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Constitutive Expression of Human Double-Stranded RNA-Activated p68 Kinase in Murine Cells Mediates Phosphorylation of Eukaryotic Initiation Factor 2 and Partial Resistance to Encephalomyocarditis Virus Growth

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The cDNA encoding interferon-induced human double-stranded RNA-activated p68 kinase was expressed in murine NIH 3T3 cells by using the pcDNA1/neo vector. Several stable clones were selected which expressed either the wild-type kinase or an inactive mutant possessing a single amino acid substitution in the invariant lysine 296 in the catalytic domain II. The transfected wild-type kinase showed properties similar to those of the natural kinase, such as subcellular ribosomal localization and dependence on double-stranded RNA for autophosphorylation. Upon infection with encephalomyocarditis virus (EMCV), wild-type- but not mutant-expressing clones were found to partially resist virus growth. Such natural antiviral activity was virus specific, since no inhibition was observed in the case of vesicular stomatitis virus infection. In accord with EMCV inhibition, the wild-type p68 kinase was found to be highly phosphorylated during infection. Furthermore, its natural substrate, the small subunit of protein synthesis initiation factor eIF2, was phosphorylated. These results demonstrate that p68 kinase is activated during EMCV infection, leading to reduced virus production.

The interferon-induced, double-stranded RNA (dsRNA)-activated kinase is a cellular protein which has been implicated as one of the mechanisms responsible for inhibition of protein synthesis (through phosphorylation of the protein synthesis initiation factor eIF2). It is referred to as a p68 kinase in human cells and a p65 kinase in murine cells on the basis of M_r (15, 16). The p68 and p65 kinases have been extensively characterized by biochemical studies and recently cloned (10, 29). The dsRNA-activated protein kinase is a serine/threonine kinase that manifests two distinct kinase activities, one for autophosphorylation and the other for phosphorylation of its substrate. Activation requires binding of the protein to dsRNA molecules or, alternatively, to single-stranded RNA molecules presenting internal dsRNA structures (12, 13). The regulatory region interacting with dsRNA has been localized toward the middle of the N-terminal region of the protein (10, 21). Once phosphorylated, the p68 kinase is no longer dependent on its activator and phosphorylates its only known physiological substrate, the α subunit of eIF2. This effect has been shown by in vitro studies (11, 12) and in vivo experiments using cells previously treated with interferon to induce the dsRNA-activated kinase and then virus infected to provide for dsRNA activators (32, 37). The function of eIF2α is to allow the formation of the initiation ternary complex eIF2–GTP–Met-tRNA and the subsequent binding of Met-tRNA to the 40S subunit of the ribosomes. When eIF2 completes one round of initiation, it is ejected from the ribosomes as eIF2-GDP, which can be recycled into the active eIF2-GTP complex, a reaction catalyzed by the guanine nucleotide exchange factor eIF2B. Phosphorylated eIF2α inhibits the function of eIF2B by sequestering it and stops any further initiation step (14).

The dsRNA-activated human and murine protein kinases have a strong degree of sequence identity (38%) with the yeast GCN2 kinase in their catalytic kinase subdomains (4, 10). Recombinant p68 kinase has recently been expressed in the yeast *Saccharomyces cerevisiae* and shown to exhibit a growth-suppressive phenotype which could be attributed to its own activation in yeast cells and the subsequent phosphorylation of the *SUI* gene product, the equivalent in yeast cells of the mammalian eIF2α (4, 5). These results reveal the capacity of p68 kinase to mediate inhibition protein synthesis through phosphorylation of eIF2α. Although the yeast system provides an excellent genetic approach to an understanding of the dsRNA-activated kinase function in vivo, the exact role of this kinase in its natural environment, i.e., mammalian cells, remained to be demonstrated, as does its role in the mechanism of the antiviral action of interferon. Here we report the establishment of mammalian clones constitutively expressing the human p68 kinase and present direct evidence for selective reduction of encephalomyocarditis virus (EMCV) growth in these clones concomitantly with in vivo phosphorylation of eIF2α.

MATERIALS AND METHODS

Reagents. [γ-^{32}P]ATP, [^{35}S]methionine, and ^{125}I-labeled goat anti-mouse immunoglobulins were supplied by Amersham. Poly(I)-poly(C), heparin, and cycloheximide (CHX) were from Sigma. Mouse interferon (cytimmune mouse α/β interferon; 5×10^6 international reference units [IRU] per mg) was from Lee Biomolecular Research. Polyclonal antibodies (29) and Sepharose-coupled monoclonal antibody

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(MAb) (11) specific for the p68 kinase were as described previously. Polyclonal antibodies specific for eIF2 were kindly provided by E. Henshaw and R. Panniers.

Buffers. Buffer A contained 20 mM Tris-HCl (pH 7.6), 50 mM KCl, 400 mM NaCl, 5 mM 2-mercaptoethanol, 1% Triton X-100, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 100 U of aprotinin per ml, and 20% (vol/vol) glycerol. Buffer B contained 20 mM Tris-HCl (pH 7.6), 100 mM KCl, 0.1 mM EDTA, 100 U of aprotinin per ml, and 20% (vol/vol) glycerol. Buffer C was buffer B containing 2 mM $MnCl_2$ and 2 mM $MgCl_2$.

Cell cultures and virus. Daudi cells were grown in suspension in RPMI 1640 medium containing 10^{-5} M 2-mercaptoethanol and 10% fetal calf serum; HeLa cells and NIH 3T3 cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (J. Bio/TechGen, Les Ulis, France). NIH 3T3 clones expressing the neomycin resistance gene were grown in the same medium supplemented with 0.4 mg of G418 (Geneticin; GIBCO) per ml. The concentrations of the virus stocks were determined by direct titration on the different NIH 3T3 clones and correspond to 10^9 PFU/ml for EMCV and 2.5×10^6 PFU/ml for vesicular stomatitis virus (VSV).

Plasmid construction. The *Hin*dIII-*Eco*RI cDNA (2,473 bp) encoding the p68 kinase was reconstructed in the vector Bluescript SK M13+ (Stratagene), using the K13 and R6 inserts isolated from a λgt11 expression library (29). The cDNA was then excised by *Hin*dIII-*Bam*HI digestion and inserted into the *Hin*dIII-*Bam*HI-cut pcDNA1/neo eucaryotic expression vector (Invitrogen). The Lys-296→Pro-296 and Lys-296→Arg-296 mutants were generated by site-specific mutagenesis as described elsewhere (21).

Transfection. Murine NIH 3T3 cells were plated in 60-mm-diameter dishes at 10^4 cells per dish and used after 24 h. The culture medium was replaced with fresh medium, and after 3 h at 37°C, 10 μg of pcDNA1/neo vector carrying the wild-type or each mutant p68 kinase was precipitated on the cells by the calcium phosphate coprecipitation technique. After 4 h of incubation, the cells were subjected to a glycerol shock for 2 min, washed once with complete medium, and cultivated for 2 days. Cells were trypsinized and plated in 25-cm^2 flasks in medium containing G418 at a concentration of 200 μg/ml for the first 2 days and then at 400 μg/ml. On day 11, the surviving cells were collected by trypsinization and cloned in 24-well plates in growth medium containing 20% serum and 400 μg of G418 per ml. On day 21, isolated clones were trypsinized and grown for further study. Each transfection gave a yield of 10 to 20 drug-resistant clones.

Virus assay. The cells were plated at 2×10^5 cells per well in 24-well plates. After 36 h, they were washed once with phosphate-buffered saline (PBS) and incubated for 60 min at 37°C with 0.4 ml of PBS containing EMCV or VSV at the multiplicity of infection (MOI) required. The virus-containing medium was removed, and the cells were further incubated in 10% serum-containing medium. At 10 to 12 h after infection, the plates were frozen and thawed twice, and the virus supernatants were collected and centrifuged at 2,500 rpm to eliminate debris. The virus yields from each clone were titered on HeLa cells plated on six-well plates at 3×10^5 cells per well and used after 3 days. The virus (dilutions 10^{-2} to 10^{-7} in PBS) was adsorbed onto the cells for 60 min, the virus-containing medium was removed, and the cells were covered with Dulbecco's modified Eagle's medium containing 0.5% serum and 0.6% agarose (Indubiose A37 HAA; IBF, Villeneuve-la-Garenne, France). After 3 days of incubation at 37°C, the cells were fixed in 5% trichloroacetic acid for 30 min at 4°C, the agarose-containing medium was removed under water, and the plaques were visualized by staining with a solution of 0.1% crystal violet in 20% ethanol.

Assay of the dsRNA-dependent protein kinase. Cell extracts corresponding to 1.5×10^6 cells were incubated with 20 μl of MAb-Sepharose in 0.5 ml of buffer A for a minimum of 3 h at 4°C. MAb-Sepharose-bound kinase samples were washed batchwise first in buffer A and then in buffer B before incubation (15 min, 30°C) in buffer C containing 2 μM [γ-^{32}P]ATP (50 Ci/mmol) and poly(I)-poly(C) (in the concentration range of 0 to 1 μg/ml) or heparin (10 U/ml). The phosphorylation reactions were stopped by the addition of twofold-concentrated sample buffer (130 mM Tris-HCl [pH 6.8], 2 M urea, 8.5% sodium dodecyl sulfate [SDS], 0.1% bromophenol blue, 1.5 M 2-mercaptoethanol, 35% [vol/vol] glycerol). The samples were analyzed by polyacrylamide gel electrophoresis (PAGE) (8.5% gel). ^{32}P-labeled kinase was visualized by autoradiography.

2D gel electrophoretic analysis. For two-dimensional (2D) analysis of p68 kinase, 25 μl of cell extracts was mixed with 25 μl of SDS-PAGE sample buffer, heated (95°C, 3 min), and cooled at room temperature. Then 50 μl of ampholine mix containing 9.5 M urea, 8% Nonidet P-40, and 2% ampholines (pH 3.5 to 10; from a 40% [wt/vol] stock; LKB) was added, and the extracts were first separated by isoelectric focusing on urea-acrylamide gels (9.16 M urea, 3.77% [wt/vol] acrylamide–0.21% [wt/vol] bisacrylamide, 2% Nonidet P-40, 2% ampholines [pH 3.5 to 10]) as described previously (22, 30). The proteins were then further separated in a second dimension by SDS-PAGE (8.5% polyacrylamide). The proteins were transferred to Immobilon-P (Millipore) and analyzed by immunoblotting with specific polyclonal antibodies against the p68 kinase and ^{125}I-labeled goat anti-mouse immunoglobulins (Amersham).

For 2D analysis of eIF2, the same procedure was used except that in the first dimension, two different ranges of ampholines (one in the range of pH 4 to 6 and the other in the range of pH 5 to 8) were mixed in equal parts (9.16 M urea, 3.77% [wt/vol] acrylamide–0.21% [wt/vol] bisacrylamide, 2% Nonidet P-40, 1% ampholines [pH 4 to 6], 1% ampholines [pH 5 to 8]). The proteins were then further separated in a second dimension by SDS-PAGE (12.5% gel) and transferred to an Immobilon-P membrane (Millipore). The presence of eIF2α was analyzed by immunoblotting with specific polyclonal antibodies against eIF2α and ^{125}I-labeled goat anti-mouse immunoglobulins (Amersham).

RESULTS

Isolation of murine clones expressing the human p68 kinase. Murine cells were used in this study since the molecular masses of the human (68 kDa) and murine (65 kDa) dsRNA-activated protein kinases allow discrimination between the endogenous and the transfected kinases. NIH 3T3 cells were transfected with the expression vector pcDNA1/neo carrying cDNA expressing the p68 kinase either as wild type (active kinase) or mutated by a single amino acid substitution to proline or to arginine at the invariant lysine 296 (21). It has previously been shown for a number of other protein tyrosine or serine/threonine kinases that the Lys residue of the catalytic subdomain II is involved in the phosphate transfer reaction and that a single-point mutation involving this amino acid completely inhibits kinase activity (for references, see reference 31). After selection in G418-containing medium, we obtained approximately 10 to 20 neomycin-resistant clones from three different transfection experi-

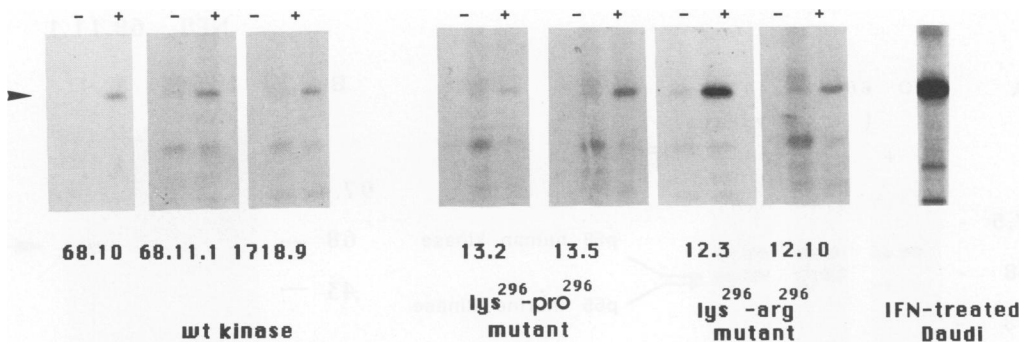

FIG. 1. Screening of stable NIH 3T3 clones expressing the wild-type or mutated human p68 kinase. The different clones were plated in 60-mm-diameter dishes at 3×10^5 cells per plate and used when they reached near confluency (10^6 cells per plate). The cells were treated with CHX (50 μg/ml) (−) and with CHX and poly(I)-poly(C) (100 μg/ml) (+) as described previously (41). After the drug treatment, the cells were washed and labeled with [^{35}S]methionine, and extracts were prepared as described in Materials and Methods. The [^{35}S]methionine-labeled p68 kinase (arrowhead) was immunoprecipitated with a specific MAb coupled to Sepharose as described previously (23) and analyzed by SDS-PAGE on 8% polyacrylamide gels. An autoradiograph of the dried gel is shown. wt, wild type; IFN, interferon.

ments. Initial attempts to detect the p68 kinase in these different clones by Western immunoblotting were impeded by reduced expression of the transfected gene. To enhance the sensitivity of detection, we devised an assay which, by increasing expression of the transfected gene, allowed selection of positive clones on a limited number of cells. For this purpose, we took advantage of the four NF-κB-responsive elements present in the cytomegalovirus promoter of the pcDNA1/neo vector (24) by amplifying expression of the transfected kinase in response to treatment of cells with CHX and the synthetic dsRNA poly(I)-poly(C). Both reagents have been reported to induce the expression of several genes, including the β-interferon gene, through NF-κB activation (9, 25, 33, 40).

Three weeks after transfection and subcloning, neomycin-resistant clones were plated (3×10^5 cells) in duplicate. One culture was retained, and the other was incubated in presence of CHX and poly(I)-poly(C) under conditions described for the induction of dsRNA-responsive genes (40). Cells were labeled in [^{35}S]methionine-containing medium, and the kinase was analyzed by immunoprecipitation with a specific MAb and subsequent gel electrophoresis. In these experiments, we used monoclonal instead of polyclonal antibodies since the latter are known to cross-react with the murine p65 kinase (29) and thus might give false-positive results in our assay. Clones positive for the presence of the p68 kinase are presented in Fig. 1. Poly(I)-poly(C)/CHX treatment resulted in the increased expression of the transfected p68 kinase, while in the absence of such treatment, kinase levels were barely detectable. This procedure allowed us to generate a series of independent clones expressing either the wild-type p68 kinase (clones 68.10, 68.11.1, and 1718.9) or the Lys→Pro (clones 13.2 and 13.5) or Lys→Arg (clones 12.3 and 12.10) mutant proteins. Some differences in the levels of the induced kinase were observed and could be attributed to different efficiencies of transfection and/or to respective NF-κB levels for each clone. Other various background bands can be detected, the nature of which is not known. They probably represent nonspecific binding to the anti-p68 kinase MAb. The most predominant band of 40 kDa disappears in some clones after the drug treatment and may represent a protein with a short half-life that is sensitive to CHX inhibition of protein synthesis.

Our induction procedure proved useful for the screening of large numbers of clones by using only a limited number of cells soon after transfection and could probably be an efficient method for screening other gene products under control of promoters containing NF-κB-responsive elements. It should be noted that poly(I)-poly(C)/CHX treatment was used only for selection of clones and not in other experiments.

Expression of the p68 kinase in stable transfectants. To further analyze the in vivo expression of the p68 kinase, we selected one clone representing each of our constructs: clone 68.11.1, obtained for the wild-type kinase, and clone 12.3, obtained for the inactive Arg-296 kinase. One clone (Neo) was selected for the expression of pcDNA1/neo alone and served as a control. Extracts were prepared from 1.5×10^6 cells in the absence or presence of interferon treatment and were assayed for the presence of the transfected kinase by Western blot analysis. The assay was performed with polyclonal antibodies in order to detect both the transfected (p68) and endogenous (p65) kinases. Results in Fig. 2A show that the wild-type and the mutant p68 kinases are constitutively expressed in clones 68.11.1 and 12.3, respectively. Treatment of both clones with murine interferon did not affect expression of the transfected kinase but did induce the murine endogenous p65 kinase to levels comparable to those induced in the Neo clone alone. The capacity to induce the murine p65 kinase following interferon treatment indicates that our cloning procedure did not affect the ability of the cells to respond to interferon. Both p65 and p68 kinases were detected in the same blot by using polyclonal antibodies raised against the human p68 kinase which cross-react with the murine kinase (29). The cDNA for the murine p65 kinase has recently been cloned and sequenced, revealing a high level of sequence homology with the human kinase (71% overall homology when conservative changes were taken into account [10]). Besides the murine p65 kinase, anti-p68 kinase polyclonal antibodies cross-react with a 80-kDa protein which is constitutively expressed in these murine cells (Fig. 2A). The nature of this protein is not known. A similar protein is also detectable in human cells. Since polyclonal antibodies were raised against pure preparations of urea-denatured p68 kinase (29), the detection of this 80-kDa protein might be due to some epitopes common to the two proteins. However, it should be noted that detection of the 80-kDa protein is not specific to polyclonal antibodies against p68 kinase, since a similar protein is also detected by

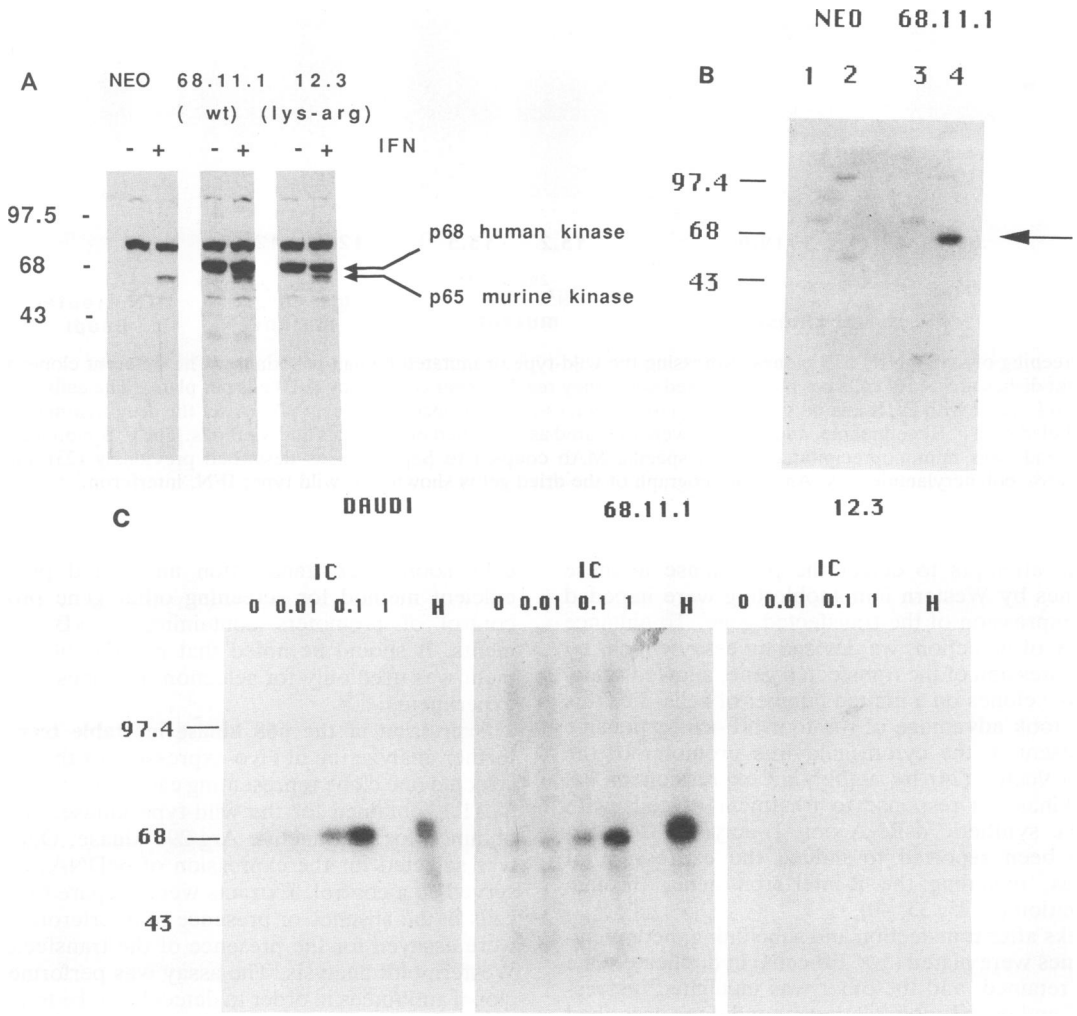

FIG. 2. (A) Constitutive expression of the p68 kinase in wild-type and mutant clones. The clones expressing the pcDNA1/neo vector alone (Neo), the wild-type p68 kinase (68.11.1; wt), or the p68 kinase carrying the Lys-296→Arg-296 mutation (12.3; lys-arg) were grown in the absence (−) or presence (+) of 500 U of mouse α/β interferon per ml for 18 h, and the cell extracts were prepared as described previously (13). Extracts corresponding to 1.3×10^6 cells were separated by PAGE (8.5% polyacrylamide gel) and analyzed by immunoblotting with polyclonal antibodies specific for human p68 kinase (29) and anti-mouse immunoglobulin antibodies coupled to horseradish peroxidase (ECL; Amersham). (B) Association of the transfected p68 kinase with the postribosomal fraction. Clones Neo (lanes 1 and 2) and 68.11.1 (lanes 3 and 4) were lysed in homogenization low-salt buffer, and mitochondrial (lanes 1 and 3) and microsomal (lanes 2 and 4) pellets were prepared (Materials and Methods). Equivalent aliquots corresponding to 1.5×10^6 cells were separated by PAGE (8.5% polyacrylamide gel) and assayed by immunoblotting (29) with polyclonal antibodies specific for the human p68 kinase and ^{125}I-labeled goat anti-mouse immunoglobulins (Amersham). An autoradiograph is shown. (C) Functional expression by the transfected kinase. MAb-Sepharose-purified p68 kinase (corresponding to 1.5×10^6 cells) was prepared from clone 68.11.1 (wild-type kinase) and from clone 12.3 (Arg-296 mutant kinase). The enzyme was assayed for autophosphorylation in the presence of different concentrations of poly(I)-poly(C) (IC; 0, 0.01, 0.1, and 1 μg/ml) or 10 U of heparin per ml (lanes H) as described in Materials and Methods (17). Extracts from interferon-treated Daudi cells (1.3×10^6 cells) were included in the assay as controls. The samples were analyzed by SDS-PAGE (8.5% gel). An autoradiograph is shown. Sizes are indicated in kilodaltons.

polyclonal antibodies raised against urea-denatured 69-kDa 2′,5′-oligoadenylate (2-5A) synthetase (not shown) (16).

Subcellular fractionation studies in Daudi (12) and HeLa (8) cells have previously shown that the natural human p68 kinase is associated with the microsomal pellet, i.e., the 100,000 × *g* pellet also containing ribosomes. Results in Fig. 2B show that the human p68 kinase expressed in clone 68.11.1 was also found to be associated with the ribosomal pellet. In these experiments, the endogenous p65 kinase was not clearly detectable since control cells (not interferon treated) were used.

The p68 kinase from the wild-type and the Arg-296 mutant clones were assayed for protein kinase activity in vitro (Fig. 2C). The transfected wild-type kinase manifested p68 kinase activity, as demonstrated by autophosphorylation in the presence of heparin or poly(I)-poly(C). In the latter case, autophosphorylation is dependent on the concentration of poly(I)-poly(C) and is one of the major properties of the p68 kinase (12). A similar pattern of activation was observed for the transfected wild-type kinase and for the natural p68 kinase (Fig. 2C). As expected, the Arg-296 mutant manifested no p68 kinase activity.

TABLE 1. EMCV and VSV yields from clones constitutively expressing the p68 kinase[a]

Expt	Clone	EMCV		VSV		IFN + EMCV	
		Log PFU	ΔLog	Log PFU	ΔLog	Log PFU	ΔLog
1	Control (Neo)	7.80		7.44			
	Wild type						
	68.11.1	6.85	0.95	7.88			
	68.10	6.65	1.15	7.39	0.05		
	Mutant						
	12.3	7.80		7.60			
	13.5	7.60	0.20	ND			
2	Control (Neo)	6.98		7.43			
	Wild type						
	68.11.1	5.00	1.98				
		5.20	1.78	7.52			
	68.10	5.27	1.71				
		5.44	1.51	ND			
	1718.9	4.88	2.10				
		5.39	1.59	7.29	0.14		
3	Control (Neo)	6.81				4.48	
	Wild type						
	68.11.1	5.87	0.94			2.69	1.79
	1718.9	5.69	1.12			3.87	0.61
	Mutant 12.3	6.81				4.67	

[a] The cells were infected with EMCV or VSV at an MOI of 0.1 PFU per cell, and the virus yields from each clone were titered on HeLa cells (Materials and Methods). Virus yields from two independent experiments are presented. In experiment 2, some of the virus yields were titered in duplicate. In experiment 3, the cells were first untreated (EMCV) or treated with 500 U of interferon per ml before EMCV infection (IFN + EMCV). ND, not determined.

Thus, the human p68 kinase can be expressed constitutively in murine NIH 3T3 cells with properties (in vitro autophosphorylation and subcellular localization) identical to those of the natural kinase expressed in human cells.

Inhibition of EMCV growth in clones expressing the human p68 kinase. We then assessed whether clones expressing the wild-type p68 kinase (68.11.1, 1718.9, and 68.10) could resist viral infection in comparison with the control Neo clone and clones expressing the mutant kinase (12.3 and 13.5). Clones were infected with EMCV at a low MOI (0.1), and after 10 h of infection (one cycle = 7 to 8 h), plates were frozen and thawed and virus yields were plaque assayed. Growth of the virus was found to be consistently lower in wild-type kinase-expressing clones than in the control Neo clone or clones expressing the mutant inactive kinase. The degree of inhibition of EMCV yield in wild-type kinase-expressing cells was variable from one experiment to the other. Table 1 gives the results of three typical experiments that illustrate the degree of minimal (0.94-log-unit) and maximal (2.1-log-unit) inhibition observed. Our results therefore show that constitutive expression of the p68 kinase can produce significant resistance to EMCV growth (90 to 99% inhibition). This antiviral state, however, remains lower than a fully antiviral state established after interferon treatment. This effect was monitored by interferon treatment prior to EMCV infection in some representative clones which showed a maximum antiviral response corresponding to 1.8- to 3.2-log-unit inhibition (Table 1, experiment 3). It is interesting to note that in the interferon-treated clones, EMCV yields were reduced 0.6 to 1.8 log units more in the wild-type than in the Neo or mutant kinase clones. Since the kinase-expressing clones showed about a log reduction in virus yields without interferon treatment, this increased inhibition in EMCV yields probably reflects additive antiviral actions between the constitutively expressed kinase and other antiviral events induced by interferon in these clones (including the induced endogenous murine kinase). It may also be possible that the transfected kinase contributes to transcriptional activation of interferon-related genes involved in antiviral action. Taken together, the significant reduction of EMCV yields in wild-type-expressing clones compared with those expressing the mutant inactive kinase clearly implicates the function of the p68 kinase in an antiviral action against EMCV.

Resistance of kinase-expressing clones to EMCV growth was also found to be MOI dependent (Fig. 3). As the MOI increased from 0.1 to 1 or 5, the wild-type-expressing clones showed decreased resistance to the virus whereas clone Neo and two clones expressing the inactive kinase supported virus growth at comparable levels for each MOI. These data suggest that clones expressing the wild-type kinase are less resistant to EMCV infection when many viral particles are involved. This situation is similar to the effect of increasing MOI on the antiviral activity of interferon. However, it should be noted that for each MOI used in the experiments described here, the virus yields obtained from the wild-type-expressing clones remained lower than the yields obtained from the controls. This result again argues in favor of constitutive EMCV resistance in the wild-type kinase-expressing clones.

The purified p68 kinase has been shown to be activated in vitro by low concentrations of dsRNA, whereas high concentrations of dsRNA render the kinase inactive (13). In view of this finding, it is possible that the decreased anti-EMCV resistance in the wild-type-expressing clones is due to inactivation of the wild-type p68 kinase by high concentrations of dsRNA-like structures that accumulate at high MOI.

VSV growth is not inhibited in clones expressing the wild-type kinase. The growth of VSV was not inhibited in any of the clones expressing the wild-type kinase compared with Neo or mutant kinase-expressing clones (Table 1). Similar distinctions between the two viruses (reduction of EMCV but not of VSV yields) were also reported for clones constitutively expressing the small 2-5A synthetase (3, 6). This specificity indicates that the antiviral action of interferon

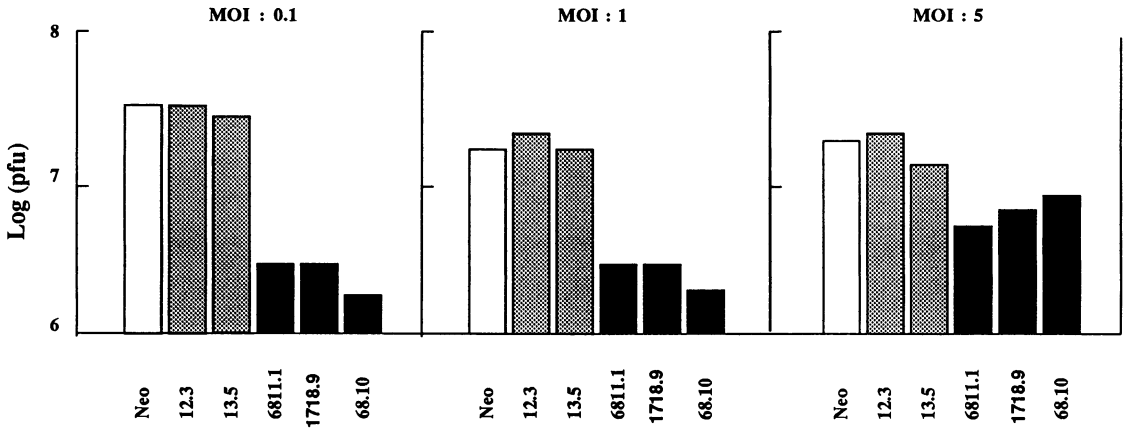

FIG. 3. Effect of MOI on EMCV yields from clones expressing the p68 kinase. Cells were infected as described in Materials and Methods. The probability of the cells being infected by more than one viral particle can be calculated according to the Poisson distribution: $P(>1) = 1 - me^{-m}(m + 1)$, where m is MOI.

against VSV is mediated by mechanisms distinct from the 2-5A synthetase or kinase pathway.

The in vivo state of phosphorylation of the transfected p68 kinase. EMCV growth was reduced in clones expressing the wild-type p68 kinase but not in clones expressing a mutant p68 kinase. This finding suggested that the expression of a functional kinase was required for the inhibition of virus growth. For this reason, we analyzed the in vivo autophosphorylation state of the p68 kinase and its ability to phosphorylate its natural substrate, eIF2. In these experiments, we used polyclonal antibodies specific for the p68 kinase and the α subunit of eIF2 to monitor by immunoblotting the phosphorylated and unphosphorylated species of each protein after resolution by 2D gel analysis (Fig. 4 and 5).

Following 2D gel analysis, the p68 kinase was revealed as several related species with similar isoelectric points in the range of 8.2 to 7.8 (22). These species represent unphosphorylated and some slightly phosphorylated p68 kinase molecules. Because of their capacity to be phosphorylated at several sites, highly phosphorylated p68 kinase molecules became resolved as highly acidic species (22, 23). We therefore used a shift toward the acidic pH value as a reference for the phosphorylated state of the wild-type and mutant p68 kinases with or without infection by EMCV (Fig. 4). In the absence of virus infection, the wild-type and mutant p68 kinases were expressed predominantly as unphosphorylated species. In addition, a minor phosphorylated form, which represents 10% of the total protein, can be

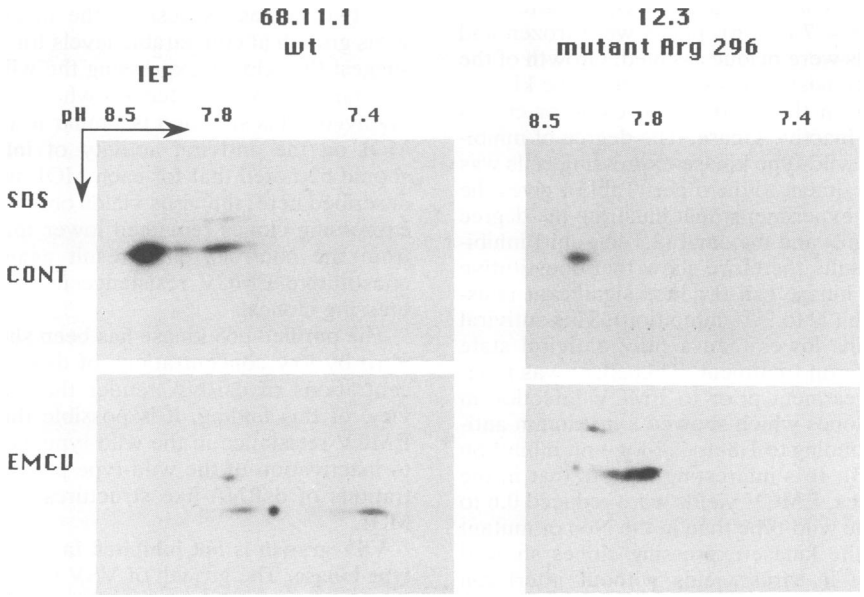

FIG. 4. In vivo phosphorylation of the p68 kinase during EMCV infection in transfected clones. Cells from clone 68.11.1 (wild-type [wt] kinase) and from clone 12.3 (Arg-296 kinase) were uninfected or infected with EMCV at an MOI of 0.1 PFU per cell. Extracts were prepared at 6 h postinfection; 25 μl of cell extracts corresponding to 1.3×10^6 cells were mixed with 25 μl of SDS-PAGE sample buffer, heated (95°C, 3 min), and separated by 2D gel electrophoresis (Materials and Methods). The proteins were transferred to Immunobilon-P (Millipore) and analyzed by immunoblotting with specific polyclonal antibodies against the p68 kinase and ^{125}I-labeled goat anti-mouse immunoglobulins (Amersham). An autoradiograph is shown. IEF, isoelectric focusing; CONT, control.

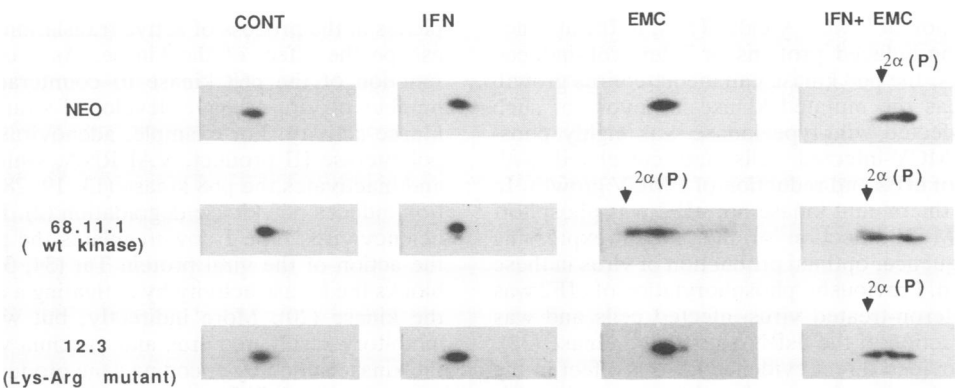

FIG. 5. In vivo phosphorylation of eIF2α during EMCV infection in transfected clones. Cells from clones Neo, 68.11.1 (wild-type [wt] kinase), and 12.3 (Arg-296 kinase) were untreated or treated with murine interferon (500 U/ml) for 18 h and not infected or infected with EMCV at an MOI of 1 PFU per cell. Extracts were prepared at 6 h postinfection; 25 μl of cell extracts corresponding to 1.3×10^6 cells from control (CONT), interferon-treated (IFN), EMCV-infected (EMC), and both interferon-treated and EMCV-infected (IFN+ EMC) cells were mixed with 25 μl of SDS-PAGE sample buffer, heated (95°C, 3 min), and separated by 2D gel electrophoresis as described in Materials and Methods. The proteins were then transferred to Immunobilon-P (Millipore). The presence of eIF2α was analyzed by immunoblotting with specific polyclonal antibodies against eIF2α and ^{125}I-labeled goat anti-mouse immunoglobulins (Amersham). An autoradiograph is shown. The arrow indicates the position of the phosphorylated (acidic) form of eIF2α [2α(P)].

observed in the pattern of the wild-type kinase (Fig. 4). Analysis of extracts prepared at 6 h postinfection with EMCV (a time when most of the virus was under active replication) revealed differences in the phosphorylation patterns of the wild-type and mutant kinase; the wild-type p68 kinase from infected cells migrated as a highly phosphorylated subspecies as expected from in vivo activation through the viral dsRNA replicative intermediates. In addition, we observed a significant decrease in the physical levels of the kinase. On the other hand, the Arg-296 mutant kinase showed only a partially phosphorylated pattern, and its level remained unchanged. Phosphorylation of the mutant kinase was not surprising since although the Lys→Arg substitution abrogates the kinase function, it leaves intact the ability of the protein to be phosphorylated at its serine and threonine residues.

We have previously observed loss of the human p68 kinase upon EMCV infection. This effect, which was attributed to trapping of the kinase in cellular compartments, not accessible to Triton X-100 extraction (8, 15). However, the mutant kinase was not lost during EMCV infection, suggesting that EMCV-mediated trapping of the kinase to membrane compartments may require activation of the kinase. The dramatic shift toward the acidic pH value indicates that the wild-type kinase is highly phosphorylated during EMCV infection.

In vivo phosphorylation of eIF2 in clones expressing the wild-type kinase upon EMCV infection. The wild-type p68 kinase (in clone 68.11.1) can be activated in vivo as it becomes highly phosphorylated upon infection with EMCV (Fig. 4). Therefore, we next analyzed the ability of this transfected p68 kinase to phosphorylate eIF2 during EMCV infection. The acidic (phosphorylated) and basic (unphosphorylated) subspecies of eIF2 were separated on isoelectric focusing gels. We expected that in clone 68.11.1, which expressed the wild-type p68 kinase, eIF2 would become phosphorylated during EMCV infection regardless of interferon treatment, whereas clone 12.3, which expressed an inactive kinase, should behave as the Neo clone does. In these latter two clones, eIF2 may become phosphorylated in interferon-treated and virus-infected cells as a result of the induction and activation of the endogenous murine p65 kinase. The results (Fig. 5) show that this is indeed the case. In EMCV-infected clone 68.11.1, eIF2 was resolved as two subspecies, one basic (60%) and one acidic (40%), representing the unphosphorylated and phosphorylated forms, respectively (Fig. 5). However, in EMCV-infected clone 12.3, eIF2 was resolved only as a major basic unphosphorylated species. When clone 68.11.1 was first treated with interferon and then infected, the percentage of phosphorylated eIF2 increased to 50% of the total eIF2. Such additional phosphorylation may be attributed to the endogenous murine p65 kinase, which is induced by interferon (Fig. 1A) and becomes activated along with the constitutive wild-type p68 kinase. In EMCV-infected clone 12.3, eIF2 was phosphorylated only after interferon treatment, similarly to the Neo clone and as a result of interferon induction of the endogenous mouse p65 kinase and its subsequent activation during EMCV infection.

DISCUSSION

We have constitutively expressed the human dsRNA-dependent p68 kinase in murine NIH 3T3 cells under the control of the cytomegalovirus promoter. Transfection of the p68 kinase cDNA either as wild type or as a catalytic subdomain II mutant cDNA allowed the generation of several clones expressing either a functional kinase or its inactive homolog.

The transfected p68 kinase was found to be associated with the ribosomal fraction, as described for the natural human p68 kinase (8, 12). Moreover, it possessed the same dependence on activation by dsRNA or heparin as did the natural p68 kinase (17). Therefore, we can assume that the transfected recombinant human p68 kinase expressed in NIH 3T3 clones has retained the intrinsic properties of its natural human counterpart. Since this enzyme is expressed in the absence of other interferon-induced gene products, NIH 3T3 clones therefore provided a useful system with which to determine the role of the p68 kinase in the mechanism of the antiviral action of interferon.

Clones expressing the wild-type but not the mutant kinase

exhibited a reduction of EMCV yields. Thus, in the absence of other interferon-induced proteins or interferon-induced metabolic changes, the p68 kinase can mediate virus growth reduction, whereas the mutated kinase is devoid of such effect. The transfected wild-type kinase was highly phosphorylated in EMCV-infected cells and correlated with phosphorylation of eIF2 and reduction of EMCV growth. In contrast, neither the mutant kinase nor eIF2 was phosphorylated during EMCV infection of the mutant-expressing cells. As a consequence, optimal production of virus in these cells was observed. Previously, phosphorylation of eIF2 was detected in interferon-treated virus-infected cells and was attributed to the action of the dsRNA-activated kinase (32). Here we have provided direct evidence for this effect in the absence of any other interferon-induced proteins. Accordingly, the wild-type kinase was activated during EMCV infection, leading to autophosphorylation and phosphorylation of eIF2. These results show that the dsRNA-activated protein kinase is directly implicated in eIF2 phosphorylation, resulting in reduced virus growth.

The inhibitory action of the wild-type p68 kinase was restricted to EMCV, as no reduction was observed for the growth of a rhabdovirus, VSV. Such a differential antiviral action has been observed previously in various cells expressing the transfected 40- to 46-kDa 2-5A synthetase in which the growth of EMCV and mengovirus was reduced, whereas that of VSV and herpes simplex virus type 2 was not affected (3, 6, 36). Taken together, these observations indicate that the two interferon-induced dsRNA-activated enzymes, the p68 kinase and the 2-5A synthetase, restrict their antiviral action to certain viruses. This effect is probably a consequence of the replication cycle of each virus.

The primary site of the antiviral action of interferon on EMCV is at the translational level, whereas that on VSV may occur at the level of primary transcription (1, 27) or/and viral protein synthesis (39). Virus maturation may also be affected (26, 38). A differential antiviral response was also reported in interferon-treated HeLa cells, in which the p68 kinase was shown to be reduced during a heat shock stress; cells remained antiviral for VSV but not for EMCV (7). Thus, loss of the p68 kinase was associated with loss of anti-EMCV resistance. In clones expressing the wild-type kinase, the resistance to EMCV was at a lower efficiency than in clones expressing the 2-5A synthetase (3, 6, 36). This difference is most probably due to amplification of the action of 2-5A by synthesis of 2′-5′-linked oligoadenylates which then activate a latent endoribonuclease responsible for degradation of RNA (16).

Direct observations by 2D gel electrophoresis experiments to monitor the state of phosphorylation of eIF2 showed that about 40% of the α subunit can be phosphorylated by 6 h postinfection, a time when most of the viral replicative dsRNA intermediates of the virus accumulate. It has been previously estimated that 30% phosphorylation of eIF2 would be sufficient to arrest total cellular protein synthesis (14). If this was the case in our wild-type kinase-expressing clones, then viral protein synthesis should have been completely blocked, leading to a profound inhibition of virus yield. However, our results show only 0.9- to 2.1-log inhibition of EMCV yields. The reason for this contradiction may be due to the differences between cell lines investigated. For example, it is plausible that the generation of active eIF2-GTP complexes in NIH 3T3 cells is more efficient because of the presence of higher eIF2B pools. It may also be possible that although most of the protein initiation is arrested in cells, a few polysomes are sequestered by the virus complexes in the process of active translation and may therefore escape the effect of the kinase. As a consequence of the function of the p68 kinase to counteract virus growth, a number of viruses have developed strategies to block p68 kinase activity. For example, adenovirus encodes an RNA polymerase III product, VAI RNA, which complexes with and inactivates the p68 kinase (13, 19, 28). Poliovirus infection induces p68 kinase degradation (2). Human immunodeficiency virus type 1 downregulates the p68 kinase through the action of the viral protein Tat (34, 35). Influenza virus blocks the kinase activity by activating a cellular inhibitor of the kinase (20). More indirectly, but with the same final inhibitory result, reovirus and vaccinia virus downregulate the kinase action by encoding gene products that bind to and sequester the dsRNA activator of the p68 kinase (18, 42).

Under routine culture conditions, we did not find any slow-growth phenotype for the wild-type p68 kinase-expressing clones compared with the Neo control or with clones expressing the mutated inactive p68 kinase. Furthermore, FACScan analysis showed that the clones expressing the wild-type kinase manifest a tendency to grow somewhat better than the Neo clone, with four divisions rather than three in 3 days (29a). The normal growth conditions of our clones expressing the kinase indicate that the NIH 3T3 cells do not express sufficient activators of the kinase. This situation may, however, be different for other cell lines. It is possible that transfection of vectors expressing the wild-type kinase in cells containing high levels of natural activators for the p68 kinase would affect the establishment of stable clones. In the latter case, the activated p68 kinase will become autophosphorylated and catalyze phosphorylation of eIF2 pools, leading to a slow-growth phenotype. In accord with this view, recent data have shown that expression of the wild-type p68 kinase in yeast cells results in a dramatic slow-growth phenotype due to the activation of the kinase by natural dsRNA structures present in yeast cells and to phosphorylation of the *SUI* gene product, the yeast homolog of the mammalian eIF2 (4). It is possible that the degree of activity of the p68 kinase on eIF2 depends on its serine/threonine phosphorylation ratio. It has been reported previously that the phosphorylated serine/threonine ratio changes after interferon treatment (22). This may also be the case during EMCV infection and in yeast cells. It is therefore of interest to analyze the phosphorylated serine/threonine ratio of the functional p68 kinase.

In conclusion, we have obtained constitutive expression of the human p68 kinase in murine NIH 3T3 cells, the expression of which affects EMCV growth and correlates with phosphorylation of eIF2. The possibility that the p68 kinase also inhibits yeast growth through phosphorylation of eIF2 emphasizes the important role of this enzyme in mechanisms regulating protein synthesis. In a stress situation, such as amino acid starvation in yeast cells or virus infection in mammalian cells, kinases responsible for eIF2 phosphorylation may become directly activated and consequently turn off protein synthesis. In the case of virus infection in mammalian cells, the kinase levels may not be sufficient to counteract virus infection. However, once kinase levels are increased by interferon treatment, the enzyme can be activated during virus infection, resulting in inhibition of virus.

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