Characterization and Regulation of the 58,000-Dalton Cellular Inhibitor of the Interferon-induced, dsRNA-activated Protein Kinase*

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The P68 protein kinase is a serine/threonine kinase induced by interferon treatment and activated by double-stranded RNAs (dsRNAs). Once activated, the kinase phosphorylates its natural substrate, the α subunit of eukaryotic initiation factor 2 (eIF-2) leading to potential limitations in functional eIF-2 and decreases in protein synthesis initiation. We have recently purified from influenza virus-infected cells a P68 kinase inhibitor, found to be a 58-kDa cellular protein. We have now investigated the mechanisms by which the 58-kDa inhibitor regulates P68 kinase activity and how the inhibitor itself is controlled. The 58-kDa inhibitor did not function by degrading or sequestering the dsRNA activator of P68 but could repress phosphorylation of eIF-2 by an already activated protein kinase. Utilizing antibody prepared against a 58-kDa-specific peptide, we showed that the 58-kDa proteins from infected and uninfected cells were present in equivalent amounts. Although kinase inhibitory activity could not be detected in crude uninfected cell extracts, ammonium sulfate treatment unmasked this activity and allowed purification of the cellular inhibitor with identical chromatographic properties as that from influenza virus-infected cells. Finally, we have identified and partially purified a specific inhibitor of the 58-kDa protein which we refer to as an “anti-inhibitor.” Based on these data, we present a model depicting the complex regulation of the interferon-induced protein kinase in eukaryotic cells.

The interferon-induced P68 protein kinase (referred to as P68 from its molecular weight of 68,000 in human cells, but also referred to as DAI, dsI, or Pl/eIF-2 kinase; Refs. 1–4) is a member of the serine/threonine kinase family (5). P68 kinase activity is independent of CAMP and cGMP but is markedly stimulated by the divalent cation Mn2+ and by ATP (6). Two kinase activities are characteristic of P68: the first is dsRNA1 induced activation (autophosphorylation) and the second is the phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF-2) (7, 8). This latter activity prevents the recycling of phosphorylated eIF-2-GDP to eIF-2-GTP by the guanine nucleotide exchange factor eIF-2B (9–12), leading to limitations in functional eIF-2. Thus activation of the kinase triggers a series of events which culminates in an inhibition of protein synthesis initiation.

The activation of the P68 protein kinase has been suggested to play a role in the interferon response to virus infection, but no definitive proof has yet been obtained (6, 13). Even though RNAs encoded by both RNA and DNA viruses have the capacity to activate the protein kinase (14–18), several viruses have evolved mechanisms to counteract the potentially harmful effects caused by this activation, thereby escaping the inhibitory effects of interferon (6, 13, 19–26). There is also accumulating evidence that the kinase may play a pivotal role in the regulation of cellular gene expression, in the absence of interferon induction or virus infection. For example, Petryshyn and colleagues (27–29) have reported the kinase may be important in controlling growth arrest prior to differentiation into adipocytes. Furthermore, it has been suggested that the P68 kinase may play a role in the stress-heat shock response (30) or the transcriptional regulation of select proto-oncogenes or β-interferon gene (31, 32). Finally, it is significant that the human P68 kinase shares homology with the yeast GCN2 kinase which also phosphorylates the eIF-2α subunit and regulates mRNA-specific translational control in yeast (33).

We recently purified from influenza virus-infected cells a cellular 58-kDa protein which inhibits P68 activity (21, 34). If the kinase is involved with the different cellular processes described above, such a regulator of P68 kinase activity also might have profound effects on cellular gene expression in addition to its effects on viral gene expression. In the current study we have analyzed the function of the inhibitor as well as regulation of the 58-kDa protein itself. Unlike the adenovirus inhibitor VAI RNA (39, 35, 36), the 58-kDa protein blocked the eIF-2-phosphorylating activity of the P68 kinase, when added after the kinase was already activated. Western blot analysis utilizing peptide-derived antibody demonstrated that the inhibitor was present in equal amounts in uninfected and virus-infected cells. We found that although inhibitory activity was undetectable in crude uninfected cell extracts, a protein with chromatographic properties identical to the originally purified protein can be activated after fractionation of extracts with ammonium sulfate. Finally, we have identified a specific inhibitory activity against the 58-kDa protein itself, suggesting the regulation of the P68 kinase even in uninfected cells is complex.

MATERIALS AND METHODS

Cells and Virus—The Madin-Darby bovine kidney (MDBK) cells were grown in monolayers as described previously (37). The WSN
strain of influenza virus was grown on MDBK cells and titrated by plaque assay in Madin-Darby canine kidney cells as earlier described (37).

Purification of the P68 Inhibitor—Monolayers of uninfected MDBK cells were washed two times with ice-cold Hank's balanced salt solution (38). For 20 min at 30°C after this, the cell extract (500 KCl, 1 mM dithiothreitol, 2 mM MgCl₂, 100 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100. The cytoplasmic extracts were then centrifuged at 100,000 × g for 1 h in a Beckman Ti 60 rotor. The supernatant (S100) was fractionated by ammonium sulfate precipitation into the 0-40%, 40-60%, and 60-80% samples. For further inhibitor purification, the 40-60% fraction was resuspended in Buffer B: 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 0.1 mM EDTA, 1.0 mM phenylmethylsulfonyl fluoride, and 5% glycerol supplemented with 100 mM KCl and dialyzed against the identical buffer. The dialyzed sample was applied to a Centriprep 30 (Millipore), and dialyzed against Buffer B containing 25 mM KCl. PFLC Mono S chromatography was performed as earlier described (21). Sedimentation was performed on a 10-30% glycerol gradient containing buffer B plus 25 mM KCl. The gradient was centrifuged at 49,000 rpm for 2 h in a Beckman SW 55 rotor. Fractions were collected, spot-dialyzed, and assayed for kinase inhibitory activity.

P68 Kinase Inhibition Assays—Crude fractions were assayed for kinase inhibitory activity utilizing a histone phosphorylation assay. Fractions were mixed with an interferon-treated 293-cell extract (as described (22)) for 20 min at 30°C after which P68 was immunoprecipitated using the monoclonal antibody to P68 (38) as earlier described (34). The P68 protein kinase was then analyzed for function by its ability to phosphorylate exogenously added histones as extensively described elsewhere (34). In addition to the histone assays, more purified inhibitory fractions were assayed as follows. Pure P68 (200 µg/ml) was treated with activator, poly(I):poly(C) (0.010 µg/ml) and incubated at 30°C for 10 min. Following this incubation, 100 µl of the kinase inhibitory fractions (contain- ing 2.5 µg/ml) was added in the presence of 1 µM [γ-32P]ATP (424 Ci/mmol) and incubated for an additional 10 min. Finally the natural P68 substrate, purified eIF-2 (0.5 µg/ml), kindly provided by Brian Safer (University of Washington). Two New Zealand White rabbits were immunized at 21-day intervals with 200 µg of the peptide-keyhole limpet hemocyanin complex. Three weeks after the last injection, antibodies were collected, spot-dialyzed, and assayed for kinase inhibitory activity.

Preparation of Antibody against the Synthetic Peptide of the 58-kDa Inhibitor—To obtain protein sequence information, approximately 5 µg of glycerol gradient-purified inhibitor was separated on SDS-PAGE, electroblotted onto nitrocellulose filter paper, and stained with Ponceau S. Western immunoblots were preincubated with the kinase inhibitory fractions (containing the 58-kDa protein) for 10 min at 30°C. This mixture was then incubated with the 293 interferon-treated extracts (providing the P68 source) after which the kinase was immunoprecipitated using the P68 monoclonal antibody and kinase function-assayed utilizing histones as the substrate.

RESULTS AND DISCUSSION

Recovery of Kinase Inhibitory Activity and Identification of the 58-kDa Inhibitor in Uninfected MDBK Cells—We previously purified a P68 inhibitor from influenza virus-infected cells presumed to be cellular, since the 58-kDa protein did not react with influenza virus-specific antibody (21). In our earlier experiments we could not, however, detect inhibitory activity in crude uninfected cell extracts. A possible explanation for this was obtained when ammonium sulfate fractionation was performed on the 100,000 × g supernatant from influenza virus-infected and uninfected cells and P68 kinase inhibitory activity measured in a histone phosphorylation assay (Fig. 1A). This assay included mixing fractions with an interferon-treated 293-cell extract (which served as a source of P68). After mixing, the kinase was immunoprecipitated with the P68 monoclonal antibody (38) and activity measured by its ability to phosphorylate exogenously added histones. It should be noted that histones are a suitable substrate to test kinase activity in vitro and importantly can be purchased commercially in contrast to highly purified eIF-2. Whereas only the crude virus-infected supernatant had kinase inhibitory activity (compare lanes A and B), the 40–60% ammonium sulfate fractions from both infected and uninfected extracts had equivalent inhibitory activity (Fig. 1A, compare lanes C and D). This suggested that ammonium sulfate treatment may be “unmasking” the inhibitory activity or perhaps altering the conformation of the inhibitor.

To test whether the inhibitory activity was similar to that originally described for infected cells, we performed PFLC Mono Q chromatography on the 40–60% ammonium sulfate fraction from uninfected cells (Fig 1B, +AS). The major inhibitory activity was recovered from the column at 280 mM KCl, precisely where the activity eluted from virus-infected cells (21). A second minor peak of activity was eluted off at 320 mM, again similar to the infected cell extracts; this activity was not further characterized. We also applied the 100,000 × g material from uninfected cells to a FPLC Mono X column and recovered a 14% SDS-polyacrylamide gel.
g supernatant from uninfected cells directly to the Mono Q column, but activity could not be recovered from extracts which were not first subjected to ammonium sulfate fractionation (Fig 1B, -AS). We went on to purify the inhibitor from uninfected cells following the protocol originally described and found the uninfected cell inhibitor to have identical chromatographic properties as that purified from virus-infected cells (21), including sedimentation on a glycerol gradient (data not shown).

To unequivocally identify and compare the kinase inhibitor in uninfected and influenza virus-infected cells, a peptide (ISTLYELGDHEL) was synthesized based on the amino acid sequence obtained from microsequencing highly purified 58-kDa protein and peptide antibody prepared as described under "Materials and Methods." The levels of the inhibitor in virus-infected and uninfected cells were determined by Western blot analysis using the peptide antibody. A 58-kDa protein was detected in both crude extracts (Fig. 2, lanes A and B). Importantly the proteins were of identical size and present in equal amounts. Other more abundant cellular proteins were nonspecifically detected but these proteins were also reactive with preimmune serum (lanes C and D). As an additional control, the antibody was preexposed with excess 58-kDa-specific peptide prior to incubation with the immunoblot (lanes E and F). As predicted, the 58-kDa protein was no longer detectable, whereas the nonspecific polypeptides were still reactive with the antibody. It is important to note that the levels of the 58-kDa protein, present in the ammonium sulfate-fractionated material from infected and uninfected cells, also were identical (data not shown). We also insured that the peptide antibody recognized the 58-kDa inhibitor purified to homogeneity (Fig 2B). These data, taken together, demonstrated that the 58-kDa inhibitory protein isolated from uninfected cells is probably identical to that purified from influenza virus-infected cells and that its activity, but not its synthesis, was induced by influenza virus infection. Examination of the protease digestion pattern of the 58-kDa proteins from infected and uninfected cells will be required to determine unequivocally that the two proteins are identical.

**The Mechanism of Action of the Cellular 58-kDa Kinase Inhibitor and Comparisons to Viral-encoded Inhibitors**—We next determined how this 58-kDa protein functioned to regulate P68 kinase activity. It was reported previously (21) that the 58-kDa kinase inhibitor had no detectable protease or phosphatase activity, which could account for observed decreases in kinase activity. Other explanations might include that the kinase inhibitory activity represented an ATPase or a ribonuclease which would eliminate the dsRNA activator. We were, however, unable to detect either of these activities (data not shown). Since it has been suggested that both reovirus and vaccinia virus down-regulate the P68 kinase by sequestering the dsRNA by a virus-encoded RNA binding protein (23, 24, 25), we tested whether Mono S-purified material possessed similar properties. If the inhibitor functioned in this way, addition of increasingly high amounts of dsRNA to an in vitro assay should essentially inactivate or neutralize the inhibitor. As shown graphically in Fig. 3, low concentrations of dsRNA activate and high amounts inhibit the activation of purified P68 (in the absence of inhibitor) as has been observed previously (6, 39, 40). In contrast, the kinase was almost completely repressed when the inhibitor was present in the reactions at the highest concentrations of dsRNA added to the reaction, suggesting that the inhibitor does not act by sequestering the activator.

As stated previously P68 has two activities: the kinase first activates itself via an autophosphorylation reaction and then catalyzes the phosphorylation of eIF-2 α (6). The best characterized kinase inhibitor, adenosine VAI RNA, can block kinase activation and activity only if it is present in the reaction prior to dsRNA addition (35, 39). Once the kinase is activated, VAI RNA can no longer function to block eIF-2 α phosphorylation. To test whether the 58-kDa protein functioned similarly, the inhibitor was added to an in vitro reaction after the kinase was activated by dsRNA. In these experiments highly purified P68 kinase and its natural substrate, eIF-2, were utilized in contrast to the earlier described histone assay. Under these conditions the inhibitor successfully blocked the phosphorylation of the eIF-2 α subunit (Fig. 4, compare lanes C and D). Indeed the levels of inhibition were comparable to those seen when the inhibitor was added prior to dsRNA addition (lanes A and B). We speculate that both types of inhibitory activity provide additional safeguards to the cell.

**Fig. 3.** The protein kinase inhibitor does not function by sequestering the dsRNA activator. Purified P68 protein kinase (0.02 μg) was preincubated with buffer alone and then the listed concentrations of poly(1):poly(C) (open circles: -58 kDa) or with the Mono S purified inhibitory fraction (0.65 μg) and then poly(1):poly(C) (closed circles: +58 kDa). The kinase assay was performed as described earlier in the presence of {gamma}32P)ATP and the extent of P68 phosphorylation determined from laser densitometry of the phosphorylated radioactive P68 bands obtained after SDS-PAGE and autoradiography. The peak level of P68 autophosphorylation was arbitrarily given the value of 100%. 

**Fig. 2.** Identification of the 58-kDa kinase inhibitor in infected and uninfected cells utilizing peptide antibody and Western blot analysis. A, cell extracts from uninfected cells (lanes A, C, and E) or from influenza virus-infected cells (lanes B, D, and F) were electrophoresed on a SDS, 14% PAGE, blotted onto nitrocellulose, and probed with the specific 58-kDa peptide antibody (lanes A and B), preimmune antiserum (lanes C and D), or with peptide antibody which was preexposed with a 100-fold molar excess of 58-kDa-specific peptide (lanes E and F). B, Western blot analysis also was performed on the glycerol gradient-purified inhibitor (21). Proteins were visualized utilizing the Amersham ECL chemiluminescence system. Positions of molecular weight standards are shown on the right of each panel.
the results of a kinase assay during which purified P68 was incubated with poly(I):poly(C) (0.05 μg/ml), purified eIF-2 (0.5 μg), and [γ-32P]ATP in the absence of inhibitor. B depicts the kinase assay in which the Mono S inhibitory fraction was preincubated with the purified protein kinase prior to addition of poly(I):poly(C), purified eIF-2, and [γ-32P]ATP. In lane C the P68 protein kinase was preincubated with poly(I):poly(C) and [γ-32P]ATP prior to an additional incubation with purified eIF-2 and [γ-32P]ATP. The assay in lane D was performed as in lane C except that the Mono S inhibitory fraction was added after the kinase was preincubated with poly(I):poly(C).

(and the virus) in the event that P68 autophosphorylation cannot be totally prevented. The additional radiolabeled band present in lanes B and D likely represents phosphorylation of a cellular protein present in the inhibitory Mono S fraction. This protein is probably not the inhibitor, since when other types of kinase inhibition assays were performed with glycerol gradient-purified material (21), this protein was not detected. Parenthetically, we still have not ascertained whether the 58-kDa protein is phosphorylated in the cell. In summary these data show that the cellular inhibitor works differently than the adenovirus VAI RNA and the reovirus and vaccinia virus-encoded inhibitors (see model, Fig. 6). Finally, it is relevant to note that previous work from our laboratory demonstrated the 58-kDa inhibitor likely functioned stoichiometrically on the P68 kinase (21) and probably in an irreversible manner (34).

Identification of a Specific Anti-inhibitory Activity in Uninfected Cell Extracts—It still remained to be determined how the inhibitor was activated during virus infection. Since the physical levels of the inhibitor did not increase during virus infection and the inhibitory activity itself could be activated by ammonium sulfate fractionation, we speculated that another factor, which functioned to inhibit the inhibitor, may have been dissociated from the 58-kDa protein during the high salt treatment. An alternative explanation was that ammonium sulfate treatment altered the inhibitor in some way. To discriminate between these two possibilities, we performed mixing experiments with the different ammonium sulfate fractions (all from uninfected cell extracts). In these series of experiments the fractions containing putative “anti-inhibitor” activity were preincubated with fractions containing the 58-kDa inhibitor prior to performing the histone phosphorylation activity assay (Fig. 5A). We used the 40–60% ammonium sulfate fraction as a source of inhibitor, which was previously found to contain the strongest activity (21). When the 40–60% fraction alone was reacted with the interferon-treated 293-cell extract, there was, as expected, a dramatic inhibition of activity compared with when the extract was reacted with buffer alone (Fig. 5A, compare lanes 1 and 2). We then prereacted the 40–60% fraction with increasing amounts of the 0–40% ammonium sulfate fraction, the 60–80% fraction, or BSA (as control) before adding the 293 interferon-treated extracts and assaying kinase function. Addition of either BSA (lanes 3–6) or the 0–40% ammonium sulfate fraction (lanes 7–10) to the 40–60% fraction did not reduce kinase inhibitory activity. In contrast, addition of increasing amounts of the 60–80% fraction (lanes 11–14) partially repressed the kinase inhibitory activity of the 40–60% fraction.

To provide additional evidence for this anti-inhibitory activity, we attempted a further purification by passing the 60–80% ammonium sulfate fraction over a FPLC Mono Q column and performing batch elution at increasing salt concentrations. We repeated the histone kinase inhibition assay utilizing these fractions combined with a more purified inhibitor fraction containing the 58-kDa inhibitor (the peak 280 mM KCl fraction from the FPLC Mono Q column referred to as Mono Q-P58; see Fig. 1). In the absence of any other fractions, the Mono Q-P58 inhibited P68 kinase activity (Fig. 5B, compare lanes 1 and 2). When the Mono Q stepwise-eluted fractions were tested for anti-inhibitory activity, only the 100 mM KCl flow-through fraction possessed this activity (lanes 5 and 6). These fractions inhibited the inhibitor as well as
the column starting material, the 60–80% ammonium sulfate fraction (lanes 3 and 4). Little activity was detected in the other column fractions. From the series of experiments described above, we conclude that a specific anti-inhibitory factor(s) is present in uninfected cells, which can regulate activity of the 58-kDa inhibitor and consequently the kinase itself.

Model of P68 Kinase Regulation in Eukaryotic Cells—Clearly many questions remain to be answered. For example, what is the nature of the anti-inhibitor and how does it work? Since the activity was found in the 100 mM KCl fraction of the Mono Q column (Fig. 5B), and since the 58-kDa inhibitor does not function by sequestering its activator (Fig. 3), it is unlikely simply to be dsRNA which functions to override the inhibition of the P68 kinase. Although the data in the present report does not yet provide final details, it does allow us to propose a model for the regulation of the interferon-induced P68 kinase (Fig. 6). A depicts the scenario in the absence of any regulation when the kinase is activated by dsRNAs, and as a result protein synthesis initiation is inhibited. B shows the results of P68 down-regulation; after virus infection and synthesis of viral-specific dsRNAs, the 58-kDa inhibitor (P58), which had been in an inactive complex with its own inhibitor (the anti-inhibitor referred to as I-P58), becomes dissociated and can then block the autophosphorylation and/or activity of the kinase possibly through a direct interaction with the kinase. The end result is that protein synthetic rates are not compromised. Alternatively, in the absence of viral infection, if the kinase does regulate cell growth and differentiation (27–29), it is possible that the 58-kDa inhibitor would become dissociated or associated with its inhibitor in response to a specific signal to control kinase activity levels and overall protein synthesis rates as needed. Regulation of the kinase by activators and inhibitors is unlikely to be an all or none phenomenon. A more subtle regulation is more probable given that small changes in kinase activity may have big effects on protein synthesis. It also should be stated that specific phosphatases, e.g. the type 1 phosphatase (41), may also regulate kinase activity, although this has not been directly demonstrated in an in vivo analysis.

Studies characterizing the anti-inhibitor and its mode of action will be facilitated once the gene for the 58-kDa inhibitor protein itself has been identified. We are in the process of obtaining the cDNA utilizing the microsequence information obtained from the protein purification. Functional analysis of P68 regulation will be further aided now that the gene encoding the protein kinase has been cloned (42) and the kinase expressed both in vitro in reticulocyte lysate (43) and in Escherichia coli (44). Finally, it is important to mention two other reports describing cellular regulators of the protein kinase which appear to be distinct from that described in this study. Saito and Kawakita (45) described an inhibitor partially purified from human FL cells with an apparent molecular mass of greater than 160,000 daltons, whereas Judware and Petryshyn (29) have identified a 15-kDa protein partially purified from 3T3-F442A cells and hypothesized to play a role in the ability of these cells to undergo adipose conversion. It would be of interest to determine how these two proteins regulate the P68 protein kinase and whether they act similarly to the 58-kDa inhibitor.

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REFERENCES

Fig. 6. Model of P68 regulation. Key: P68, interferon-induced protein kinase; P58, cellular 58-kDa inhibitor of the kinase; I-P58, the anti-inhibitor or specific inhibitor of P58; eIF-2, eukaryotic initiation factor 2 α subunit; squares denote inactive molecules and circles active circles. A, P68 activation: dsRNAs induce the autophosphorylation of the kinase which then leads to eIF-2 α phosphorylation and protein synthesis inhibition. B, P68 inhibition: during viral infection (or other environmental signals), the anti-inhibitor (J-P68) becomes dissociated from the kinase inhibitor (P58). The inhibitor, now in an active form, can function either to block the autophosphorylation of the kinase, or if the kinase is already activated, can block the phosphorylation of eIF-2 α. The end result is that protein synthesis is not compromised.
Regulation of the Kinase Cellular Inhibitor