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Purification and partial characterization of a cellular inhibitor of the interferon-induced protein kinase of M_r 68,000 from influenza virus-infected cells

(protein kinase/influenza/eukaryotic initiation factor 2/translation/interferon)

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ABSTRACT **A number of eukaryotic viruses have evolved mechanisms to downregulate activity of the interferon-induced, double-stranded RNA-activated protein kinase (referred to as P68 based on its M_r of 68,000 in human cells). This control is essential because once activated, the P68 kinase phosphorylates its natural substrate, the α subunit of the eukaryotic protein synthesis initiation factor 2 (eIF-2), limiting functional eukaryotic protein synthesis initiation factor 2 available for protein synthesis initiation. We have previously shown that influenza virus encoded a specific mechanism to repress the autophosphorylation and activity of P68. Using *in vitro* assays for P68 inhibition, we now have purified, to near homogeneity, the P68 repressor from influenza virus-infected cells. The purified product inhibited both the autophosphorylation of P68 as well as phosphorylation of the α subunit of eukaryotic protein synthesis initiation factor 2 by the kinase. We tested for both protease and phosphatase activity but found neither activity associated with the purified inhibitor. Surprisingly we found the purified repressor, which had an apparent M_r of ≈58,000, was a cellular and not a viral-encoded protein. Possible mechanisms by which influenza virus activates this cellular regulator of the protein kinase, thereby minimizing potential antiviral effects of interferon, are discussed.**

To avoid decreases in protein synthetic rates during infection, animal viruses have devised strategies to downregulate activity of the interferon-induced, double-stranded RNA-activated protein kinase (referred to as P68 from its M_r of 68,000 but also referred to by others as DAI or P1/eIF-2 kinase) (1–4). The P68, a serine/threonine kinase, is characterized by two distinct kinase activities: the first involves an autophosphorylation (activation) reaction, and the second involves a protein kinase activity on exogenous substrates (5, 6). The virus-induced control of P68 is essential because viral-specific RNAs synthesized during infection have the potential to activate the P68 kinase (7–11). Once activated, P68 phosphorylates its natural substrate, the α subunit of protein synthesis eukaryotic initiation factor 2, eIF-2. Phosphorylation of the eIF-2 α subunit blocks the eIF-2B-mediated exchange of GDP in the inactive eIF-2–GDP complex with GTP required for catalytic utilization of eIF-2 (12–14). These events lead to limitations in functional eIF-2, which is an essential component of protein synthesis initiation and is normally required to bind initiator Met-tRNA (via the ternary complex eIF-2–GTP–Met-tRNA) to the initiating ribosomal subunit before mRNA is bound (15).

The regulation of P68 is best understood in the adenovirus system primarily because of the characterization of the adenovirus mutant dl331 (16). During infection by the dl331 mutant, which is deficient in the synthesis of the adenovirus-encoded RNA polymerase III product, virus-associated RNA 1, all protein synthesis is blocked due to the activation of P68 and phosphorylation of eIF-2 α subunit (17–22). We demonstrated that virus-associated RNA 1 functioned to block the activation of the kinase by forming a complex with P68 (17). In contrast to adenovirus, both reovirus and vaccinia viruses appear to downregulate P68 by encoding gene products that may bind to and sequester the double-stranded RNA activator of the kinase (23–25). Poliovirus has devised yet another strategy to minimize the damaging effects of kinase activation: early after virus infection the kinase is significantly degraded (7). Finally, we recently have shown that the human immunodeficiency virus HIV-1 may mediate downregulation of the protein kinase via action of the *tat* gene product (26).

The first indication that influenza virus encoded mechanisms to regulate P68 came from studies analyzing cells doubly infected with influenza virus and the adenovirus mutant dl331. When mutant dl331-infected cells were superinfected with influenza virus, dramatic suppression of the protein kinase activity normally detected during mutant infection was observed (27). We then reported a similar suppression of kinase activity in cells infected by influenza virus alone (28). In the present report we describe the purification and characterization of an unusual P68 repressor from influenza virus-infected cells. The inhibitor, which was purified by using *in vitro* P68 inhibition assays, was unexpectedly found to be a cellular and not a viral protein.

MATERIALS AND METHODS

Cells and Virus. The Madin–Darby bovine kidney (MDBK) cells (29) and the adenovirus type 5 human embryonic cell line 293 (30) were grown in monolayers as described (28). The WSN strain of influenza virus was grown and titrated as earlier described (29).

Purification of the P68 Inhibitor. Monolayers of MDBK cells (2×10^{10} cells; 800 T150 flasks) were infected with influenza virus at a multiplicity of infection (moi) of ≈10 plaque-forming units per cell for 4 hr. The infected cells were washed twice with ice-cold Hanks' balanced salt solution and lysed in buffer A: 50 mM Tris·HCl, pH 7.5/50 mM KCl/1 mM dithiothreitol/2 mM $MgCl_2$/aprotinin at 100 μg per ml/1 mM phenylmethylsulfonyl fluoride/1% Triton X-100. The cytoplasmic extracts were then centrifuged at 100,000 × *g* for 1 hr in a Beckman Ti 70.1 rotor. The supernatant (S100) was fractionated by ammonium sulfate precipitation (40–60%). The ammonium sulfate precipitate was resuspended in buffer

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Abbreviations: P68, interferon-induced protein kinase; eIF-2, eukaryotic protein synthesis initiation factor 2.

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B: 20 mM Tris·HCl, pH 7.5/1 mM dithiothreitol/0.1 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride/5% glycerol supplemented with 100 mM KCl and dialyzed against the identical buffer. The dialyzed sample was applied to a Mono Q HR 10/10 column. Bound proteins were eluted with a 100-ml linear gradient of 100–500 mM KCl in buffer B. The kinase inhibitory material eluted at ≈280 mM KCl. Active fractions were pooled, concentrated by using a Centriprep 30 concentrator (Amicon), and dialyzed against buffer B containing 25 mM KCl. The dialyzed fraction was applied to a heparin-agarose column with bound material sequentially eluted with buffer B containing 100, 300, and 500 mM KCl. The kinase inhibitory activity was found in the 300 mM KCl fraction, which was then concentrated and dialyzed against buffer B/25 mM KCl. The dialysate was loaded onto a Mono S HR 5/5 column with bound material subsequently eluted with buffer B/250 mM KCl. To achieve the final purification, the active Mono S fraction was layered onto a 10–30% glycerol gradient containing buffer B/25 mM KCl. The gradient was centrifuged at 49,000 rpm for 21 hr in a Beckman SW 55 rotor. Fractions were collected, spot dialyzed, and assayed for kinase inhibitory activity as described in the text.

P68 Kinase Inhibition Assays. Throughout the purification, fractions were assayed for kinase activity by using a histone phosphorylation assay. Fractions were mixed for 20 min at 30°C with cell-free extracts (28) prepared from interferon-treated 293 cells disrupted with Triton X-100 (as P68 source) after which P68 was immunoprecipitated by using the monoclonal antibody to P68 (31) as described (28). The P68 protein kinase was then analyzed for function by its ability to phosphorylate exogenously added histones as extensively described elsewhere (28). In addition to the histone assays, the highly purified inhibitory fractions were assayed as follows. Pure P68 (32) was preincubated with inhibitory fractions for 10 min at 30°C in buffer C (17 mM Tris·HCl, pH 7.5/75 mM KCl/0.1 mM EDTA/1.0 mM dithiothreitol/aprotinin at 8 μg per ml/0.1 mM phenylmethylsulfonyl fluoride/2 mM $MgCl_2$/2 mM $MnCl_2$/0.3 mg of bovine serum albumin per ml/8% glycerol). After this incubation, activator, poly(I)·poly(C) (0.010 μg/ml) was added in the presence of 1 μM [γ-^{32}P]ATP (424 Ci/mmol; 1 Ci = 37 GBq) and incubated for an additional 10 min. Finally, the natural P68 substrate, purified eIF-2 (0.5 μg), provided by Brian Safer (National Institutes of Health), was added for an additional incubation of 10 min at 30°C. The reaction was terminated by addition of 2× disruption buffer (1× disruption buffer: 70 mM Tris, pH 6.8/0.5 M 2-mercaptoethanol/2% SDS/10% (vol/vol) glycerol), the mixture was boiled, and the phosphorylated proteins were analyzed on SDS/14% polyacrylamide gel.

Western (Immunologic) Blot Analysis. Western blot analysis was performed according to the procedure of Towbin (33). For the P68 protease assay, the blot was treated with monoclonal antibody to P68, followed by an incubation with ^{125}I-labeled anti-mouse IgG. For detection of influenza virus-specific proteins, the blot was treated with a mixture of antisera to purified influenza virions, which included antibodies to the polymerase proteins, the nucleocapsid protein, and the hemagglutinin protein. We also included monospecific antibodies to the matrix and two nonstructural proteins, (all provided by Peter Palese and Mark Krystal; Mt. Sinai Medical School, New York), neuraminidase and the nucleocapsid proteins (provided by Robert Webster; St. Jude's Children's Research Hospital, Memphis, TN). After incubation, ^{125}I-labeled protein A was added to detect the viral proteins. It is important to note that initially these antisera were evaluated separately with crude extracts to ensure our assays were adequately sensitive to detect the viral-specific proteins.

RESULTS

***In Vitro* Assay for the Inhibition of P68 Activity in Influenza Virus-Infected Cells.** To identify the repressor of the protein kinase in influenza virus-infected cells, it was essential to develop an *in vitro* assay that quantitatively measured inhibitory activity. The assay included mixing fractions isolated during the purification procedure with an interferon-treated 293 cell extract (which served as the source of P68). After being mixed, the kinase was immunoprecipitated, and activity was measured by its ability to phosphorylate exogenously added histones. To quantitate activity, the histones were excised from the gel. Influenza virus-infected MDBK cells served as the starting material for the purification procedure because the virus replicates efficiently in this cell line, but since MDBK is a bovine and not a human cell line, the species-specific monoclonal antibody does not recognize the bovine kinase. The protein kinase immunopurified from 293 cell extracts, which were previously mixed with crude mock-infected extracts, efficiently phosphorylated histones (Fig. 1). In contrast, when 293 cell extracts were mixed with crude influenza virus-infected extracts, P68 had significantly reduced activity. To obtain a preliminary indication as to the nature of the P68 inhibitor, crude mock and influenza virus-infected extracts were either heated at 65°C or treated with 50 μg of trypsin before mixing with the interferon-treated 293 cell extract. Both treatments had little effect on mock extracts but significantly diminished the inhibition of P68 activity present in influenza extracts (Fig. 1), suggesting the repressor was likely a protein or perhaps a component of a ribonucleoprotein complex.

Purification of the P68 Inhibitor. The details of the purification protocol are described in detail in *Materials and Methods*. Influenza virus-infected MDBK cells were harvested and disrupted with 1% Triton X-100 4 hr after infection. After centrifugation of the cytoplasmic extract at 100,000 × *g*, the S100 supernatant was subjected to ammonium sulfate precipitation. The dialyzed 40–60% ammonium sulfate fraction was then applied to a fast protein liquid chromatography Mono Q anion-exchange column. P68 repressor activity eluted off the column at ≈280 mM KCl. The peak fractions were added to a heparin–agarose cation-exchange column. Activity was found in the 300 mM KCl fraction, which was subsequently concentrated, dialyzed, and applied to fast protein liquid chromatography Mono S column after which kinase inhibitory activity was detected in the 250 mM KCl batch-eluted fraction. Before the final glycerol gradient step, the active fractions were analyzed both for their specific activity (Table 1) and their polypeptide content (Fig. 2*A*). The specific activity of the P68 inhibitor

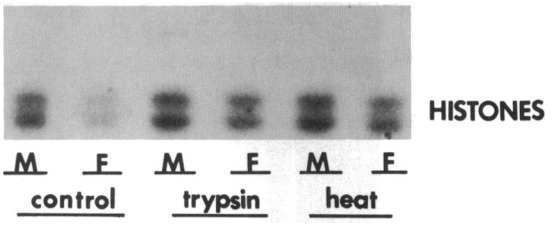

FIG. 1. *In vitro* assay for the measurement of P68 kinase activity. P68 was immunopurified from interferon-treated 293 cell extracts mixed with either mock (M)- or influenza virus (F)-infected MDBK cell extracts. Where noted, both mock- and influenza virus-infected extracts, before mixing with the 293 cell extracts, were either untreated (control), heat treated (65°C for 1 min), or treated with 50 μg of trypsin for 30 min at 37°C (followed by addition of a 2-fold excess of soybean trypsin inhibitor to inactivate the trypsin). The bands representing histones were excised from the gel and counted: control M, 3658 cpm and F, 920 cpm; heat M, 4225 cpm and F, 3428 cpm; trypsin M, 4279 cpm and F, 3554 cpm.

Table 1. Summary of purification of the P68 inhibitor

Purification step	Volume, ml	Protein,* mg	Total activity,† units	Specific activity, units/mg	Purification, -fold	Yield, %
Cytoplasmic extract	438.00	2,847.000	83,000	29.2		100.00
100,000 × *g*	430.00	2,193.000	64,500	29.4	1.0	77.70
Ammonium sulfate	30.00	600.000	24,900	41.5	1.4	30.00
Mono Q	5.00	16.000	4,000	250.0	8.6	4.80
Heparin-agarose	3.50	1.820	2,590	1,420.0	48.6	3.10
Mono S	2.80	0.180	1,148	6,370.0	230.5	1.40
Glycerol gradient	1.68	0.013	269	20,690.0	708.0	0.32

*Protein was measured by the Micro BCA protein assay (Pierce) except for Mono S and glycerol gradient fractions in which protein was estimated from silver-stained gels.

†One unit of activity is defined as the amount of protein required to cause 1% inhibition in the phosphorylation of exogenously added histones by the P68 protein kinase.

increased from ≈30 units per mg of protein in the crude factors to >6300 units per mg in the peak Mono S fraction, reflecting a 230-fold increase during the purification procedure. The silver-stained SDS/polyacrylamide gel revealed that the Mono S fraction, although not pure, was highly enriched for several polypeptides (Fig. 2*A*, lane 6). The arrow in Fig. 2*A* denotes the position of the M_r 58,000 polypeptide identified as the putative inhibitor after the final glycerol gradient centrifugation described below.

To ensure that the partially purified Mono S fraction possessed *bona fide* P68 inhibitory activity, we developed a kinase inhibitory assay using highly purified P68 and eIF-2, its natural substrate (see *Materials and Methods*). Because the purified P68 had minimal endogenous activity, activator double-stranded RNA was added to the reaction. This provided the opportunity to test whether the inhibitor blocked the activation as well as the activity of P68, assumed likely if the repressor acted directly on the kinase. The results (Fig. 2*B*) clearly demonstrated that the partially purified inhibitor blocked both the autophosphorylation (activation) of P68 as well as its kinase activity on the α subunit of eIF-2. We next evaluated whether the observed decreases in P68 activity were from degradation of the P68 by the partially purified preparation (Fig. 2*C*). There was no detectable decrease in physical kinase levels using assay conditions identical to those used for the inhibition assay described in Fig. 2*B*. In addition, we tested for possible phosphatase activity in the purified fraction that would result in the dephosphorylation of P68, eIF-2, or both but found no such activity (data not shown).

Because the peak Mono S fraction contained several polypeptides, it was necessary to subject this fraction to glycerol gradient centrifugation to obtain a pure inhibitory preparation. A mixture of standard proteins were centrifuged in a parallel gradient to allow size determination. The peak of activity sedimented in fractions 16–19 (Fig. 3*A*), which were located between the bovine serum albumin (M_r 68,000) and ovalbumin (M_r 44,000) M_r standards. These fractions were pooled, and the polypeptide content was analyzed on a silver-stained SDS/polyacrylamide gel (Fig. 3*B*), and its activity was confirmed by using the *in vitro* assay with purified P68 and eIF-2 (Fig. 3*C*). The kinase inhibitory activity was associated with essentially one polypeptide, with a M_r of ≈58,000 (see arrow, Fig. 3*B*). Fortunately, the majority of the other polypeptides found in the Mono S fraction were found in fractions migrating faster than those containing inhibitory activity (data not shown).

The purified product inhibited, in a dose-dependent manner, both P68 autophosphorylation and eIF-2 α subunit phosphorylation. A half-maximal effect was obtained with ≈1.6 μg/ml (27 nM), whereas maximal activity (≈80% inhibition) was achieved with 3.2 μg/ml (54 nM) (Fig. 3*C*, lanes 4 and 5, respectively). Specific activity of the final product of M_r 58,000 was ≈20,700 units/mg, which reflected an overall >700-fold purification compared with the starting material (see Table 1). It is important to note that the sedimentation

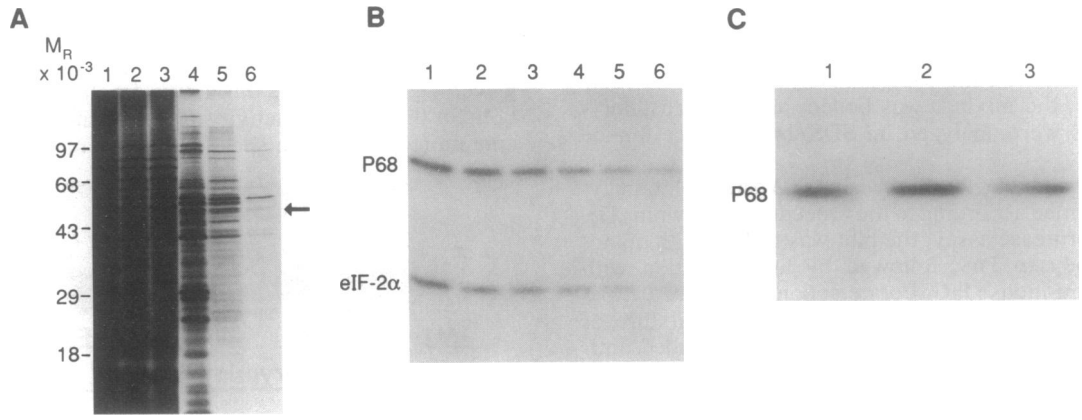

FIG. 2. SDS/PAGE analysis of the purified inhibitor fractions and measurement of the kinase inhibitory activity present in the Mono S peak fraction: (*A*) SDS/14% PAGE of active fractions from the purification procedure. Lanes: 1, cytoplasmic extract of influenza virus-infected cells; 2, 100,000 × *g* supernatant; 3, 40–60% ammonium sulfate fraction; 4, Mono Q peak; 5, heparin-agarose column peak; and 6, Mono S peak. Protein bands were visualized by silver staining. The arrow on the right denotes the position of the putative M_r 58,000 inhibitor present in the Mono S fraction (see Fig. 3). (*B*) Kinase inhibitory activity of the peak Mono S fraction. Highly purified P68 was preincubated with 0.0 μl (lane 1), 0.1 μl (lane 2), 0.5 μl (lane 3), 1.0 μl (lane 4), 2.0 μl (lane 5), and 4.0 μl (lane 6) of the partially purified fraction after which P68 activation and eIF-2 α subunit phosphorylation activity were measured as described. The protein concentration of the Mono S fraction was ≈0.060 mg/ml. (*C*) Lack of protease activity associated with the P68 inhibitor. Pure P68 was incubated with 25 μl of the Mono S fraction with equal aliquots removed after incubation for 0 min (lane 1), 15 min (lane 2), and 30 min (lane 3) under standard kinase assay conditions. The physical levels of the protein kinase, electrophoresed on a SDS/14% PAGE, were measured by Western blot.

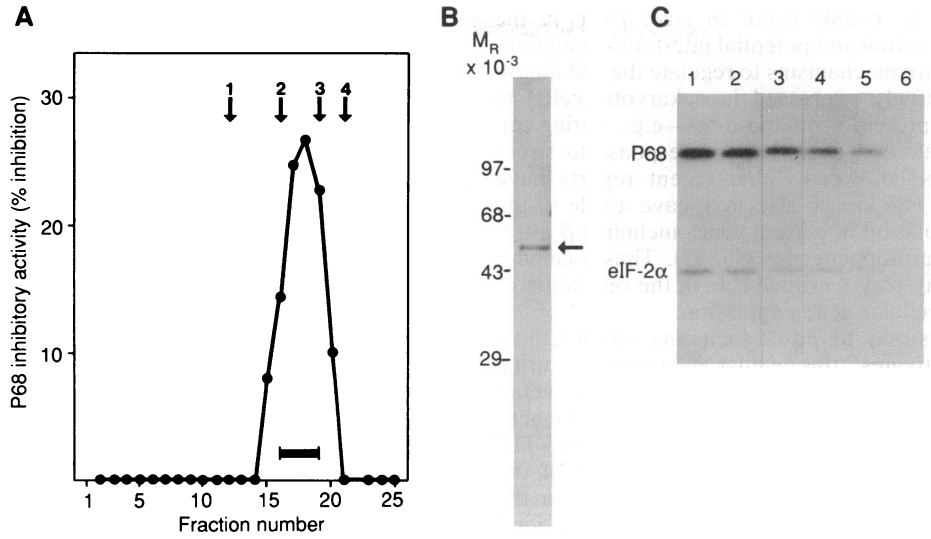

FIG. 3. Glycerol gradient analysis of the P68 inhibitor. (*A*) Glycerol gradient profile of the Mono S fraction layered onto a 10–30% glycerol gradient and spun at 49,000 rpm for 21 hr at 4C. Fractions were assayed for inhibition of P68 autophosphorylation using purified P68 and the *in vitro* assay. The positions of M_r standards analyzed on a parallel gradient are shown by arrows on top: 1, γ globulin (M_r 158,000); 2, bovine serum albumin (M_r 68,000); 3, ovalbumin (M_r 44,000); 4, myoglobin (M_r 17,000). Active fractions were pooled as shown by the brackets. (*B*) SDS/14% PAGE analysis of pooled active gradient fractions as detected by silver staining. The arrow indicates the M_r 58,000 polypeptide with the position of M_r standards shown on the left. (*C*) Activity of the purified M_r 58,000 polypeptide. The gradient peak was analyzed for inhibitory activity using purified P68 (≈0.020 μg) and eIF-2 (0.50 μg). Purified P68 was preincubated with 0.0 μl (lane 1), 0.5 μl (lane 2), 2.0 μl (lane 3), 8.0 μl (lane 4), and 16.0 μl (lane 5) of purified material after which P68 and eIF-2 α subunit phosphorylation was measured as described. Lane 6 shows the result of an assay in which the purified P68 kinase was omitted but which contained 8.0 μl of inhibitor. Concentration of the purified inhibitor was ≈0.008 mg/ml.

coefficient of ≈3.9–4.0S was consistent with the inhibitor being a monomeric polypeptide neither complexed with other proteins nor RNA (as part of ribonucleoprotein particle). Further, because the protein of M_r 58,000 was assayed only with purified components, we conclude it likely functions alone to repress P68 activity.

Identification of the M_r 58,000 Inhibitor as a Cellular Protein. An important question was whether the purified inhibitor was an influenza virus-encoded protein. We therefore performed Western blot analysis on the active fractions isolated during the purification procedure through the Mono S step using a mixture of influenza virus-specific antisera. This mixture included antiserum prepared against purified virions, as well as monospecific antisera to the matrix protein, the nonstructural proteins, the neuraminidase protein, and the nucleocapsid protein. The results (Fig. 4) show that while influenza virus-specific proteins were readily detected in the less-purified fractions (lanes 2–5), viral proteins were essentially undetectable after the Mono Q column (lanes 6 and 7). Indeed, most of a potential candidate, nucleocapsid protein, was found in the Mono Q column flowthrough (lane 8), which consistently lacked P68 inhibitory activity. It can be argued that the viral proteins are present in the active fractions but modified in such a way that would make them unreactive with standard monospecific antiserum. We consider this unlikely because purification experiments using [^{35}S]methionine-labeled extracts similarly revealed that virus-specific proteins could not be detected in peak fractions after the Mono Q column (data not shown). Reconstitution experiments, using *in vitro* transcribed and translated influenza viral gene products similarly failed to provide evidence that a viral protein functioned directly to inhibit the protein kinase (data not shown). Finally, recent experiments have shown that a P68 inhibitor, with identical chromatographic properties, can be isolated from uninfected MDBK cells (see *Discussion*). Taken together, these results strongly suggest the P68 repressor purified from virus-infected cells is a cellular and not a viral protein.

DISCUSSION

In the current report, we have described the purification and initial characterization of a cellular inhibitor of the interferon-induced kinase from influenza virus-infected cells; to our knowledge a specific cellular regulator of the P68 protein kinase has not been previously reported. Although a recent study (34) described the dephosphorylation of P68 *in vitro* by a manganese-dependent type 1 phosphatase, it remains un-

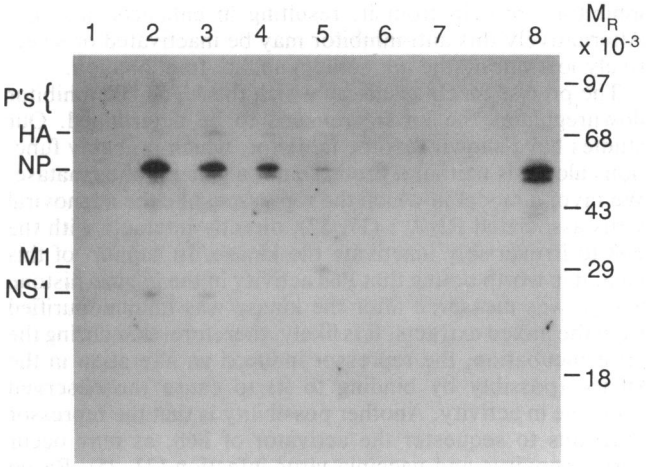

FIG. 4. Western blot analysis of the active purified fractions. The fractions prepared from the purification procedure were electrophoresed on a SDS/14% PAGE, blotted onto nitrocellulose paper, and probed with the mixture of influenza virus-specific antisera as described in text. Viral proteins were visualized after hybridization with ^{125}I-labeled protein A. Lanes: 1, as control, mock-infected cytoplasmic extract; 2, virus-infected cytoplasmic extract; 3, 100,000 × *g* supernatant; 4, ammonium sulfate peak fraction; 5, Mono Q peak; 6, heparin-agarose peak; 7, Mono S peak; 8, Mono Q flowthrough fraction. The positions of influenza virus-specific proteins are shown on the left and M_r standards on the right. M1, matrix protein; NS1, nonstructural protein; HA, hemagglutinin protein; NP, nucleocapsid protein; P's, polymerase proteins.

clear whether similar events occur *in vivo*. Even in the absence of virus infection and potential interferon induction, cells probably require mechanisms to regulate the P68 kinase (which is constitutively expressed in eukaryotic cells) in order to modulate protein synthetic rates—e.g., during cell division or differentiation or perhaps in response to stress (35). Further, it is noteworthy that recent reports have suggested that the P68 kinase also may have a role in the transcriptional regulation of certain genes including β interferon and several protooncogenes (36, 37). Thus a cellular inhibitor of P68 may play a critical role in the regulation of multiple levels of cellular gene expression.

Several questions may be posed including why and how influenza virus "activates" this cellular P68 repressor during infection. The kinase must be downregulated to avoid overall decreases in protein synthesis levels resulting from the activation of P68 during infection by viral-specific RNAs (7–11). In addition, others have suggested that regulation of the kinase and eIF-2 phosphorylation may play a role in the selective cessation of host protein synthesis during viral infections, but there is as yet no direct evidence to support these speculations (38). Interestingly, influenza is relatively insensitive to the antiviral effects of interferon unless the host cells possess the *Mx* gene (39), in which case viral transcription but not translation is blocked (40). On this basis, one might speculate that viruses such as influenza and adenovirus (also insensitive to interferon), have devised strategies to combat the negative antiviral effects of interferon. One necessary component would be the downregulation of P68 activity, particularly because kinase levels increase several-fold in response to interferon induction (6). The more difficult question to answer is how the cellular P68 inhibitor is activated during infection. We are unable to observe kinase inhibitory activity in either crude uninfected extracts or in the S100 fraction of uninfected cells (Fig. 1; ref. 28). However, once the uninfected extracts are subjected to ammonium sulfate precipitation, P68 repressor activity is recoverable (data not shown). It is conceivable that the high salt treatment may in some way mimic virus infection and alter the conformation of the inhibitor or perhaps dissociate an "anti-inhibitor" protein from it, resulting in enhanced activity. Alternatively this anti-inhibitor may be inactivated or selectively lost during the ammonium sulfate fractionation.

The precise mechanisms by which the M_r 58,000 inhibitor downregulates the kinase remains to be determined. Our studies have shown that the inhibitor, which probably functions alone, is neither a protease nor a general phosphatase. We favor a model in which the repressor, like the adenoviral virus-associated RNA 1 (17, 32), directly interacts with the P68 to irreversibly inactivate the kinase. In support of this idea, it is worth noting that P68 activity in the *in vitro* histone assays was measured after the kinase was immunopurified from the mixed extracts. It is likely, therefore, that during the prior incubation, the repressor induced an alteration in the kinase (possibly by binding to it) to cause the observed decrease in activity. Another possibility is that the repressor functions to sequester the activator of P68, as may occur during reovirus and vaccinia virus infection (23–25). Based on the previous discussion and the fact that activator is not present during our *in vitro* histone assays, we consider this explanation unlikely. Large-scale purifications from infected and uninfected cells need to be accomplished to compare the P68 inhibitors and obtain sufficient material for microsequencing the repressor to then obtain a cDNA clone.

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