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Degradation of the Interferon-Induced 68,000-M_r Protein Kinase by Poliovirus Requires RNA

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Control of the interferon-induced double-stranded RNA (dsRNA) activated protein kinase (referred to as P68 because of its M_r of 68,000 in human cells) by animal viruses is essential to avoid decreases in protein synthetic rates during infection. We have previously demonstrated that poliovirus establishes a unique way of regulating the protein kinase, namely by inducing the specific degradation of P68 during infection (T. L. Black, B. Safer, A. Hovanessian, and M. G. Katze, J. Virol. 63:2244–2251, 1989). In the present study we investigated the mechanisms by which P68 degradation occurred. To do this we used an in vitro degradation assay which faithfully reproduced the in vivo events. Although viral gene expression was required for P68 degradation, the major poliovirus proteases, 2A and 3C, were found not to be directly involved with P68 proteolysis. However, the protease responsible for P68 degradation required divalent cations for maximal activity and probably has both an RNA and a protein component since trypsin and ribonuclease abrogated the activity. Despite this requirement for divalent cations and RNA, activation of the kinase was not required for proteolysis since a catalytically inactive P68 was still degraded. Mapping of P68 protease-sensitive sites by using in vitro translated truncation and deletion mutants revealed that sites required for degradation resided in the amino terminus and colocalized to dsRNA-binding domains. Finally, we found that preincubation of cell extracts with the synthetic dsRNA poly(I-C) largely prevented P68 proteolysis, providing additional evidence for the critical role of RNA. On the basis of these data, we present a hypothetical model depicting possible mechanisms of P68 degradation in poliovirus-infected cells.

Poliovirus adversely affects cellular gene expression by inhibiting DNA, RNA, and protein synthesis in virus-infected cells (4, 5, 13). The best-characterized effect on cellular metabolism is the selective host cell mRNA translational shutoff induced by poliovirus (18, 28, 30). The inhibition of cellular protein synthesis occurs primarily at the level of translation initiation and correlates with proteolysis of a 220,000-Da protein (P220 [30]), a component of eukaryotic initiation factor eIF-4F which facilitates the binding of capped mRNAs to the 43S initiation complexes (for recent reviews, see references 39, 61, and 83). As yet, however, the exact function of P220 remains unclear (83). Poliovirus does not require a functional P220 for viral mRNA translation since the mRNAs are uncapped and the untranslated region contains a ribosomal landing pad which allows for internal initiation of protein synthesis (80).

We previously have reported on the degradation of another cellular protein in poliovirus-infected cells (15): the double-stranded RNA (dsRNA)-activated protein kinase referred to as P68 because of its M_r of 68,000 in human cells (41, 62). Like P220, the P68 kinase plays a critical role in the regulation of translation. P68, also referred to as DAI, P1/eIF-2 kinase, eIF-2-PKds, dsI, and dsRNA-PK, is one of at least 30 genes induced by treatment with interferon (for reviews, see references 41, 56, 73, 74, and 77). P68 is a serine-threonine kinase activated by dsRNA in the presence of ATP and divalent cations (Mg^{2+} or Mn^{2+}) (32, 42). Upon activation the P68 kinase becomes autophosphorylated and phosphorylates its natural substrate, the alpha subunit of eIF-2 (41). Phosphorylation of the eIF-2 alpha subunit blocks the eIF-2B-mediated exchange of GDP in the inactive eIF-2–GDP complex with GTP, which is required for catalytic utilization of eIF-2 (38, 39, 50, 65, 72). These events lead to limitations in functional eIF-2, which is an essential component of protein synthesis and which is required to bind initiator $tRNA^{Met}$ to the 40S ribosomal subunit before mRNA is bound (39, 44, 61). Thus activation of the kinase triggers a series of events which culminate in an inhibition of protein synthesis initiation. Since virus-specific RNAs (12, 27, 57, 70, 78), including poliovirus dsRNAs (15, 19, 29, 69), have the capacity to activate P68, viruses must downregulate kinase action (45, 58); poliovirus accomplishes this by inducing kinase degradation (15). In addition to the P68 and P220 proteins, at least 10 other yet to be identified cellular proteins were shown by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis to be degraded in poliovirus-infected cells (84).

Proteolysis plays a central role not only in the regulation of cellular gene expression but also, even more so, in the control of viral gene expression in poliovirus-infected cells. Indeed, the major poliovirus proteases, 2A, 3C, and 3CD, are responsible for cleavage of the poliovirus polyprotein into both structural and enzymatic regulatory components (for reviews, see references 36, 52, and 64). The poliovirus protease 2A, which cleaves at Tyr-Gly amino acids, cotranslationally cleaves the precursor to release P1, the precursor to the structural polypeptides. Almost all the other processing events are caused by the major viral protease, 3C, or its precursor protease, 3CD, which cleave the polyprotein primarily at Gln-Gly sites, although other sites can also be recognized. As might be expected, the mechanisms of the viral polyprotein cleavages are better understood than the proteolytic mechanisms involved in the degradation of cellular proteins. P220 is probably cleaved by a cellular protease which is activated in an unknown fashion by the poliovirus protease 2A (37, 51, 86). It was recently demonstrated that protease 3C may be responsible for the inacti-

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vation of the transcription factor TFIIIC, but direct cleavage by 3C has not yet been demonstrated, and the action of 3C was not shown to be direct (23). In the work described in this report we have investigated the molecular mechanisms involved in the degradation of the P68 protein kinase in poliovirus-infected cells. Although we observed that neither protease 2A nor protease 3C was directly responsible for P68 proteolysis, we did find that degradation required both an RNA and protein component. Furthermore, mapping experiments revealed that the amino terminus contained the protease-sensitive sites which were essentially identical to previously described dsRNA-binding domains. A hypothetical model of P68 degradation by poliovirus, which may include an interplay between viral dsRNAs and a cellular protease, will be presented.

MATERIALS AND METHODS

Cells and virus. HeLa cells were grown in monolayer in Dulbecco's modified Eagle's medium containing 10% calf serum (CS). Suspension HeLa cells were grown in Joklik's modified minimal essential medium supplemented with 10% CS. Poliovirus type 1 (Mahoney strain), a generous gift from Nahum Sonenberg and Jerry Pelletier, was grown in suspension HeLa cells. For preparation of virus stocks, suspension HeLa cells were infected at a multiplicity of infection of 5 for 10 h in Joklik's modified minimal essential medium containing 2% CS. The infected cells were pelleted by centrifugation at 1,000 × *g* and resuspended in a minimum volume of Joklik's modified minimal essential medium containing 2% CS. The poliovirus titer was determined by plaque assay on HeLa cells as described previously (15).

In vitro assays for P68 degradation. Extracts were prepared from suspension HeLa cells which had been either mock or poliovirus infected for 5 h. Cells were washed with ice-cold Hanks' balanced salt solution and then disrupted in lysis buffer (10 mM Tris-HCl [pH 7.5], 50 mM KCl, 1 mM dithiothreitol, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 0.1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1% Triton X-100). As a source of radiolabeled P68, extracts were prepared from HeLa cells labeled with [^{35}S]methionine (500 μCi/ml) and treated with human lymphoblastoid alpha and beta interferon for 16 h. The [^{35}S]methionine-labeled extract was mixed with either the mock- or poliovirus-infected extract for 15 min at 30°C. After mixing, radiolabeled P68 was immunoprecipitated from the mixture by using P68 monoclonal antibody (MAb)-Sepharose and subjected to SDS-PAGE. For the analysis of the effects of the poliovirus proteases 2A, 3C, and 3CD on P68, [^{35}S]methionine-labeled, interferon-treated HeLa cell extracts were mixed with *Escherichia coli* extracts, incubated for 15 min at 30°C, and then subjected to immunoprecipitation with the P68 MAb followed by SDS-PAGE analysis.

For the in vitro degradation assays with in vitro-translated P68 and secreted embryonic alkaline phosphatase (SEAP) proteins, the cDNAs for P68 (see below) and SEAP were transcribed as described previously (49). The resulting RNA was quantitated, and equimolar amounts of RNA were used to program wheat germ translation extracts (Promega) in the presence of [^{35}S]methionine. Equimolar amounts of labeled protein were then mixed with mock- or poliovirus-infected extracts for 15 min at 30°C. The labeled protein was immunoprecipitated with the polyclonal P68 or SEAP antibody and analyzed by SDS-PAGE.

P68 protein kinase constructs. The cloning of plasmid pBS-8.4R containing the complete coding region for the P68 protein has been described previously (62). The P68 cDNA was subsequently cloned into pcDNAI/Neo (Invitrogen) by using the *Hin*dIII and *Pst*I sites of P68 (49). Full-length P68 was synthesized after linearization with *Eco*RV (see Fig. 6, lane P68-1). Truncated P68 proteins were synthesized in wheat germ extracts by using RNA generated by linearization of the cDNA with *Mae*I (P68-3), *Ban*I (P68-5), *Eae*I (P68-6), and *Eco*RI (P68-7). A catalytically inactive P68 kinase with a lysine-to-arginine change in domain II (P68-2) or a stop codon at position 296 (P68-4) was prepared as previously described (8, 49). Constructs with internal deletions at 156 to 243 (P68-11) or 91 to 243 (P68-12) within the P68 kinase amino terminal were also synthesized by using site-directed mutagenesis as previously described (49). For the construct lacking the entire amino terminus (P68-10), an *Nde*I restriction site was introduced at the *Ban*I site as described previously (7). A cDNA clone lacking amino acids 1 to 96 (P68-9) was obtained from Eliane Meurs (7). DNA sequence analysis to verify all mutations was performed by using the dideoxy-chain termination method (75). A cDNA clone with a 12-amino-acid deletion at positions 39 to 50 (P68-8) was obtained from Brian Williams (31) and subsequently cloned into the pcDNAI/Neo vector. The SEAP cDNA, obtained from Bryan Cullen (10), was subcloned into pcDNAI/Neo.

Antibody and immunoprecipitation analysis. Antiserum to P220 was a kind gift of Nahum Sonenberg, and SEAP antiserum was purchased from Dako Co. Monoclonal antibody to P68 was prepared as described previously (53). P68 polyclonal antibody was prepared by injecting rabbits with P68 purified from extracts obtained from *E. coli* BL21 (DE3)pLysS, which contains a chromosomal copy of phage T7 RNA polymerase under control of the *lac* promoter (8). To express P68 in this system, the P68 kinase cDNA was cloned into the pET T7 vector which contains a phage T7 promoter (8, 81). For immunoprecipitations, extracts were diluted in buffer I (20 mM Tris-HCl [pH 7.5], 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 20% glycerol, 1% Triton X-100). The extracts were reacted for 2 h at 4°C with P68 MAb-Sepharose, polyclonal antibody prepared against P68 (for experiments in Fig. 6), or polyclonal SEAP antiserum. For the immunoprecipitations with polyclonal antisera, protein A-Sepharose was added and the mixture was incubated for an additional 1 h at 4°C. The precipitates were then washed four times with buffer I and three times with buffer II (10 mM Tris-HCl [pH 7.5], 100 mM KCl, 0.1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 20% glycerol). The washed immunoprecipitates were boiled in 2× electrophoresis disruption buffer (49) and analyzed by SDS-PAGE.

Assay for activity of *E. coli*-expressed proteases 2A, 3C, 3CD, and 3D. *E. coli* extracts containing expressed poliovirus protease 2A was obtained from Liz Wyckoff and Ellie Ehrenfeld. *E. coli* was transformed with plasmids containing the protease 2A gene cloned in the correct and opposite orientations fused to a portion of the *E. coli trpE* gene (2, 86). The bacteria were pelleted and resuspended in buffer consisting of 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4), 10 mM KCl, 1.5 mM $MgCl_2$, and 0.5 mM dithiothreitol. The cells were lysed by sonication, and cell debris were removed by centrifugation (2, 86). For P220 degradation experiments, the *E. coli* extracts were mixed with HeLa cell extract supernatants centrifuged at 10,000 × *g* (S10 supernatants) and incubated for 1 h at 30°C. The mixture was separated by SDS-PAGE and analyzed by Western immunoblot analysis with the P220 antibody as

described below. Extracts were assayed for their ability to degrade P68 as described above.

E. coli extracts containing expressed Mahoney poliovirus proteins 3C, 3CD, and 3D were a kind gift from Steve Plotch (9). The proteins were expressed under the control of the phage T7 promoter in *E. coli* BL21(DE3)pLysS. This strain contained a chromosomal copy of T7 RNA polymerase under *lac* promoter control. The synthesis of the poliovirus proteins was therefore inducible by treatment with isopropyl-β-D-thiogalactopyranoside (IPTG). Approximately 500 ml of transformed *E. coli* was lysed in 10 ml of buffer consisting of 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, and 1 mM dithiothreitol (9). The soluble fraction was assayed for its ability to cleave P68, as described above, and also a natural substrate of 3C, the 2C3AB portion of the poliovirus polyprotein. The 2C3AB portion of the poliovirus polyprotein, which contains a 3C cleavage site, cloned under phage T7 promoter control was obtained from Steve Plotch (9). The DNA was linearized with *Eco*RI and used in an in vitro transcription reaction. The RNA was translated in wheat germ extracts, and the radiolabeled 2C3AB protein was mixed for 15 min at 30°C with increasing amounts of *E. coli* extract containing expressed 3C, 3CD, and 3D. The cleavage of 2C3AB was quantitated by analysis of the mixture by SDS-PAGE.

Partial purification of P68 proteolytic activity from poliovirus-infected cell extracts. HeLa suspension cells (2×10^9) were either mock or poliovirus infected for 5 h at a multiplicity of infection of 20 PFU per cell. After infection, cells were lysed in 10 ml of lysis buffer for 10 min on ice. Nuclei and membranes were removed by centrifugation at 4,000 × *g* for 10 min. Degradation activity was followed by assaying fractions in the in vitro assay for P68 degradation as described above. Pooled mock- or poliovirus-infected extracts were ammonium sulfate fractionated sequentially at 20, 40, 60, and 80% saturation. The pellets were resuspended and dialyzed against lysis buffer containing 5% glycerol. The active fraction was subjected to centrifugation for 5 min at 10,000 × *g*; the resulting pellet was resuspended in lysis buffer containing 8 M urea in place of the Triton X-100 detergent. The sample was centrifuged at 10,000 × *g* for 5 min, and the supernatant was assayed for activity with and without dialysis to remove the urea. After dialysis of the supernatant to remove the urea, the activity was pelleted and the pellet was again suspended in lysis buffer containing 8 M urea.

Western blot analysis. Unlabeled protein extracts prepared at 5 h postinfection as described above were separated by SDS-PAGE and subsequently transferred to nitrocellulose in Towbin's buffer (25 mM Tris-HCl [pH 8.3], 0.192 M glycine, 20% methanol) containing 0.1% SDS. After transfer, the blot was blocked for 1 h with rocking in phosphate-buffered saline (PBS) containing 5% nonfat dry milk. The blot was washed twice in PBS and then incubated for 1 h in PBS containing 25% fetal calf serum, 0.5% Triton X-100, and a 1:2,000 dilution of P68 MAb or P220 polyclonal antibody. After the primary antibody treatment, the blot was washed three times in PBS with 0.5% Triton X-100. The blot was then incubated for 1 h in PBS containing 25% fetal calf serum, 0.5% Triton X-100, and a 1:3,000 dilution of Amersham goat anti-mouse or anti-rabbit antiserum coupled to horseradish peroxidase as the second antibody and washed three times in PBS with 0.5% Triton X-100. Bands were visualized by treating the blot for 1 min in Amersham enhanced chemiluminescence reagents and exposing the blot to film or by incubation with ^{125}I-protein A.

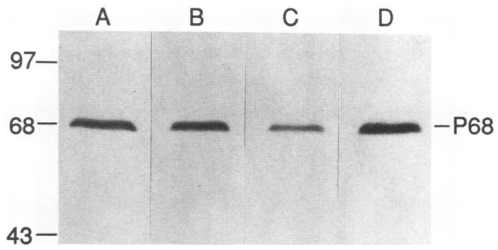

FIG. 1. Degradation of the P68 kinase during poliovirus infection requires viral gene expression. Unlabeled extracts prepared at 3.5 h after mock infection in the absence (lane A) or presence (lane B) of 1.5 mM guanidine HCl and 3.5 h after poliovirus infection in the absence (lane C) or presence (lane D) of 1.5 mM guanidine HCl were electrophoresed on an SDS–10% polyacrylamide gel. P68 protein levels were then determined by Western blot analysis with the P68 MAb and the Amersham enhanced chemiluminescence technique.

RESULTS

Poliovirus gene expression is required for P68 protein kinase degradation in poliovirus-infected cells. Both the P68 protein kinase and the P220 component of the cap-binding protein complex are translationally important proteins which are specifically degraded during poliovirus infection. It was demonstrated that, in cells infected with poliovirus in the presence of guanidine hydrochloride (an inhibitor of poliovirus replication), cleavage of P220 still occurred (18). To determine whether viral replication and gene expression were required for P68 degradation, we examined the levels of the protein kinase in guanidine-treated poliovirus-infected cells. We first looked at the effects of guanidine on the rate of cellular and viral protein synthesis in mock- and virus-infected cells and found that there was little effect on the protein pattern in mock-infected cells but that guanidine treatment of poliovirus-infected cells caused a marked decrease in virus-specific protein synthesis along with an increase in the cellular protein synthesis (14). Unlike the P220 protein, the P68 protein kinase was not degraded in cells infected with poliovirus in the presence of guanidine as determined by Western blot analysis (Fig. 1; compare lanes C and D). Pulse-chase experiments confirmed that the decreases in kinase levels observed in lane C were due to protein degradation (14). These data revealed that viral gene expression was required for P68 degradation and that the mechanisms regulating the cleavage of P68 and P220 are probably distinct.

Poliovirus-encoded proteases 2A, 3C, and 3CD are not directly responsible for the degradation of the P68 protein kinase. Since viral gene expression was required for P68 proteolysis, we examined whether poliovirus protease 2A, earlier shown to be involved in P220 degradation (51, 86), was involved. To directly answer this question, we tested the ability of protease 2A expressed in *E. coli* (2, 86) to degrade P68 in vitro. We confirmed that the extracts contained active protease 2A by assaying for P220 cleavage (Fig. 2A). Extracts from *E. coli* transformed with the gene for protease 2A in the correct (lane B) and reverse (lane C) orientations were mixed with HeLa cell S10 extracts. Western blot analysis revealed that only the *E. coli* extracts containing protease 2A in the correct orientation were able to efficiently cleave P220. The *E. coli* extracts were then

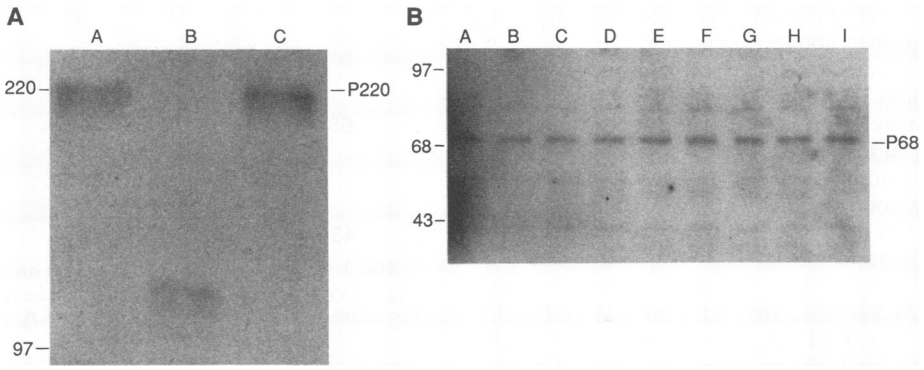

FIG. 2. *E. coli*-expressed poliovirus protease 2A does not degrade P68 in vitro. (A) HeLa cell S10 extracts (4 μl) were mixed with buffer alone (lane A) and 2 μl of *E. coli* extract containing the protease 2A gene in the correct orientation 2AR (lane B) and in the reverse orientation 2AW (lane C). The mixture was incubated for 1 h at 30°C and then electrophoresed on an SDS–6.5% polyacrylamide gel. The P220 protein was detected by Western blot analysis with rabbit antibody to P220 and ^{125}I-protein A. The positions of molecular weight standards (in thousands) are on the left. (B) [^{35}S]methionine-labeled, interferon-treated HeLa cell extracts were mixed with 2 μl of buffer alone (lane A), 2 μl of 2AR (lane B), 2 μl of 2AW (lane C), 5 μl of buffer alone (lane D), 5 μl of 2AR (lane E), 5 μl of 2AW (F), 8 μl of buffer alone (lane G), 8 μl of 2AR (lane H), or 8 μl of 2AW (lane I) for 60 min at 30°C. P68 was immunoprecipitated from the mixture with the MAb to P68 and analyzed on an SDS–10% polyacrylamide gel.

mixed with [^{35}S]methionine-labeled HeLa cell extracts, and kinase levels were analyzed by immunoprecipitation as described in Materials and Methods (Fig. 2B). There was no degradation of the P68 kinase by the 2A-containing extracts at any concentration used. In confirmation of these results, we determined that P68 degradation efficiently occurred in cells infected with a poliovirus mutant (11) defective in the protease 2A and P220 degradation (14). We next examined the possible involvement of the other major poliovirus protease, 3C (63), in the degradation of P68 during poliovirus infection. We also assayed the precursor protease 3CD for P68 proteolysis since it has been reported to have a cleavage specificity different from that of protease 3C (16). These assays were performed as described in detail in Materials and Methods. Neither *E. coli* extracts containing 3C nor those containing 3CD caused a decrease in the P68 protein concentration even at 50-fold the amount required to cleave their natural substrate (14). These data, taken together, strongly suggest that neither protease 2A, 3C, nor 3CD was directly responsible for P68 degradation during poliovirus infection.

Proteolysis of the P68 protein kinase requires divalent cations and an RNA and protein component. To identify the protease responsible for P68 degradation, which, from our observations described above, was likely to be cellular in origin, we attempted biochemical purification by using our in vitro assay. Initially, extracts were subjected to sequential ammonium sulfate fractionation at 20, 40, 60, and 80% saturation. The 20% ammonium sulfate fraction from poliovirus-infected cell extracts contained the majority of the P68 proteolytic activity; importantly, P68 degradation activity was not present in any of the ammonium sulfate fractions from mock-infected cell extracts, again showing the specific nature of the degradation of P68 by poliovirus-infected cell extracts (Fig. 3). Further attempts at purification, however, were hindered by the extremely low solubility of the protease, which was solubilized only by treatment with 8 M urea (14). In addition, we were unsuccessful at recovering protease activity from either fast protein liquid chromatography (FPLC) Mono Q or Mono S columns because of the insolubility and possible multicomponent nature of the protease (see below). As an alternative approach to characterizing the P68 protease, we determined whether well-defined protease inhibitors prevented P68 degradation in vitro. Inhibitors of serine, cysteine, metallo- and aspartic acid type proteases were tested, but only EDTA significantly and reproducibly diminished degradation of the P68 kinase. This inhibition was reversed by addition of $MnCl_2$ or $MgCl_2$ but not LiCl (Fig. 4A). Treatment of the extracts with ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) had no effect on the degradation, indicating that Ca^{2+} was not essential for degradation (data not shown). To further determine the nature of the protease responsible for P68 degradation, we tested the sensitivity of extracts to pretreatment with heat, trypsin, and ribo- or deoxyribonucleases. Poliovirus-infected cell extracts pretreated with trypsin, RNase A, or RNase III were no longer able to degrade P68 (Fig. 4B). Importantly, heating these virus-infected extracts (at 30, 37, or 65°C) without protease or nuclease present was not sufficient to abolish activity (Fig. 4B). In contrast to the results obtained with RNase A and RNase III, pretreatment with RNase H or DNase I had little effect on degradation of P68 (Fig. 4C). The ability of RNase A and RNase III to

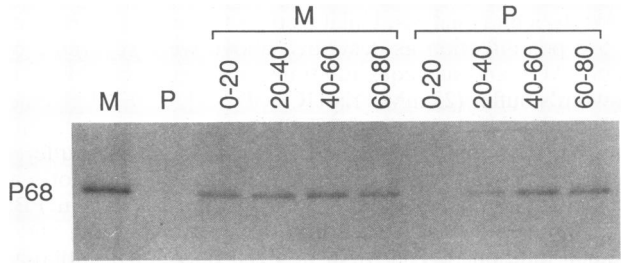

FIG. 3. Ammonium sulfate fractionation of P68 proteolytic activity from poliovirus-infected cell extracts. Mock-infected (M) and poliovirus-infected (P) extracts were assayed for P68 degradation activity as crude extracts (left two lanes) or after fractionation into the 0 to 20%, 20 to 40%, 40 to 60%, or 60 to 80% ammonium sulfate fractions. These fractions (approximately 1 mg per reaction) were incubated with [^{35}S]methionine-labeled extracts, and the mixture was subjected to immunoprecipitation with the P68 MAb followed by SDS-PAGE (10% polyacrylamide) and autoradiography.

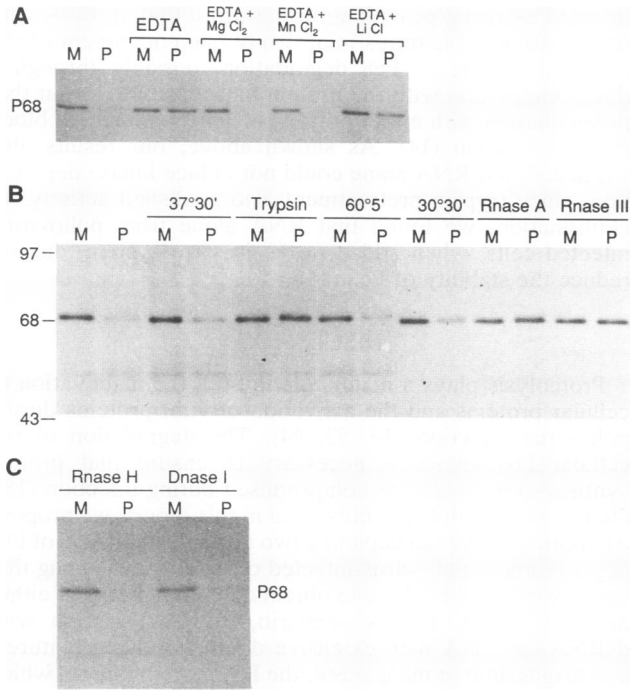

FIG. 4. Degradation of P68 requires divalent cations and both an RNA and protein component. (A) The in vitro degradation assay was carried out by incubating [^{35}S]methionine-labeled extracts with mock-infected (M) and poliovirus-infected (P) cell extracts in the presence of 4 mM EDTA alone or 4 mM EDTA with either 6 mM $MgCl_2$, 6 mM $MnCl_2$, or 6 mM LiCl. P68 levels were analyzed by immunoprecipitation followed by SDS-PAGE and autoradiography. (B) Mock-infected (M) and poliovirus-infected (P) extracts were pretreated with 25 μg of trypsin (30 min at 37°C; followed by addition of 50 μg of soybean trypsin inhibitor), 10 μg of RNase A incubated at 300 mM KCl at 30°C for 30 min, 1 μl of RNase III (300 μg/ml; generously supplied by Donald Court, National Cancer Institute Frederick Cancer Research Facility) incubated at 130 mM NH_4Cl at 30°C for 30 min, or heating alone at the stated temperatures and times. The extracts were subsequently assayed for activity in the P68 degradation assay by being mixed with radiolabeled HeLa cell extracts followed by immunoprecipitation analysis. Molecular weight markers (in thousands) are indicated on the left of the panel. (C) Extracts were pretreated with either 2 μg of DNase I (30 min at 30°C) or 2 U of RNase H (20 min at 37°C) and assayed as described above.

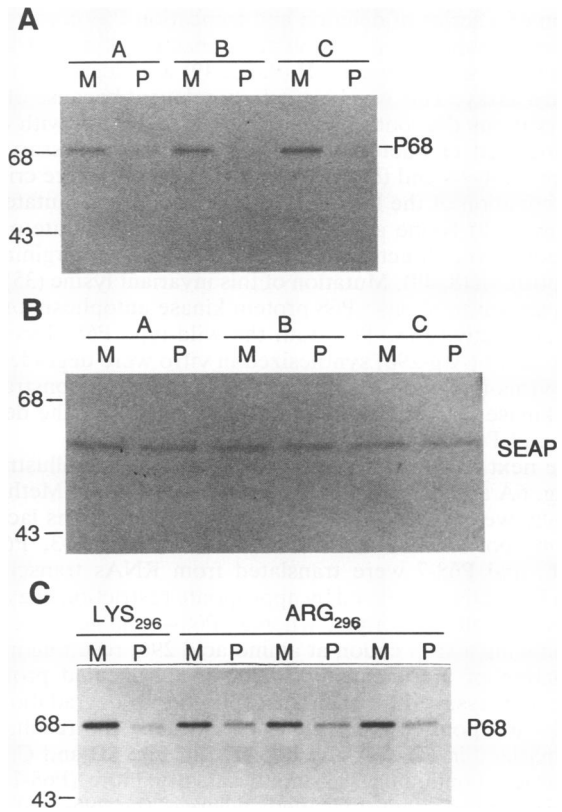

FIG. 5. Degradation of in vitro-translated wild-type and mutant P68 kinases by poliovirus-infected cell extracts. (A) [^{35}S]methionine-labeled P68 protein synthesized in wheat germ translation extracts was mixed with 25 μl (lanes A), 50 μl (lanes B), or 100 μl (lanes C) of mock-infected (M) or poliovirus-infected (P) extracts (80 mg/ml) for 15 min at 30°C. P68 was immunoprecipitated from the mixture with the P68 MAb and analyzed on an SDS–10% polyacrylamide gel. (B) [^{35}S]methionine-labeled SEAP protein synthesized in wheat germ translation extracts was mixed with mock- and poliovirus-infected extracts as described in panel A. The labeled SEAP protein was immunoprecipitated from the mixture with rabbit antibody to SEAP. (C) In vitro P68 degradation assay performed on in vitro-translated wild-type kinase, Lys-296 (P68-1; Fig. 6), mixed with 50 μl of mock-infected (M) or poliovirus-infected (P) extracts or a catalytically inactive kinase, Arg-296 (P68-2; Fig. 6), mixed with 25, 50, or 100 μl (from left to right) of mock-infected (M) or poliovirus-infected (P) cell extracts. P68 was immunoprecipitated with P68 MAb and analyzed on an SDS–10% polyacrylamide gel. The molecular weight markers (in thousands) are indicated on the left of each panel.

inhibit proteolysis strongly suggested that an RNA component with both single-stranded and double-stranded characteristics was required for P68 degradation since RNase III had a dsRNA cleavage specificity (68, 70), whereas RNase A cleavage should have been single-stranded RNA specific under the high-salt conditions tested (3). The lack of inhibition by DNase I and RNase H demonstrated that DNA or DNA-RNA hybrids were not involved in P68 degradation. To summarize, efficient P68 degradation by poliovirus required both a heat-stable protein and RNA component together with divalent cations for maximal activity.

The amino terminus contains the protease-sensitive sites of the P68 protein kinase. To gain further insights into the molecular mechanisms of P68 degradation in poliovirus-infected cells, we determined whether activation of the kinase was required for degradation and, further, which regions of the P68 kinase were important. To accomplish this, it was necessary to test whether P68, synthesized in an in vitro transcription-translation system, was efficiently degraded in the in vitro assay. The cDNA for the P68 kinase was transcribed in vitro with phage T7 RNA polymerase, and the resulting RNA was translated in wheat germ extracts as described in Materials and Methods and by Katze et al. (49). Degradation of the [^{35}S]methionine-labeled in vitro-translated P68 protein kinase by poliovirus-infected extracts (Fig. 5A) was as efficient as the degradation of in vivo-labeled P68 (Fig. 3). As control, SEAP protein was expressed in vitro and mixed with increasing amounts of mock- and poliovirus-infected cell extracts (Fig. 5B). SEAP was not degraded by either the mock- or virus-infected cell extracts, providing further confirmation of the specificity of P68 degradation by poliovirus. Before analyzing the degra-

dation of a series of deletion and truncation P68 constructs, we tested whether a catalytically inactive kinase with a mutation in kinase domain II (8, 35, 49) was degraded in the in vitro assay. This was particularly relevant because of our observations that both divalent cations and RNA with double-stranded characteristics appeared to be necessary for P68 proteolysis and that these components also were critical for activation of the kinase. The P68 kinase was mutated at the invariant lysine at amino acid 296 by degenerate oligonucleotide site-directed mutagenesis, creating an arginine at this position (8, 49). Mutation of this invariant lysine (35) has been shown to abolish P68 protein kinase autophosphorylation and activity (8, 49). Both the wild-type P68 (Lys-296) and mutant (Arg-296) synthesized in vitro were degraded by poliovirus-infected cell extracts (Fig. 5C), demonstrating that kinase activation was not a prerequisite for the degradation of P68 by poliovirus.

We next analyzed the series of P68 constructs illustrated in Fig. 6A and described in detail in Materials and Methods. Initially we expressed a series of truncated proteins lacking various portions of the carboxyl terminus. P68-3, P68-5, P68-6, and P68-7 were translated from RNAs transcribed from P68 cDNAs cleaved by appropriate restriction enzymes to give runoff transcripts, whereas P68-4 was generated by introducing a stop codon at amino acid 296, resulting in the formation of a truncated protein. The truncated proteins were then assayed for their susceptibility to degradation by mock- and poliovirus-infected cell extracts; the results are summarized in Fig. 6A and depicted in Fig. 6B and C. P68 constructs containing 385 amino acids or more (P68-1 and P68-3) were efficiently cleaved, whereas the 296- and 243-amino-acid constructs (P68-4 and P68-5; Fig. 6C) were less efficiently degraded and those containing either 155 or 90 amino acids (P68-6 and P68-7) were no longer degradable (Fig. 6B). To further define the critical regulatory sequences within the amino half of the P68 protein kinase, we analyzed a set of amino-terminal deletions, P68-8 through P68-12 (Fig. 6A and D). As predicted from the truncation data, the construct lacking nearly the entire amino half of the protein kinase (P68-10), as well as P68-11 and P68-12, was not efficiently degraded. Somewhat unexpectedly, we found that the 12-amino-acid deletion at residues 39 to 50 (P68-8) and a deletion of the first 96 amino acids (P68-9) also eliminated degradation. We conclude that although the carboxyl terminus is dispensable for degradation, multiple domains within the amino terminus may be critical for activity. It is relevant that studies by our group (6–8, 49) and others (20, 31, 59, 66) have revealed that the amino terminus also contains the sites critical for dsRNA binding (see Discussion). Indeed, in our in vitro dsRNA-binding assays, we have found that constructs P68-1 through P68-4 efficiently bound the activator reovirus dsRNAs, P68-5 bound dsRNA but with somewhat reduced efficiency, but P68-6 through P68-12 did not bind RNA (6–8, 49). Thus the regions critical for dsRNA binding and proteolysis appear to colocalize to the amino terminus.

Preincubation with dsRNA can block the poliovirus-mediated degradation of the P68 protein kinase. Both our RNase data and the protease site-mapping data have implicated RNA as playing a key role in the proteolysis of the P68 protein kinase. We were able to provide additional evidence for the involvement of RNA by pretreating [^{35}S]methionine-labeled extracts (containing the P68 kinase) with 1 or 10 μg of the synthetic dsRNA poly(I-C) per ml before mixing it with mock- or poliovirus-infected extracts (Fig. 7). Preincubation with poly(I-C) significantly interfered, in a dose-dependent manner, with the P68 degradation by virus-infected extracts, providing further proof that an RNA with double-stranded features is probably one component of the pathway leading to P68 degradation, possibly through a direct interaction with the protein kinase. It is relevant that preincubation with either poly(A) or poly(U) failed to block P68 degradation (14). As shown above, our results also suggested that RNA alone could not induce kinase degradation, since trypsin pretreatment also abolished activity. In confirmation, we found that RNA alone from poliovirus-infected cells, when added in the in vitro system, did not reduce the stability of P68 (14).

DISCUSSION

Proteolysis plays a major role in both the inactivation of cellular proteins and the activation of viral proteins during poliovirus infection (36, 52, 64). The degradation of the cellular P68 kinase is necessary to ensure that protein synthesis levels are not compromised during infection (15). On the basis of the data presented in this report, we propose a hypothetical model depicting two possible pathways of P68 degradation in poliovirus-infected cells, acknowledging that more work must be done to obtain definitive proof for either model (Fig. 8). In one scenario, upon interaction with dsRNA (or RNA with extensive double-stranded features) and magnesium or manganese, the P68 protein kinase, which is composed of an amino terminus containing both the protease-sensitive sites and the dsRNA-binding domains and a carboxyl terminus comprising catalytic domains, undergoes a conformational change. This alteration in structure then enables a cellular protease to degrade the kinase. We have never observed discrete P68 cleavage products, as are found for P220 (30), suggesting that after an initial cleavage the kinase is completely destabilized. In an alternative model, the dsRNA and protease act in concert in the presence of divalent cations, possibly as a ribonucleoprotein complex, to proteolyze the kinase directly and completely. Both these models suggest that the specificity of the reaction or the targeting of P68 results from the kinase interaction with dsRNA or RNA with extensive double-stranded regions. Although our RNase A inactivation data show that the RNA may indeed have single-stranded regions, the RNase III, protease mapping (Fig. 6), and dsRNA inhibition (Fig. 7) experiments support the concept that the RNA involved in proteolysis has extensive double-stranded features. It is again important to mention that neither poliovirus dsRNA nor the synthetic poly(I-C) dsRNA, when added to uninfected extracts, induces P68 degradation (14), and the trypsin inactivation data revealed that a protein component, probably the protease, is also required for proteolysis (Fig. 4). We conclude, therefore, that poliovirus infection must result in the activation of a latent heat-stable cellular protease, which then either acts together with dsRNA or attacks the kinase after dsRNA has bound to P68.

Clearly, additional questions remain to be answered. For example, what is the nature of the RNA participating in the cleavage of P68? A possible candidate is the virus-specific RNA formed in poliovirus-infected cells (5, 19, 29), which, as we have previously demonstrated, can efficiently activate the protein kinase, presumably owing to its extensive double-stranded structure (15). Although it is true that the activation step is not required for P68 degradation (Fig. 6), we have shown that poliovirus RNA replication is necessary for kinase degradation (Fig. 1). At present, however, we cannot rule out the possibility that cellular RNAs with extensive double-stranded regions also may be components

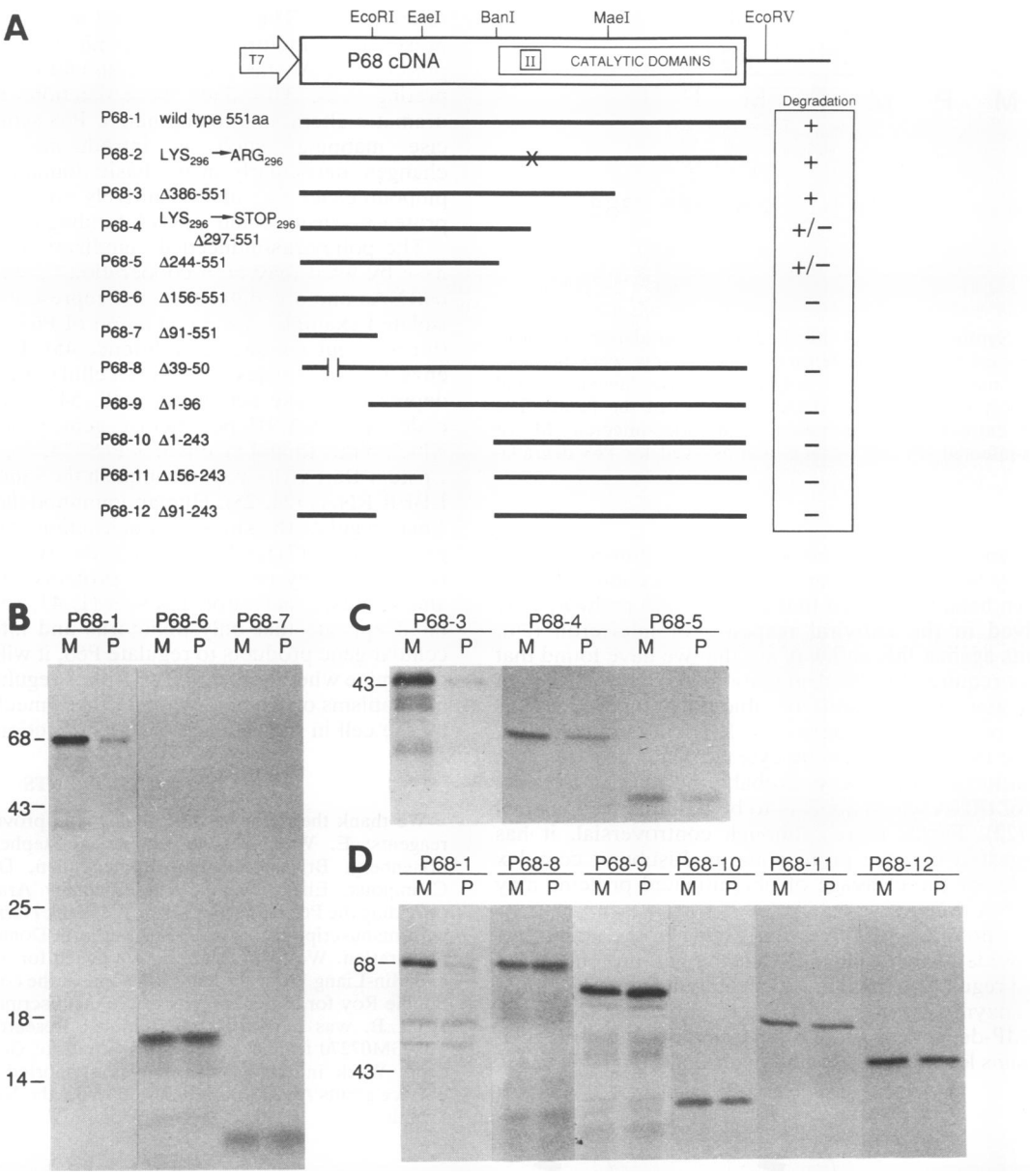

FIG. 6. Mapping the protease-sensitive sites of the P68 protein kinase. (A) The P68 cDNA is illustrated with selected restriction sites used to produce runoff RNAs and truncated kinase proteins. The P68-1 through P68-12 constructs are described in detail in Materials and Methods. The amino acid residues deleted in each protein are indicated. Degradation was quantitated by laser densitometry. Symbols: +, >90% degraded; −, <10% degraded; ±, approximately 50% degraded. (B to D) Each construct described in panel A was translated in vitro in the presence of [^{35}S]methionine, mixed with either mock-infected (M) or poliovirus-infected (P) extracts, and then immunoprecipitated with the P68 polyclonal antiserum which recognized all the in vitro translated proteins. Molecular weight markers (in thousands) are indicated on the left.

of the degradation pathway. As yet we do not understand the role played by the divalent cations. Since we have established that these divalent cations are not necessary for binding of dsRNA to P68 (33), it is likely that they are required for protease activity itself, as has been found for metalloproteases whose action also is inhibited by EDTA (17). Finally, the identity of the protease remains elusive. It is probably cellular in origin, since we have taken great efforts to eliminate a role for the major poliovirus-encoded proteases, 2A, 3C, and 3CD (Fig. 2), although their action on P68 in an indirect manner cannot be absolutely ruled out. Attempts to biochemically purify the P68 protease by conventional methods have thus far been unsuccessful for two reasons. First, the protease was extremely insoluble, requiring 8 M urea to liberate activity. Second, the multicomponent nature (consisting of both a protein and an RNA component) of the protease has made recovery of activity from FPLC anion- or cation-exchange columns difficult to achieve. One attractive hypothesis is that the widely studied ubiquitin pathway (21, 22, 40, 67) is responsible for P68 degradation, particularly since this pathway has been recently shown to be responsible for proteolysis of a cellular

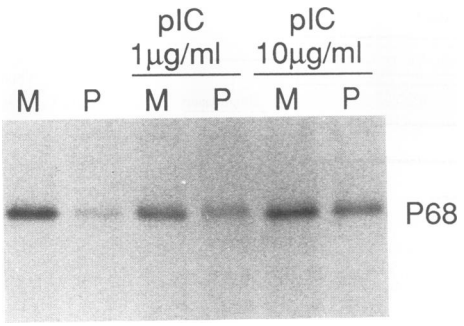

FIG. 7. Synthetic dsRNA blocked P68 degradation in vitro. [^{35}S]methionine-labeled, interferon-treated extracts were preincubated for 10 min at 30°C with no poly(I-C) (first two lanes), or 1 and 10 μg of poly(I-C) per ml as depicted on top of the figure. The pretreated extract was then mixed with mock-infected (M) or poliovirus-infected (P) cell extracts and assayed for P68 degradation.

protein in another viral system and since components of this system may be altered during some viral infections (26, 76). It has even been speculated that the ubiquitin pathway may be involved in the antiviral response of interferon (26). Arguments against this pathway are that we have found that ATP is not required for P68 degradation and that we have not observed any mobility shift or ubiquitination of the P68 protein in our in vitro system (14). It is curious, however, that RNase treatment of reticulocyte extracts interferes with the ubiquitinization pathway, probably because of the degradation of tRNA which appears to be required for maximal activity (22). Furthermore, although controversial, it has been suggested that the proteosome multisubunit complex responsible for the cleavage of ubiquitinated proteins may contain RNA (82).

That the protease-sensitive sites reside in the amino terminus provide further evidence that the P68 protein kinase has both a regulatory domain and a catalytic domain responsible for enzymatic function. Both protein kinase C and the cyclic GMP-dependent protein kinases also contain regulatory domains located at their amino termini (for a review, see reference 79). The data presented in this report suggest the presence of multiple amino-terminal domains critical for proteolysis. At this point, one should be cautious in interpreting these data since these deletions may give rise to dramatic alterations to the native P68 structure; more precise mapping, such as introducing single-amino-acid changes, particularly in the basic domains, is necessary to pinpoint exact sites and definitively prove the identity of the protease-sensitive and dsRNA-binding domains.

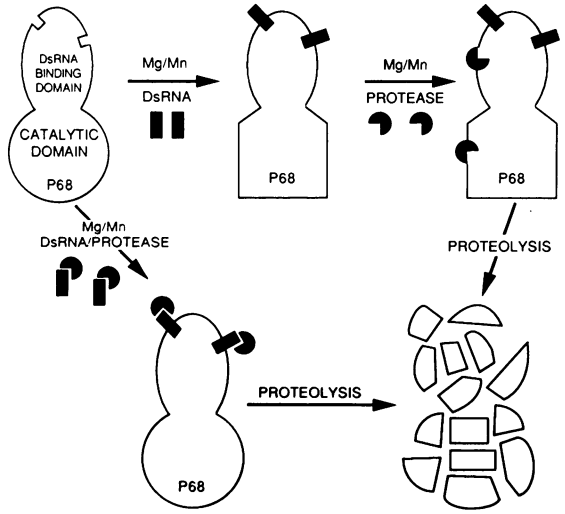

FIG. 8. Hypothetical models depicting the mechanism of P68 degradation during poliovirus infection. See the text for details.

The poliovirus-induced destabilization of the protein kinase by what may be a cooperation between virus-specific dsRNAs and a cellular protease represents a novel but not isolated example of the regulation of P68 by animal viruses (for a recent review, see reference 45). For example, influenza virus activates a 58-kDa cellular protein which then depresses kinase activity (47, 48, 54, 55). Adenovirus encodes an RNA III polymerase gene product, VAI RNA, which binds to and inactivates P68 (34, 46, 58, 60), whereas Epstein-Barr virus may accomplish the same by action of the EBER RNAs (24, 25). Human immunodeficiency virus type 1 may regulate the kinase through action of the *tat* regulatory gene product (71), whereas both reovirus and vaccinia virus regulate P68 by encoding viral proteins which may bind to and sequester activator dsRNAs (1, 43, 85). Although thus far it appears that only poliovirus and influenza virus use cellular gene products to regulate P68, it will be of interest to determine whether other virus groups regulate P68 by similar mechanisms or, indeed, whether these mechanisms are used by the cell in the absence of virus infection.

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