and to its cellular equivalent, p60c-sty, was found to be myristic acid attached in amide linkage to the N-terminal amino acid, glycine; here higher length precursors were found to be shortened before incorporation (Buss & Sefton, 1985). Similarly, myristylation by way of an amide bond was detected at the N-terminal glycine residues of the pre-S1 proteins of various hepadnaviruses (Persing et al., 1987), of polyoma virus and SV40 virus (Streuli & Griffin, 1987) and of picornaviruses (Chow et al., 1987). Growth of picornaviruses in the presence of palmitic acid (n-hexadecanoic acid) resulted in incorporation of myristic acid at the N terminus of VP4, i.e. palmitic acid was metabolized before esterification (Chow et al., 1987).

Here we describe myristylation of structural proteins of rotaviruses, a genus of the Reoviridae family. Rotaviruses are the main cause of acute gastroenteritis of infants and young children and of the young of a wide variety of mammalian species and of birds (McNulty, 1978; Flewett & Woode, 1978; Holmes, 1983). The genome, consisting of 11 segments of double-stranded RNA, forms a core which is enclosed by a characteristic double-layered capsid. The major structural proteins are VP2 (core), VP6 (inner capsid) and VP7 and VP3/VP4 (outer capsid). There is now agreement that VP3/VP4 should be called VP4 (Liu et al., 1988). VP4 is post-translationally cleaved into VP5* and VP8* (Estes et al., 1981). Minor structural proteins are VP1 and VP3 (core) and VP11 (outer capsid) (McCrae & McCorquodale, 1982). For the designation of proteins (except VP5*) the nomenclature of McCrae & McCorquodale (1982) is used.

Confluent monolayers of BS-C-1 cells in roller bottles (Falcon; no. 3027, 850 cm²) were infected with the tissue culture-adapted bovine rotavirus (BRV; U.K. Compton strain) or with human rotavirus (HRV) strain Wa (subgroup II; serotype 1) at an m.o.i. of 1 to 5 and propagated in Eagle's MEM in the presence of trypsin (Sigma; type IX at 1 µg/ml). [9,10(n)-3H]Myristic

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Fig. 1. Proteins in particles of BRV purified from CsCl density gradients after equilibrium ultracentrifugation. Lanes 2 and 5, lower band of 1.38 g/ml density; lanes 3 and 6, middle band of 1.36 g/ml density; lanes 4 and 7, upper band of 1.30 g/ml density; lane 1, BRV-infected cell proteins. Labelling of preparations in lanes 1 to 4 was with [35S]methionine, in lanes 5 to 7 with [3H]myristic acid. Separation was on a 15% polyacrylamide gel using Laemmli's (1970) buffer system. The gel was then fixed, treated with Enhance and autoradiographed. The viral proteins (VP1 to VP10) are indicated on the left; the nomenclature of McCrae & McCorquodale (1982) was used. VP5* is a cleavage product of VP4 (Estes et al., 1981; Liu et al., 1988).

acid (sp. act. 48 Ci/mmol; Amersham) and [9,10(n)-3H]palmitic acid (sp. act. 54 Ci/mmol; Amersham) were dried under an nitrogen stream and redissolved in 10 ml of culture medium; virus was grown in the presence of 1 mCi of myristic acid or palmitic acid per bottle which was added after the adsorption step. In parallel, cultured virus was labelled with 500 μCi of [35S]methionine (sp. act. 1415 Ci/mmol; Amersham) per bottle as described (Hundley et al., 1985). When full c.p.e. was reached, cultures were frozen and thawed twice and the virus was semi-purified by differential ultracentrifugation through a sucrose cushion as described (Follett & Desselberger, 1983). Virus was then extracted with Freon and banded in CsCl gradients (McCrae, 1985). Bands of densities 1.38, 1.36 and 1.30 g/ml (representing RNA-containing single-shelled, RNA-containing double-shelled and empty virus particles, respectively) were collected, diluted with phosphate-buffered saline, and the virus was re-pelleted; the pellets were resuspended in 10 to 20 μl of water. Aliquots were mixed with different electrophoresis loading buffers and the proteins separated on 15% polyacrylamide gels using Laemmli's (1970) buffer system. Reproducible labelling with myristic acid was obtained, and a typical result is shown in
Fig. 2. Proteins of rotavirus particles purified from CsCl density gradients and separated on a gel as described in the legend to Fig. 1. Lane 1, upper band particles labelled with [35S]methionine (see lane 4, Fig. 1); lanes 2 to 4, lower band particles labelled with [3H]myristic acid. The samples in lanes 1, 2 and 4 were boiled for 10 min in protein dissociation mixture (PDM) containing 2-mercaptoethanol (Sabara et al., 1987), the sample in lane 3 was heated to 37 °C for 10 min in protein running mixture containing 6 M-urea but no mercaptoethanol (Sabara et al., 1987), and the sample in lane 4 was treated with 1 M-hydroxylamine (pH 9.0) for 1 h at room temperature before being boiled in PDM. Lanes 5 and 6 contain [3H]myristic acid-labelled proteins of lower band virus and [35S]methionine-labelled proteins of upper band virus, respectively. After electrophoretic separation of proteins, this part of the gel was soaked in 1 M-hydroxylamine for 1 h at room temperature. Gels were then fixed, treated with En3Hance and autoradiographed. Viral proteins (VP1 to VP7) are indicated on the right. VP5* is a cleavage product of VP4. VP6t is the trimer of VP6 and is marked with an arrow in lane 3.

Fig. 1; the rotavirus proteins VP2 and VP6 were reproducibly labelled with myristic acid. The viral protein VP6, seen as a monomer of M, 45K on gels under fully denaturing conditions, occurs as a trimer in the virus particle (Gorziglia et al., 1984; Sabara et al., 1987). When electrophoresis conditions preserving this structure were chosen (Sabara et al., 1987), it was found that the myristic acid label of VP6 concentrated in a band of approximately 135K (Fig. 2) further demonstrating specific bonding to VP6. The myristic acid label of the rotavirus proteins could not be removed by treatment with 1 M-hydroxylamine pH 9.0 (either by treatment of a small aliquot before electrophoresis or by soaking of the gel in 1 M-hydroxylamine after electrophoresis) thus indicating (Fig. 2) that the myristic acid moiety was covalently linked to VP2 and VP6 by an amide linkage (Chow et al., 1987).

To determine the identity of the attached fatty acid, rotavirus preparations labelled with [3H]myristic acid or [3H]palmitic acid were hydrolysed with 6 M-HCl for 16 h at 110 °C, and the liberated fatty acid was extracted twice with toluene and analysed by ascending chromatography on C18 reverse-phase thin-layer chromatography (TLC) plates (Whatman) in acetic
Fig. 3. Identification of fatty acid linked to rotavirus proteins. Rotavirus was labelled with [3H]myristic acid or [3H]palmitic acid, purified in a CsCl gradient (lower band), hydrolysed with 6 m-HCl at 110 °C for 18 h and extracted twice with toluene. The toluene extracts were concentrated to volumes of 10 µl by exposure to a nitrogen stream and separated by ascending chromatography on a C18 reverse-phase TLC plate (Whatman) using acetic acid : acetonitrile (1:1). The arrow indicates the direction of chromatography. Lane 1, [3H]myristic acid marker; lane 2, [3H]palmitic acid marker; lane 3, mixture of [3H]-labelled fatty acid markers of lanes 1 and 2; lane 4, toluene extract of [3H]myristic acid-labelled particles of BRV; lane 5, toluene extract of [3H]palmitic acid-labelled particles of BRV; lane 6, toluene extract of [3H]myristic acid-labelled particles of HRV strain Wa (subgroup II, serotype 1).

acid : acetonitrile (1:1) (Chow et al., 1987) using [3H]myristic acid and [3H]palmitic acid as markers. Alternatively, reverse-phase HPLC using a Micro-Bondapack C18 column (Waters Associates) was carried out using an acetonitrile : trifluoroacetic acid (TFA) gradient. The column was equilibrated with 0·1% TFA in water : 0·05% TFA in acetonitrile (95:5) and a linear gradient was applied to reverse the buffer components to 5:95 within 8 min. The column eluents were continuously measured for 3H content using a Ramona Raytest flow-through scintillation counter. Data were stored on an IBM PC running under the Ramona Radio-Chromatography System program version 10.1 (Raytest Instruments) (Darling et al., 1987). It was found that the liberated fatty acid always comigrated with the myristic acid marker (Fig. 3); palmitic acid was thus metabolized to myristic acid before esterification into viral proteins. Identical results were obtained from the HPLC data (not shown). In picornavirus-infected cells similar observations have been made (Chow et al., 1987).

The rotavirus proteins VP2 and VP6 were found to contain myristic acid in an amide linkage. This could have been to the ε-NH₂ group of a lysine residue or the N-terminal amino acid of the protein. In most cases of myristylation of viral proteins this has been in an N-terminal glycine residue (Table 1 of Chow et al., 1987; Table 2 of Paul et al., 1987). The sequence of the gene coding for VP6 of BRV (RF strain), determined by Cohen et al. (1984), is highly conserved in comparison with the sequence of the VP6 gene of SA11 virus (Estes et al., 1984). There is no N-terminal glycine in the VP6 sequence but lysines in positions 9 and 11; the first glycine is found in position 21 from the N terminus and is followed by a serine in position 25. This corresponds to a consensus sequence for myristylation (Gly-X-X-X-Ser/Thr) proposed by Chow et al. (1987) and would make it a good candidate for myristylation, provided that the first 20 amino acids of VP6 were post-translationally cleaved off like a leader peptide. It remains to be seen whether this is the case. Proteolytic removal of a leader peptide to produce an N-terminal glycine for myristylation is necessary in cardio- and aphthoviruses (Chow et al., 1987) and there is some speculation that this may also be the case for the VP4 equivalent of hepatitis A virus (Cohen et al., 1987; Chow et al., 1987).

The finding that VP2 and VP6 of rotaviruses are myristylated raises the question of the functional significance of this modification. VP2 is the major core protein and VP6 the major inner capsid protein of rotaviruses (McCrae & McCorquodale, 1982; Arias et al., 1982). Both
may act as scaffolding proteins for the formation of virus particles; this function was proposed for the myristylated VP2s of SV40 and polyoma viruses (Streuli & Griffin, 1987) and for the myristylated VP4s of picornaviruses (Chow et al., 1987; Paul et al., 1987). Mutants of polyoma virus bearing selected deletions in the VP2 gene multiply poorly (Cole et al., 1977). A mutant Mason–Pfizer monkey virus (MPMV), in which the normally myristylated glycine residue of the gag precursor protein was replaced by a valine using in vitro mutagenesis, was completely non-infectious (Rhee & Hunter, 1987); the gag precursors were synthesized, but not myristylated and not cleaved from the mature protein, and immature particles accumulated in the cytoplasm but were not released. This result indicated a function in intracellular transport to the plasma membrane for myristylated proteins of the MPMV. As rotaviruses are not shed by budding, this function for myristylation is less likely than a scaffolding role. It can be expected that analysis of mutants and progress in the manipulation and expression of rotavirus genes will further elucidate the function of myristylation of rotavirus proteins.

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REFERENCES


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