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Reovirus Polypeptide σ3 and N-Terminal Myristoylation of Polypeptide μ1 Are Required for Site-Specific Cleavage to μ1C in Transfected Cells

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N-myristoylated viral polypeptide μ1 was produced in COS cells transfected with a transient expression vector containing a DNA copy of the reovirus M2 gene. The μ1 product was specifically cleaved to polypeptide μ1C in cells that were cotransfected with the reovirus S4 gene and that expressed polypeptide σ3. Studies with site-specific mutants of the M2 gene demonstrated that conversion of μ1 to μ1C was dependent on myristoylation and the presence of the proteolytic cleavage sequence asparagine 42-proline 43 in μ1, as well as on the presence of polypeptide σ3. The μ1C product and polypeptide σ3 formed complexes that were immunoprecipitated by σ3-directed antibody, and a myristoylation-negative M2 double mutant, G2A-N42T, yielded μ1 that did not undergo cleavage to μ1C or bind σ3. However, the N42T single mutant did form immunoprecipitable complexes with σ3, indicating that binding can occur in the absence of cleavage. Polypeptide σ3 alternatively can bind double-stranded RNA and in COS cells stimulates translation of reporter chloramphenicol acetyltransferase mRNA translation, presumably by blocking double-stranded RNA-mediated activation of the eukaryotic initiation factor 2 α subunit kinase which inhibits the initiation of protein synthesis. Consistent with these observations and with the formation of μ1C-σ3 complexes, coexpression of M2 with S4 DNA prevented the translational stimulatory effect of polypeptide σ3.

Cellular metabolism is usually redirected by animal viruses that cause either transforming or lytic infections (7). A notable example is the replacement of host mRNA translation by viral protein synthesis in mouse L cells infected with mammalian reoviruses (40). This serotype-dependent phenotype segregates with viral genome segment S4 (35) which codes for polypeptide σ3, a double-stranded RNA (dsRNA)-binding protein (15, 33). Thus, polypeptide σ3 is pleiotropic, i.e., it is a main component of the reovirus outer capsid, where it is present in 1-1 complexes with the M2 gene-encoded μ1 cleavage product, polypeptide μ1C (17), and it is also implicated in the switch from cellular to viral protein synthesis after infection.

A common defense mechanism used by animal cells against virus infection is based on the interferon-induced, dsRNA-activated inhibitory kinase (DAI) (14, 32). Its activation by dsRNA-mediated autophosphorylation leads to phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF-2α), resulting in a general block in translation initiation. This defense is counteracted in adenovirus-infected cells by the virus-encoded polIII product, VA1 RNA, which prevents DAI activation, sparing sufficient translational capacity to support late viral protein synthesis and completion of the replicative cycle (24). Other animal viruses have evolved similar but distinct mechanisms for escaping the cellular interferon response and DAI-mediated down regulation of translation. For example, vaccinia virus (30, 38) and influenza virus (22) both counter the inhibitory action of DAI but do so via proteins that are viral and cellular, respectively.

Transient expression of transfected genes in COS cells can also result in DAI activation and decreased translation of plasmid-derived mRNAs (1, 18). As in virus-infected cells, coexpression of adenovirus VA1 RNA prevents inhibition by blocking DAI-catalyzed phosphorylation of eIF-2α. Similarly, reovirus polypeptide σ3 expression increases chloramphenicol acetyltransferase (CAT) mRNA translation in S4/CAT DNA cotransfected COS cells, presumably by binding dsRNA activators of DAI (9). This translational enhancement by σ3, compared with decreased host protein synthesis in reovirus-infected cells, may seem paradoxical. However, in infected cells, σ3 associates with polypeptide μ1C to form complexes that apparently do not bind dsRNA (15). Consequently, the relative expression levels of the S4 and M2 genes and the ratio of free to complexed σ3 could explain the serotype-dependent, different extents of host translation alteration in reovirus-infected cells.

In addition to binding the viral μ1C protein and/or dsRNA, polypeptide σ3 contains a Zn finger motif and a sequence similar to that of the putative catalytic site of the picornavirus 2A and 3C proteases (8, 33), enzymes which are inhibited by Zn^{2+} (27). These are intriguing observations because polypeptide μ1 is specifically cleaved by an unidentified cellular or viral protease to form μ1C-σ3 complexes (17, 21, 29). It is also of some interest that polypeptide μ1, like poliovirus polyprotein (4), is cotranslationally modified by the N-terminal addition of the 14-carbon saturated fatty acid, myristic (*n*-tetradecanoic) acid (26). In an effort to determine whether myristoylation is functionally significant for μ1 cleavage and to assess any relationship between translational effects of σ3 and the formation of μ1C complexes, we have analyzed COS cells transfected with DNA copies of the S4 gene and wild-type versus mutant M2 genes. The results indicate that μ1 is proteolytically processed to μ1C in S4/M2 DNA cotransfected cells and that processing is dependent on both the presence of σ3 and N-terminal myristoylation, as well as the specific Asn-42-Pro-43 cleavage sequence in μ1.

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Furthermore, S4/M2 cotransfection results in σ3-μ1C complex formation and decreased σ3-mediated stimulation of CAT expression in COS cells.

MATERIALS AND METHODS

Cells and vectors. COS-7 and COS-1 cells were obtained from the American Type Culture Collection and grown as described previously (10). Expression vectors pt3S4 and pRSV-CAT contain the reovirus serotype 3 S4 gene or the bacterial CAT gene inserted into pBC12BI downstream of the Rous sarcoma virus long terminal repeat as described previously (9).

Subcloning of M2 DNA. The serotype 3 M2 gene cloned into the *Pst*I site of pBR322 (kindly provided by W. K. Joklik) was amplified with *Taq* polymerase (Cetus Corp.) by polymerase chain reaction (PCR), using two 30-residue oligomers constructed to add unique *Xho*I and *Eco*RV sites at the 5′ and 3′ ends, respectively. PCR incubations were for 1 min at 94°C, followed by 2 min at 55°C and 3 min at 72°C, repeated for 30 cycles. The PCR product was passed through a Qiagen column (Qiagen, Inc.), digested with *Xho*I and *Eco*RV, and ligated (T4 DNA ligase; New England Biolabs) to *Xho*I-*Eco*RV-digested, gel-purified DNA derived from pt1S4, a modified pBC12BI construct containing the reovirus type 1 S4 gene bordered by *Xho*I and *Eco*RV sites (34). The ligation mixture was used to transform *Escherichia coli* MC1061.

M2 mutagenesis. Site-directed mutations corresponding to amino acid positions 42 and/or 43, the site of μ1 cleavage to μ1C, were generated by PCR using Vent polymerase (New England Biolabs). The wild-type asparagine at position 42 (N; codon = AAT) was converted to threonine (T; codon = ACT), histidine (H; codon = CAT), glutamic acid (E; codon = GAA), or glutamine (Q; codon = CAA); the wild-type proline at position 43 (P; codon = CCC) was mutated to alanine (A; codon = GCC). For each mutant a pair of 24-nucleotide oligomers constructed to span the mutation site was used for PCR amplification (13). All mutations were confirmed by sequence analysis.

Transfections, radiolabeling, and CAT assays. COS-7 cells were transfected exactly as described previously (9). For COS-1 cultures, cells were plated at a concentration of 6.5×10^5 cells per 60-mm-diameter dish. After incubation for 16 to 18 h, plates were rinsed twice with prewarmed phosphate-buffered saline (PBS) and transfected by exposure overnight to 2.5 ml of Dulbecco modified Eagle medium (DMEM) containing 0.1 M Tris (pH 7.3), 5 mg DEAE-dextran, and 5 μg of the indicated DNA. The cells were then rinsed with PBS, and 2 ml of DMEM containing 10% dimethyl sulfoxide and 10% fetal calf serum (FCS) was added for 2 min followed by the addition of 5 ml of DMEM containing 10% FCS and 0.1 mM chloroquine. This solution was replaced 2.5 h later with DMEM containing 10% FCS, and cultures were incubated for 48 h at 37°C. Radiolabeling with [^{35}S]methionine and CAT assays were as described previously (9).

[^{3}H]myristate labeling. COS-1 cell cultures were radiolabeled 48 h after transfection by adding to each plate 2.2 ml of DMEM containing 5% FCS, 1% dimethyl sulfoxide, and 1 mCi of [^{3}H]myristic acid (New England Nuclear; specific activity = 22.4 Ci/mmol, lyophilized and reconstituted in 10 μl of ethanol). After 4 h at 37°C, cells were harvested and analyzed.

Immunoprecipitation analyses. Immunoprecipitation procedures were as described previously (6), using rabbit polyclonal antiserum raised against a lysate of reovirus type 3-infected MA104 cells or against gel-purified type 3 polypeptide σ3. Precipitates were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (7.5% polyacrylamide). For the ^{3}H-myristoylated samples, the 7.5% gel was soaked in water for 5 min followed by 30 min in Autofluor (National Diagnostics) before being dried and exposed to film for 1 week at −70°C between two intensifying screens.

Membrane-binding experiments. COS-1 cells, transfected and ^{35}S-labeled as described above, were harvested by the method of Hamaguchi and Hanafusa (12). Briefly, dishes were placed on ice and washed twice with PBS before adding 0.5 ml of CSK buffer {10 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.8), 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.3 M sucrose, 1 mM phenylmethylsulfonyl fluoride, 1% Trasylol, 1 mM Na_3VO_4, 10 μM Na_2MoO_4, and 1% Triton X-100}. Plates were rocked for 1 min, the supernatant was removed, and another 0.5 ml of CSK buffer used to re-treat the cells; the supernatants were then pooled. To the same dish was added 1 ml of radioimmunoprecipitation assay (RIPA) buffer, and the cultures were harvested. Both the CSK and RIPA extracts were clarified by centrifugation at 15,000 × *g* for 30 min at 4°C, and the CSK supernatant was adjusted to 1% deoxycholate–10 mM Tris (pH 8.0) before immunoprecipitation and gel analysis of the samples were done.

As an alternative to the CSK versus RIPA buffer solubility method, differential centrifugation was used to study membrane association. For these experiments, cells were harvested in 1 ml of 40 mM Tris (pH 7.4)–1 mM EDTA–0.15 M NaCl, transferred to a microcentrifuge tube, and spun for 2 min at 15,000 × *g*. The cell pellet was reconstituted in 1 ml 0.25 M Tris (pH 7.8) and subjected to three freeze-thaw cycles before being centrifuged for 1 h at 50,000 × *g*. The supernatant, S1, was carefully removed, and 1 ml of RIPA buffer was added to the membrane-containing pellet fraction which was then vortexed, incubated for 3 min at room temperature, and clarified by centrifugation at 15,000 × *g* for 10 min. Both the S1 and the clarified pellet fractions were assayed by immunoprecipitation and gel analysis.

RESULTS

Effect of M2 gene coexpression on S4 DNA-mediated stimulation of CAT synthesis. Reovirus structural polypeptide σ3, which can bind dsRNA or polypeptide μ1C (15, 17, 21, 33), may modulate translation in virus-infected cells by preventing dsRNA activation of the eIF-2α kinase (DAI) (9, 16). To determine whether M2 coexpression in COS cells would affect the stimulation of CAT mRNA translation by polypeptide σ3 (9), COS-7 cells were cotransfected with transient expression vector pBC12BI containing CAT or the S4 or M2 gene. Plasmids containing inverted S4 sequences were used as controls. The presence of polypeptide σ3 in S4-transfected cells stimulated CAT expression severalfold compared with that of cells containing either the M2 gene or S4 DNA in the inverted orientation (Fig. 1). Relative CAT levels in S4 DNA-cotransfected cells remained high in the presence of the inverted S4 DNA; however, when M2 DNA was present with the S4 gene the stimulatory effect by polypeptide σ3 was prevented (Fig. 1). This finding suggested that the products of the M2 gene might be forming complexes with σ3 in transfected cells and consequently eliminating enhancement of CAT expression, a possibility compatible with the hypothesis that σ3 in protein complexes

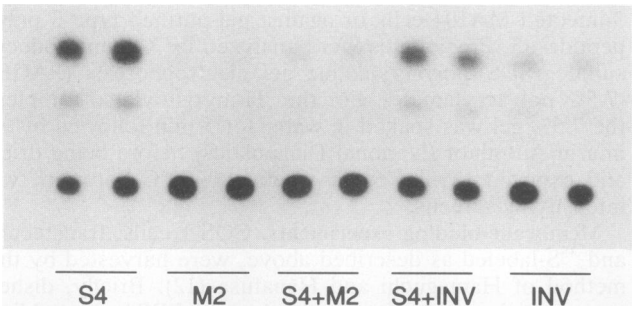

FIG. 1. CAT expression in COS cells cotransfected with reovirus genes. COS-7 cells were transfected with 0.2 μg of CAT expression vector DNA plus 2 μg of the same vector with S4, M2, or inverted S4 sequences in place of CAT (lanes S4, M2, and INV, respectively) or with 0.2 μg of CAT DNA plus 1 μg each of S4 plus M2 or S4 plus inverted S4 DNA (lanes S4+M2 and S4+INV, respectively). Extracts were prepared at 48 h after transfection and were assayed for CAT activity as described previously (9).

not binding dsRNA would not interfere with DAI-mediated down regulation of translational initiation.

Cleavage of μ1 to μ1C in M2 DNA-transfected COS cells coexpressing polypeptide σ3. To ensure that polypeptides σ3 and μ1 were produced from the respective S4 and M2 genes under these conditions, transfected COS cells were labeled with [^{35}S]methionine and extracts were analyzed by immunoprecipitation with reovirus-directed antibody followed by gel electrophoresis. Protein bands corresponding to σ3 and μ1 were evident in cells transfected with the S4 and M2 genes, respectively (Fig. 2, lanes 1 and 2). Cells transfected with both genes contained an additional immunoprecipitable polypeptide that migrated in the position of authentic μ1C, the μ1 cleavage product (Fig. 2, compare lane 8 with lane 7). In other experiments, the relative amount of cleaved product was greater, and the intensity of the μ1C band exceeded that

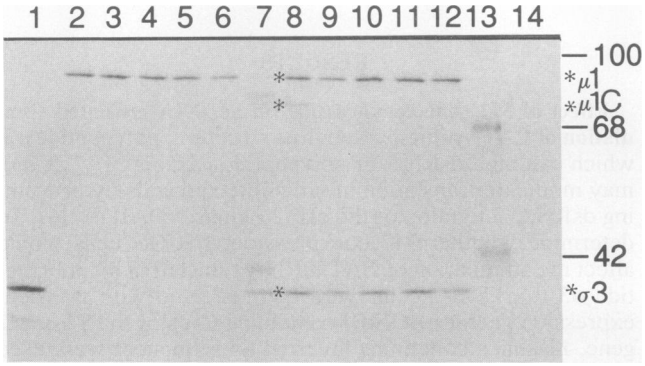

FIG. 2. Immunoprecipitation of reovirus proteins transiently expressed in COS cells. Extracts of transfected COS-1 cells were immunoprecipitated with rabbit antibody made against reovirus-infected cells and analyzed by PAGE. Each lane corresponds to 20% of the cell lysate from a 60-mm-diameter culture which had been transfected with 5 μg of S4 DNA (lane 1), M2 DNA (lane 2), or the following M2 mutant DNAs: N42T/P43A (lane 3), N42H/P43A (lane 4), P43A (lane 5), and N42H (lane 6). Lanes 8 to 12 contain extracts from cells that were transfected with 2.5 μg of S4 DNA plus 2.5 μg of the same M2 DNAs as described for lanes 2 to 6. Lane 7 contains [^{35}S]methionine-labeled proteins immunoprecipitated from reovirus-infected L cells. Lane 13, ^{14}C-labeled protein markers, with molecular sizes (in kilodaltons) on the right; lane 14, mock-transfected cells.

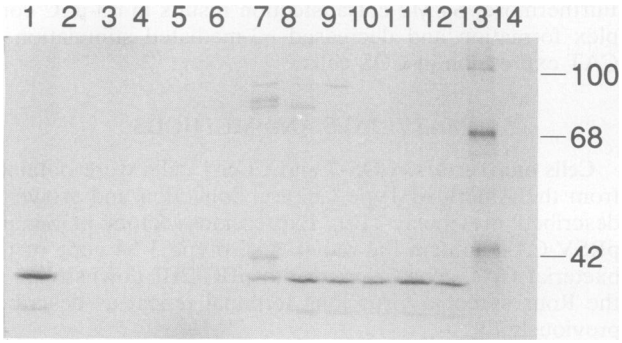

FIG. 3. Formation of protein complexes in COS cells. Aliquots of the same extracts used for Fig. 2 were immunoprecipitated with rabbit antibody directed against reovirus polypeptide σ3 and analyzed by PAGE. The lanes are the same as described in the legend to Fig. 2.

of μ1 (data not shown). A μ1C band was not present in samples of cells transfected with M2 DNA alone (Fig. 2, lane 2), suggesting that polypeptide σ3 is involved in the processing of μ1 to μ1C.

To investigate the origin of the putative μ1C polypeptide, M2 point mutations were generated at amino acids 42 and 43. Since cleavage of μ1 to μ1C occurs between Asn-42 and Pro-43 (17, 29), amino acid changes incompatible with μ1 cleavage would eliminate the putative μ1C from gel profiles. Initially, two double mutants were constructed: N42T/P43A and N42H/P43A. As shown in Fig. 2, the mutant viral proteins were made at levels similar to those of the wild-type μ1 polypeptide (compare lanes 3 and 4 with lane 2). However, in contrast to the wild type, neither of the double mutants yielded the μ1C band in cells cotransfected with the S4 gene (Fig. 2, compare lanes 9 and 10 with lane 8). To determine whether residues 42 and 43 are both recognized for μ1 cleavage, two single amino acid mutants, P43A and N42H, were also tested. As observed with the double mutants, μ1 was synthesized in cells containing mutant M2 and cotransfected with S4 DNA; however, the mutant μ1 polypeptides were not cleaved (Fig. 2, compare lanes 11 and 12 with lane 8). The results indicate that both amino acids in the sequence Asn-42-Pro-43 are important for proteolytic processing of μ1 to μ1C.

Complex formation of σ3 with μ1 and μ1C. The same extracts were incubated with a polypeptide σ3-specific antibody to determine whether σ3 formed complexes with the μ1 and μ1C products of the wild-type and mutant M2 genes in transfected COS cells. Extracts of cells transfected with S4 DNA alone yielded σ3 (Fig. 3, lane 1), and the antibody did not cross-react with the polypeptide μ1 present in M2 transfected cells (lane 2). However, cells transfected with both S4 and M2 DNA yielded polypeptide σ3 and immunoprecipitable M2 products, mainly μ1C, indicating formation of σ3 complexes (Fig. 3, lane 8). Consistent with complex formation, boiling prior to antibody treatment yielded σ3 but no M2-encoded polypeptides in the immunoprecipitates (data not shown). Among the several different M2 mutants, including N42Q (see Fig. 5A and B, lanes 6), only the μ1 polypeptides that contained threonine in place of asparagine 42 formed protein complexes that were sufficiently stable to be specifically immunoprecipitated with the σ3-directed antibody (Fig. 3, lane 9). However, as shown in Table 1, although wild-type M2 cotransfection blocked the stimulation of CAT expression by S4 DNA, neither of the N42

TABLE 1. Effect of reovirus genes on CAT expression[a]

Cotransfected genes	CAT activity (%)
S4	47 ± 2
M2	15 ± 1
N42T	19 ± 3
N42H	12 ± 1
S4 + M2	17 ± 1
S4 + N42T	31 ± 5
S4 + N42H	31 ± 4

[a] COS-7 cells were cotransfected in duplicate with 0.2 μg of CAT expression vector plus 2 μg of S4 or M2 or mutant M2 (N42T and N42H) DNA or with 1 μg each of S4 plus either M2 or mutant M2 DNA. Extracts were assayed 48 h later for CAT activity as described previously (9). Films were quantitated by densitometry, and values are expressed as percent conversion of [^{14}C]chloramphenicol to the acetylated forms.

mutants was as effective, suggesting that μ1C may be important for forming complexes that interfere with σ3 binding to dsRNA.

Myristoylation of μ1 and requirement for σ3 binding and cleavage to μ1C. The μ1 polypeptide of reovirus type 3 is N-terminally myristoylated (26), and it was of interest to determine what role, if any, this modification plays in σ3 complex formation and/or cleavage to μ1C. A myristoylation-minus M2 mutant DNA was generated by changing the glycine codon at position two to an alanine triplet. The corresponding mutation in the poliovirus VP0 polyprotein prevented both N-terminal myristoylation and proteolytic processing of the viral polyprotein (19). The reovirus G2A mutant polypeptide μ1 was tested by labeling mutant M2-transfected cells with [^{3}H]myristic acid. As shown in Fig. 4, the wild-type μ1 protein but not the G2A mutant was myristoylated. In addition, when the G2A M2 mutant DNA was cotransfected into COS-1 cells with S4 DNA, μ1 was synthesized but not cleaved to μ1C (Fig. 5A, lane 3). It should be noted that the mutant DNA retained the same cleavage sequence as that of the wild-type M2 product which was cleaved (Fig. 5A, lane 2).

Mutant μ1 polypeptide, containing changes at both positions 42 (N → T) and 43 (P → A), retained σ3 binding activity (Fig. 3, lane 9). Similarly, the N42T single mutant formed complexes that were precipitable with σ3 antibody (Fig. 5B, lane 4). However, like the double mutant, the N42T single mutant μ1 was not cleaved (Fig. 5A, lane 4). To determine the effect of superimposing the myristoylation-minus mutation on the properties of the N42T mutant, a G2A/N42T double mutant was tested in cells cotransfected with S4 DNA. Loss of the myristoylation site abolished μ1 binding to σ3 (Fig. 5B, lane 5). Table 2 is a summary of the M2 mutants.

Membrane association of polypeptide μ1. Many N-myristoylated viral proteins are membrane associated, consistent with a possible role in viral entry into cells and/or virion assembly (37). Analogous to the situation with other similarly modified viral structural proteins, Nibert et al. (26) have suggested that N-myristoylation of the reovirus μ1 polypeptide is essential for membrane interactions that facilitate entry of parental infectious particles into cells. N-myristoylation of p60^{src} is required for plasma membrane attachment and cell transformation (5), and it was reported that p60$^{c\text{-}src}$ and a nonmyristoylated mutant of p60$^{v\text{-}src}$, but not wild-type p60$^{v\text{-}src}$, could be solubilized by treating cells with nondenaturing CSK buffer containing the nonionic detergent Triton X-100 (12). To determine whether the same extraction procedure would indicate membrane association of reovirus polypeptide μ1 by differentiating between wild-type μ1 and the nonmyristoylated G2A mutant, M2 DNA-transfected COS cells were extracted with CSK buffer followed by the

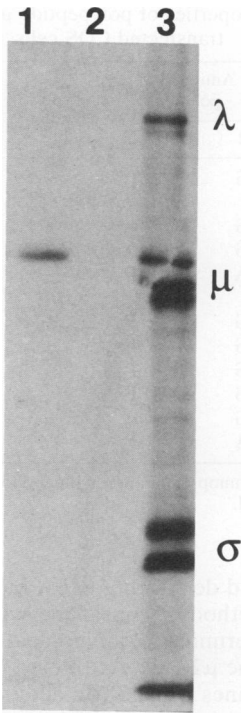

FIG. 4. N-myristoylation of polypeptide μ1 in M2 DNA-transfected COS cells. COS-1 cells were transfected with 5 μg of either M2 DNA (lane 1) or M2 G2A mutant DNA (lane 2) and labeled with [^{3}H]myristic acid as described in Materials and Methods. The entire lysate from a 60-mm-diameter dish was immunoprecipitated with antibody to reovirus-infected cells and analyzed by PAGE. Lane 3 contains [^{35}S]methionine-labeled proteins immunoprecipitated from extract of reovirus-infected L cells.

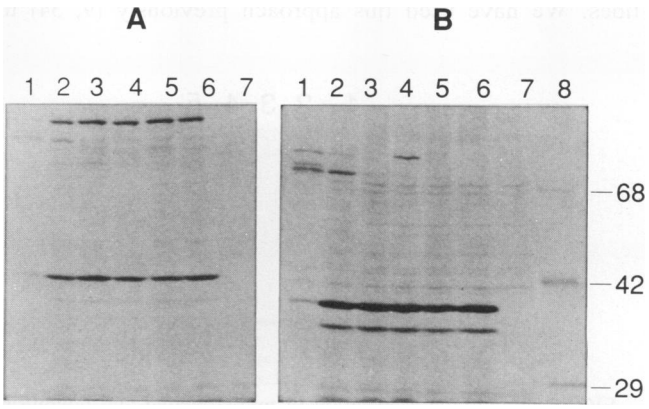

FIG. 5. Effect of myristoylation on μ1 cleavage to μ1C and on σ3 binding. Extracts of transfected cells, as described in the legend to Fig. 2, were immunoprecipitated with antibody against reovirus-infected cells (A) or against polypeptide σ3 (B). Cells were cotransfected with 2.5 μg each of S4 DNA and either M2 DNA (lane 2) or M2 mutants G2A (lane 3), N42T (lane 4), G2A/N42T (lane 5), and N42Q (lane 6). Lanes: 1, [^{35}S]methionine-labeled reovirus proteins; 7, mock-transfected cells; lane 8, ^{14}C-labeled protein markers.

TABLE 2. Properties of polypeptide μ1 in M2 DNA-transfected COS cells

M2 DNA	Amino acid position			σ3 binding[a]	Cleavage to μ1C	N-myristoylation
	2	42	43			
Wild type	G	N	P	+	+	+
Double mutants	G	T	A	+	−	ND[b]
	G	H	A	−	−	ND[b]
	A	T	P	−	−	ND[b]
Single mutants	G	T	P	+	−	ND[b]
	G	H	P	−	−	ND[b]
	G	Q	P	−	−	ND[b]
	G	E	P	−	−	ND[b]
	G	N	A	−	−	ND[b]
	A	N	P	−	−	−

[a] Determined by immunoprecipitation with antibody to polypeptide σ3.
[b] ND, not determined.

SDS-containing and denaturing RIPA buffer as described in Materials and Methods. Consistent with removal of the myristoylated N-terminal 42-amino-acid fragment from μ1, essentially all of the μ1C was extracted into the CSK buffer (Fig. 6, compare lanes 2 and 3). In addition, proportionately more of the nonmyristoylated G2A mutant μ1 polypeptide was soluble in the nondenaturing buffer compared with the wild-type myristoylated μ1 polypeptide (Fig. 6, compare lanes 4 and 5 with lanes 2 and 3). In four separate experiments, the extent of membrane association was at least 10% higher for the wild-type compared with that of the mutant μ1 protein, and similar results were obtained by differential centrifugation of cell extracts. These results suggest that polypeptide μ1 associates with cell membranes and that myristoylation enhances the association.

DISCUSSION

Transfection of individual viral genes or combinations of selected genes into mammalian cells provides an opportunity to analyze the metabolic effects of specific viral polypeptides. We have used this approach previously (9, 34) to investigate the role of the reovirus S4 gene product, polypeptide σ3, in the inhibition of host protein synthesis that occurs in virus-infected cells. Although the shutoff phenotype segregates with the S4 gene (35), transient expression of polypeptide σ3 in transfected COS cells resulted in a stimulation of CAT reporter mRNA translation. These paradoxical effects can be readily reconciled because σ3 can bind dsRNA and block activation of the inhibitory eIF-2α kinase (9, 16, 33), a property apparently abrogated by the formation of 1-1 complexes with the polypeptide μ1C products of the viral M2 gene (15, 17). Thus, the serotype-dependent inhibition in infected cells may reflect the level of polypeptide σ3 not in protein complexes and available to bind dsRNA activators of eIF-2α kinase. Consistent with this hypothesis, M2 gene cotransfection of S4-expressing COS cells yielded μ1C-σ3 complexes (Fig. 3) and prevented enhancement of CAT expression by polypeptide σ3 (Fig. 1). In addition, the S4 gene effectively substituted for the adenovirus VA1 gene in a COS cell expression system (1, 18, 24) that requires VA1 RNA to block eIF-2α kinase activation (22a). These and other findings (16) suggest that reoviruses are among the many different animal viruses that have evolved mechanisms for evading the cellular protective response to infection that is mediated by the interferon-induced, dsRNA-activated eIF-2α kinase (32).

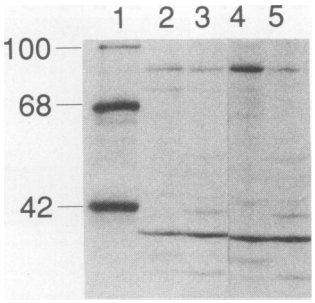

FIG. 6. Membrane association of myristoylated and nonmyristoylated μ1 polypeptides. COS-1 cells were cotransfected with 2.5 μg each of S4 and M2 DNA (lanes 2 and 3) or with 2.5 μg each of S4 and M2 G2A mutant DNA (lanes 4 and 5). Extracts were made by treatment of cells with CSK buffer (lanes 2 and 4) followed by treatment with RIPA buffer (lanes 3 and 5), and samples were immunoprecipitated with antibody to reovirus-infected cells and analyzed by PAGE, as described in Materials and Methods. Lane 1 contains ^{14}C-labeled protein markers.

A variety of eukaryotic proteins, including many viral products (4, 28, 36), are N-myristoylated by a cotranslational modification that is usually essential for biological function (11, 37). For example, assembly of infectious human immunodeficiency virus type 1 is dependent on N-myristoylation of the $Pr55^{gag}$ precursor which precedes membrane attachment and processing to virion capsid proteins (3). Similarly, N-myristoylation of poliovirus capsid protein VP4 is required for proteolytic processing and infectious virus formation (19, 23, 25). Recently it was shown that the reovirus μ1 polypeptide produced in virus-infected cells is myristoylated (26). Myristoylated μ1 was also synthesized in M2 DNA-transfected COS cells (Fig. 4). The reovirus polypeptide is probably myristoylated at the N terminus by a cellular N-myristoyl transferase since N-myristoylations all occur at a Gly residue adjacent to the initiator Met, and mutagenesis of the M2 sequence from N-Met-Gly to N-Met-Ala yielded nonmyristoylated μ1 (Fig. 4 and 5).

Like a number of other myristoylated viral capsid proteins, μ1 is processed by a specific proteolytic cleavage event—in the reovirus polypeptide, between Asn-42 and Pro-43—to produce μ1C (17, 29). The protease responsible has not been identified. However, it is intriguing to note that the major surface component of infectious reovirions consists of σ3-μ1C complexes (17) and processing of the precursor μ1 has previously been linked to binding of σ3 (21), a polypeptide that shares sequences with the conserved catalytic regions of the 2A and 3C proteases of several picornaviruses (8, 33). In transfected COS cells, cleavage of μ1 to μ1C requires the presence of polypeptide σ3 as well as an intact cleavage sequence which is conserved in the M2 genes of all three reovirus serotypes (17, 39). It is not clear whether cleavage is catalyzed by σ3 directly or whether σ3 binding exposes the cleavage site to proteolytic attack, either autocatalytically as in poliovirus VP0 conversion to VP4 and VP2 (2) or by a cellular protease. It is of interest to note that μ1 polypeptide contains a second Asn-Pro sequence, at amino acids 587 and 588 (17, 39), but it is not known whether this is the site that is cleaved in vivo to produce polypeptide δ (17).

In the case of poliovirus protein processing, protease 3C converts the viral polyprotein P1 precursor to VP0, VP3, and

VP1 by cleaving exclusively between glutamine and glycine residues (20). However, only 8 of the 13 Gln-Gly bonds in P1 are cut, presumably because they are accessible in the folded precursor. Conformation and processing of P1 clearly are influenced by N-myristoylation (19, 23), and the myristate group has been shown to increase the stability of multimeric intermediates in the assembly of infectious poliovirions (25). N-myristoylation of reovirus polypeptide μ1 may similarly influence its conformation and processing, because a myristoylation-negative mutant of μ1 expressed in COS cells did not bind σ3 and was not cleaved, although it retained the Asn-42-Pro-43 cleavage site (Fig. 4 and 5). Binding of σ3 was not sufficient to assure processing because the N42T mutant which bound σ3 was not cleaved (Fig. 5). Furthermore, σ3 binding of the N42T mutant was eliminated in the nonmyristoylated G2A/N42T double mutant (Fig. 5). From these results it appears that N-myristoylation of μ1 is important both for σ3 binding and for cleavage to produce stable σ3-μ1C complexes. It would be of interest to define the order of events leading to formation of these structural complexes and the regions of contact between the participating proteins. Coexpression of μ1 and mutant forms of polypeptide σ3, changed either within or outside the sequence resembling that of the poliovirus proteases, has not proven useful because complex formation and μ1 cleavage were both apparently abolished (36a). Analyses of additional mutants by RNA transfection (31) may help to elucidate the physiological consequences of polypeptide μ1 myristoylation in productively infected cells. By this approach it should be possible to test the importance of the hydrophobic myristate group for membrane attachment during virus entry into the cytoplasm via the endocytic pathway. Myristoylation of μ1 may also enhance cleavage and binding to σ3, directing it to outer capsid formation from dsRNA-related roles such as in cytopathogenesis and possibly genome assembly. Further studies should give new insights into the reovirus replicative process.

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