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Selective Inhibition of Virus Protein Synthesis by Prostaglandin A_1: a Translational Block Associated with HSP70 Synthesis

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Cyclopentenone prostaglandins are potent inhibitors of virus replication. The antiviral activity has been associated with the induction of 70-kDa heat shock protein (HSP70) synthesis. In this report, we describe that in African green monkey kidney cells infected with Sendai virus (SV) and treated with prostaglandin A_1 (PGA_1), SV protein synthesis was selectively blocked as long as HSP70 was being synthesized by the host cell. The block appeared to be at the translational level, as indicated by the following (i) PGA_1 had no effect on SV primary transcription, and a dramatic decrease in the abundance of SV mRNA occurred only at later stages of infection; and (ii) treatment with PGA_1 started at 6 h postinfection, at which time SV mRNA had already accumulated in infected cells, did not suppress the levels of NP mRNA, but it reduced the amount of ribosome-bound NP mRNA and caused a dramatic decrease in the level of genomic RNA. The PGA_1-induced block of SV protein synthesis appeared to be cell mediated, since it was prevented by actinomycin D, while PGA_1 had no effect on SV mRNA translation in vitro. The possibility that HSP70 could be a mediator of the antiviral effect is suggested by the fact that treatment with other classical inducers of HSP70, including sodium arsenite, cadmium, and heat shock at 42°C for 5 h, also selectively prevented SV protein synthesis as long as heat shock protein synthesis occurred. Moreover, SV protein synthesis was not inhibited by PGA_1 in murine Friend erythroleukemic cells, which lack the ability to induce HSP70 expression in response to PGA_1.

Prostaglandins (PGs) are a class of naturally occurring cyclic 20-carbon fatty acids with potent biological properties. In eukaryotic cells, they are synthesized from polyunsaturated fatty acid precursors derived from the phospholipid pool of the cell membrane in response to external stimuli, such as cell injury and inflammation (30). PGs function as microenvironmental hormones involved in the regulation of physiological and pathological processes, including inflammation (41) and the febrile response (12), cell proliferation and differentiation (31), cytoprotection (28), and virus replication (37).

The ability of PGs of the A type (PGAs) to inhibit virus replication and prevent the establishment of persistent infections was first reported in 1980 (33). It is now well established that PGs containing an α,β-unsaturated carbonyl group in the cyclopentane ring structure (cyclopentenone PGs, i.e., PGAs and PGJs) possess a potent antiviral activity against a wide variety of DNA and RNA viruses, including poxviruses (39), herpesviruses (18, 43), orthomyxoviruses (35), paramyxoviruses (1), picornaviruses (3), rhabdoviruses (5, 38), togaviruses (23), and retroviruses (4, 13, 18), in different types of mammalian cells. The mechanism of the antiviral action appears to be complex. While some authors have described a block of virus RNA transcription (5, 43), several reports have demonstrated a selective effect on the synthesis and/or maturation of specific virus proteins (32, 38, 39).

Sendai virus (SV), a nonsegmented single-stranded RNA virus of negative polarity, is one of the first virus models used for the study of PGA antiviral activity. SV, like other paramyxoviruses, consists of an inner nucleocapsid containing the genomic 15-kb RNA tightly associated with the nucleocapsid protein (NP), surrounded by an envelope composed by an internal nonglycosylated polypeptide, M, and by two viral glycoproteins protruding from the surface of the virion, the HN protein, which has both hemagglutinating and neuroaminidase activities, and the F protein, which plays an essential role in hemolysis, cell fusion, and infectivity of the virion (16). The core structure represents the template for viral mRNA synthesis as well as antigenome synthesis. The viral polymerase responsible for RNA synthesis is a complex formed by L and P proteins, which are also associated with the nucleocapsid. The polymerase complex is thought to enter the nucleocapsid template at the 3′ end and to sequentially synthesize the leader and the NP, P, M, F, HN, and L mRNAs by terminating and restarting at each of the gene junctional stop-start signals. The minus-strand genome is also a template for the synthesis of antigenomes, unmodified full-length complements of the genome, which serve as intermediates in genome replication. SV genomes and antigenomes are found only encapsidated with the NP protein, and their synthesis is dependent on ongoing protein synthesis. In a model proposed by Kolakofsky and Blumberg (20), the switch from transcription to replication would be determined by the level of intracellular unassembled NP protein.

PGA_1 has been shown to specifically alter the glycosylation and intracellular transport of the virus glycoproteins HN and F, causing a block of SV maturation and budding from infected cells (32). No inhibition of SV protein synthesis was noted 24 h postinfection (p.i.), at the time of the PGA_1-induced block of virus maturation. In this model, PGA_1 caused a dramatic (>99%) suppression of virus yield at nontoxic concentrations, which did not inhibit cellular nucleic acid or protein synthesis and induced the synthesis of a 70-kDa heat shock protein (HSP70) in both uninfected and SV-infected cells (1). The analysis of the kinetics of HSP70 induction and synthesis after PGA_1 treatment in SV-infected cells has led us to the inter-

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esting finding that SV protein synthesis is selectively and completely blocked as long as HSP70 is synthesized by the host cell and is turned on only after HSP70 synthesis returns to control levels. The block appears to be at the translational level, since primary RNA transcription is not affected by PGA_1 and, in cells treated with PGA_1 6 h p.i., SV RNA is transcribed and accumulated while the amount of SV mRNA bound to polysomes is reduced. The block of SV protein synthesis was found to be dependent on HSP70 synthesis.

MATERIALS AND METHODS

Cell culture and virus infection. The establishment and culture of African green monkey kidney (AGMK) 37RC cells have been described elsewhere (32). 37RC cells and murine Friend erythroleukemic cells (FLC; strain 745A) were grown in RPMI 1640 medium supplemented with 5% fetal calf serum (Gibco) and antibiotics at 37°C in a 5% CO_2 atmosphere. PGA_1 and PGJ_2 (Cayman Chemical Co.) were stored as a 100% ethanolic stock solution (10 mg/ml) and tested at the concentration of 4 μg/ml unless otherwise specified. Control medium contained the same concentration of ethanol (0.02%), which did not affect cell metabolism or virus replication. Sodium *m*-arsenite and cadmium chloride (Sigma Chemical Co.) were dissolved in water. For the heating procedure, confluent monolayers were submersed in a temperature-controlled water bath (Grant Instruments, Cambridge, Mass.) at 42 ± 0.01°C for 5 h.

Preparation of SV by allantoic inoculation of embryonated eggs has been previously described (32). Confluent 37RC cell monolayers or FLC in suspension were washed with phosphate-buffered saline (PBS) and infected with SV (5 hemagglutinating units [HAU]/10^5 cells) for 1 h at 37°C. After this time, virus inocula were removed and monolayers were washed three times with PBS and incubated in RPMI 1640 containing 2% fetal calf serum. All treatments were started after the 1-h adsorption period unless specified otherwise. Virus production was determined by measuring the HAU present in the medium of infected cells at different times p.i. Hemagglutinin titrations were done according to standard procedures, using human type O Rh^+ erythrocytes (32).

Protein synthesis, immunoprecipitation, and PAGE analysis. At different times after SV infection, confluent cell monolayers were labeled with [^{35}S]methionine (5 μCi/2 × 10^5 cells unless specified otherwise) in methionine-deprived medium containing 5% dialyzed fetal calf serum. Cells were usually preincubated for 15 min in methionine-free medium, and the compounds tested were not present during the labeling period. After labeling, cells were washed and lysed in lysis buffer (2% sodium dodecyl sulfate [SDS], 10% glycerol, 0.001% bromophenol blue, 0.1 M dithiothreitol, 0.0625 M Tris-HCl [pH 6.8]), and the radioactivity incorporated was determined (32). Samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) in a vertical slab gel apparatus (3% stacking gel and 10% resolving gel unless specified otherwise) and processed for autoradiography (1). Autoradiograms were quantified densitometrically by using a laser beam densitometer (Ultroscan XL; LKB) as described previously (32), and bands were expressed as relative peak areas. Virus proteins were identified on the basis of M_r and in relation to the position of viral marker proteins from unlabeled egg-grown purified SV.

For immunoprecipitation, metabolically labeled cells were lysed in radioimmunoprecipitation assay buffer (1% Nonidet P-40, 0.5% deoxycholate, 150 mM NaCl, 10 mM Tris-HCl [pH 7.4]). Nuclei were removed by centrifugation for 5 min, and the cytoplasm-containing supernatants were incubated with an anti-HSP70 monoclonal antibody (Amersham) or anti-Sendai polyclonal antibody (kindly supplied by L. Roux, University of Geneva Medical School, Geneva, Switzerland) for 12 h at 4°C and for additional 90 min in the presence of Pansorbin (Calbiochem, La Jolla, Calif.). After centrifugation, pellets were washed three times with radioimmunoprecipitation assay buffer containing 0.1% SDS and once with PBS. Proteins were eluted from pellets by boiling in gel sample buffer and analyzed by SDS-PAGE as described above.

Immunoblot analysis. Equal amounts of protein for each sample were separated by SDS-PAGE and blotted to nitrocellulose as described previously (36). After transfer, the filters were incubated with anti-HSP70 monoclonal antibody 3A3 (kindly provided by R. Morimoto, Northwestern University, Evanston, Ill.), which recognizes both the 73-kDa constitutive HSC70 and 72-kDa inducible HSP70, diluted 1:100 in TEN-Tween 20 buffer (0.05 M Tris-HCl [pH 7.4], 5 mM EDTA, 0.15 M NaCl, 0.05% Tween 20). The bound antibody was detected by a horseradish peroxidase-linked sheep anti-mouse antibody (Amersham) as previously described (36).

Isolation of SV RNA. SV genome RNA and mRNA were isolated from virus-infected cells as described by Leppert et al. (21). Briefly, cell monolayers treated with PGA_1 or control diluent were washed with PBS and lysed in buffer containing 0.15 M NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.5% Nonidet P-40. The nuclei were removed by centrifugation at 4,000 × *g* for 4 min, and the cytoplasmic extracts were layered on a preformed 20 to 40% (wt/wt) CsCl density gradient. The gradients were centrifuged for 16 h at 32,000 rpm in a Beckman SW41 rotor. After collection of the viral nucleocapsid fraction, genomic RNA (NC RNA) was recovered by phenol extraction and ethanol precipitation. The CsCl pellet fraction, containing the unencapsidated viral RNAs, i.e., mRNAs and some of the leader RNAs (21), was resuspended in distilled water and precipitated with ethanol. For total cytoplasmic RNA isolation, the RNA-containing supernatants were directly extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and then precipitated with ethanol.

Dot blot hybridization. CsCl pellet (SV mRNA) and NC RNA (20 μg) were denatured in a buffer containing 7 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 14% formaldehyde at 65°C for 15 min. Serial twofold dilutions of each sample were applied to Hybond-N membranes. Hybridization was carried out in a solution containing 50% formamide, 5 × SSC, and 10× Denhardt's solution at 42°C overnight, using as a probe either the nick-translated plasmid containing sequences of the NP gene (clone SN11) (14) or the β-actin gene. Following hybridization, filters were washed twice for 30 min at 43°C in 2× SSC–0.1% SDS and once for 15 min at 65°C in 0.1× SSC–0.1% SDS, air dried, and exposed to X-ray film.

Northern (RNA) blot analysis. Cytoplasmic RNAs were separated in 1.2% agarose–formaldehyde gels as described by Maniatis et al. (22) and transferred to Hybond-N membranes (Amersham). Hybridizations with the SN11 plasmid were carried out in the conditions described above. For detection of HSP70 mRNA, the filters were rehybridized with a nick-translated ^{32}P-labeled human HSP70 gene sequence, clone pH 2.3, kindly supplied by R. I. Morimoto. After stripping, filters were rehybridized with a ^{32}P-labeled β-actin probe as a control.

Preparation of polysomes. Analysis of polysomal RNA was performed as previously described (9). Virus-infected cells (3 × 10^8) treated with PGA_1 or control diluent were lysed in a solution containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 25 mM KCl, 2 mM dithiothreitol, 6% sucrose, 0.5% Nonidet P-40,

100 μg of cycloheximide per ml, and 0.5 U of RNasin (Promega), per ml. After separation of nuclei by centrifugation (15 min at 10,000 rpm in a Sorvall SS34 rotor), the extracts were layered on 60 to 45% discontinuous sucrose gradients in polysome buffer (50 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 25 mM KCl, 2 mM dithiothreitol, 100 μg of cycloheximide per ml, 0.5 U of RNasin per ml). Extracts in which polysomes were disrupted with 50 mM EDTA prior to centrifugation were also assayed as a control. Gradients were centrifuged in a Beckman SW28 rotor for 16 h at 26,000 rpm, and polysomal pellets were recovered in TNE (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA) as described previously (9). RNA was extracted from polysomal pellets and processed for Northern blot analysis as described above. No RNA was detected in the pellets from EDTA-treated control extracts.

In vitro translation. Cytoplasmic RNA (5 μg) was used to program an in vitro reticulocyte lysate translation system (Amersham). After the addition of [^{35}S]methionine (50 μCi/50-μl sample), samples were incubated for 30 min at 37°C. The translation products were separated on SDS–12% polyacrylamide gels.

RESULTS

Kinetics of HSP70 and SV protein synthesis. To study the kinetics of HSP70 synthesis after PGA_1 treatment in virus-infected compared with uninfected cells, confluent monolayers of 37RC cells were mock infected or infected with SV and treated with PGA_1 or control diluent after the 1-h adsorption period. Cells were pulse-labeled with [^{35}S]methionine at different times during the next 24 h. After labeling, cells were lysed; after determination of the radioactivity incorporated into trichloroacetic acid-insoluble material, radiolabeled proteins were separated by SDS-PAGE and processed for autoradiography. As previously described for uninfected 37RC cells (1), in SV-infected cells, PGA_1 treatment caused a moderate (20 to 30% of control levels) decrease in protein synthesis between 2 and 3 h after treatment; protein synthesis returned to control levels by 4 h, and no significant alterations were noted up to 24 h. At least two HSPs were induced by PGA_1 in 37RC cells, HSP70, identified by immunoprecipitation with an anti-HSP70 monoclonal antibody (Fig. 1A and B), and HSP90, identified by comparison with [^{35}S]methionine-labeled proteins from cells treated with heat shock (45°C, 20 min; data not shown). Infection with SV did not alter the cell stress response after PGA_1 treatment, and the kinetics of HSP70 synthesis were similar in SV-infected and uninfected cells (Fig. 1A). As previously described (1), HSP70 was synthesized up to 12 to 18 h after PGA_1 treatment; being extremely stable, it can be detected at high intracellular levels for at least 24 h in 37RC cells.

Under control conditions, SV protein synthesis started between 8 and 12 h p.i. and continued up to 24 h p.i., while it was not detected before 24 h p.i. in PGA_1-treated cells (Fig. 1A). Apart of a moderate and temporary reduction in the synthesis of two cellular proteins (54 and 45 kDa, respectively), PGA_1 treatment did not inhibit cellular protein synthesis at the time when SV protein synthesis was completely blocked. To determine the effect of PGA_1 on the kinetics of SV protein synthesis, SV-infected cells were labeled with [^{35}S]methionine (1-h pulse) every 2 h between 12 and 24 h p.i. (Fig. 1C). Quantitative analyses of NP and HSP70 synthesis at different times p.i. in control and PGA_1-treated cells are shown in Fig. 1D and E, respectively. The results confirmed that PGA_1 treatment, while it did not inhibit cellular protein synthesis, caused a selective and total block of virus protein synthesis which lasted for several hours (Fig. 1C and E). Interestingly, the block of viral protein synthesis appeared to be inversely correlated with HSP70 synthesis, and SV protein synthesis was turned on only after HSP70 synthesis had returned to control levels (Fig. 1E).

HSP90 was also induced in PGA_1-treated SV-infected cells (Fig. 1C). As determined by densitometric analysis, levels of synthesis higher than the control level were detected even 24 h after treatment (at a time when SV proteins were normally synthesized), suggesting that HSP90 did not interfere with virus protein synthesis.

The PGA_1-induced block of SV protein synthesis appeared to be cell mediated, since treatment with actinomycin D (AMD), which inhibits cellular but not SV RNA transcription, resulted in a return of SV protein synthesis to control levels in 37RC cells treated with PGA_1 for 12 h (Fig. 2). AMD treatment also completely prevented PGA_1-induced HSP70 synthesis.

Effect of PGA_1 on SV mRNA transcription. To determine whether PGA_1 affected the transcription or translation of SV RNA, 37RC cells were infected with SV and treated with PGA_1 or control diluent soon after the 1-h infection period. At different times p.i., SV mRNAs were isolated from the cell extracts by CsCl gradient centrifugation and analyzed by dot blot hybridization using the ^{32}P-labeled SN11 DNA probe, which recognizes a sequence of the NP gene, the first gene transcribed from the SV genome. NP mRNA could be detected from 4 h p.i. in both PGA_1-treated and control cells. PGA_1 treatment had no effect on NP mRNA accumulation up to 6 h p.i. (Fig. 3), indicating that primary RNA transcription was not blocked. Starting from 8 h p.i., no accumulation of NP mRNA was found in PGA_1-treated cells up to 12 h p.i. However, at 24 h p.i., NP mRNA levels were similar to control levels (Fig. 3). Since virus protein synthesis after primary transcription is essential for the progression of the virus replication cycle and for the amplification of virus mRNA, these results suggested that a block of the translation of the primary transcription products could consequently impair the amplification of the message. This hypothesis was reinforced by the fact that when in parallel experiments SV protein synthesis was inhibited by a transient cycloheximide treatment (10 μg/ml, starting 2 h after virus infection for the following 10 h), cellular protein synthesis returned to control level in the first hour after the removal of the translational block, while SV protein synthesis started only 20 to 24 h p.i., with a kinetics comparable to the kinetics obtained after PGA_1 treatment (data not shown).

That SV RNA primary transcription is not the target for PGA_1 antiviral activity was also shown by the fact that addition of PGA_1 6 h after virus infection, at which time NP mRNA had already accumulated in infected cells, was as effective in inhibiting SV replication as when PGA_1 was added soon after infection (control = 32 HAU/ml and PGA_1 = negative at 24 h p.i.; $n = 4$).

In a different set of experiments, to avoid the temporary (8 to 12 h p.i.) drop in SV mRNA transcription consequent to the PGA_1-induced block of SV protein synthesis, treatment with PGA_1 was started 6 h p.i. Northern blot analysis of SV RNA showed that under these conditions, NP mRNA was transcribed and, at least at 10 to 14 h after treatment, accumulated in larger amounts in PGA_1-treated than in control cells, indicating that PGA_1 was not blocking NP mRNA transcription (Fig. 4). Evaluation of HSP70 mRNA levels in the same experiment showed that HSP70 mRNA accumulated as soon as 2 h after PGA_1 treatment (8 h p.i.) up to 10 h after treatment (16 h p.i.) (Fig. 4). Accumulation of HSP70 mRNA

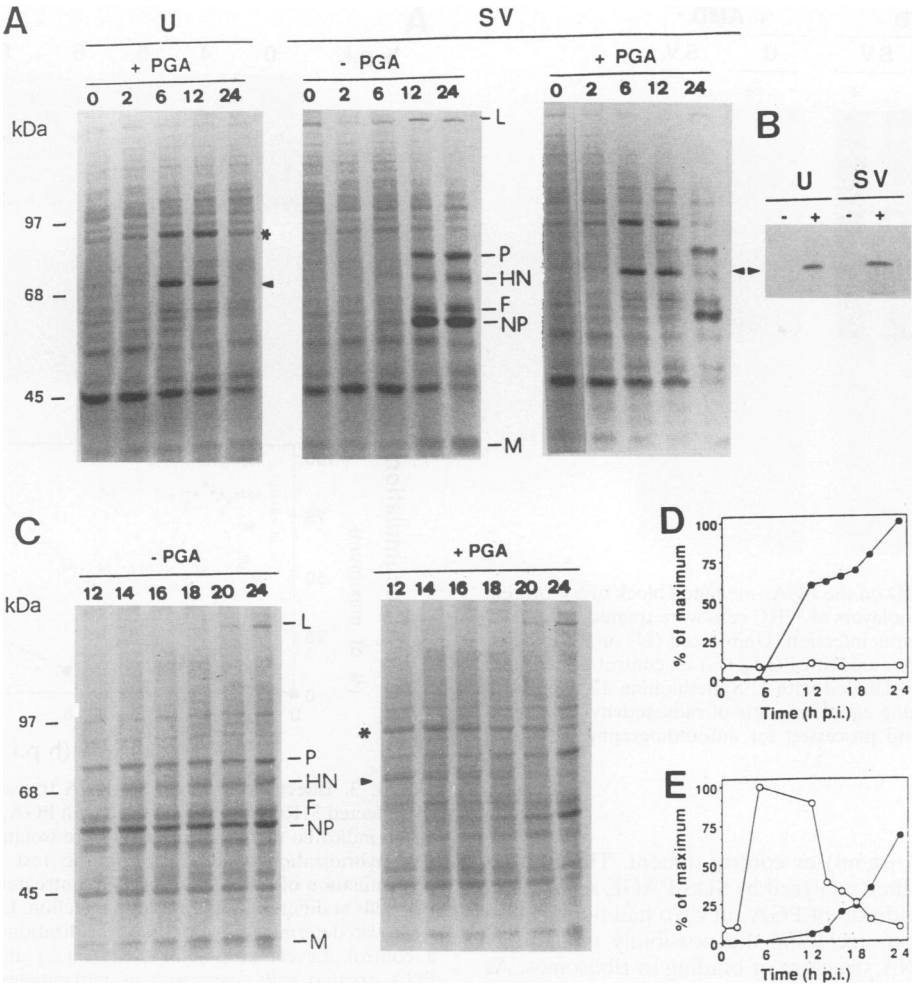

FIG. 1. Effect of PGA_1 on the synthesis of cellular and viral proteins in 37RC cells infected with SV. Uninfected (U) and SV-infected (SV) 37RC cells were treated with PGA_1 (+ PGA) or control diluent (− PGA) soon after SV infection and labeled with [^{35}S]methionine (1-h pulse) at different times p.i. (A) SDS-PAGE analysis. HSP70 is indicated by an arrowhead; HSP90 is indicated by an asterisk. (B) Identification of HSP70 in cell extracts 12 h p.i. by immunoprecipitation with anti-HSP70 monoclonal antibodies. (C) Kinetics of HSP70 and SV protein synthesis from 12 to 24 h p.i. in 37RC cells treated with PGA_1 (+ PGA) or control diluent (− PGA). Samples containing equal amounts of protein were processed for SDS-PAGE and autoradiography. (D and E) Densitometric analysis of HSP70 (○) or virus NP protein (●) synthesis in SV-infected 37RC cells untreated (D) or treated with PGA_1 (E).

in PGA_1-treated cells did not interfere with NP mRNA transcription. The SV 15-kb genomic RNA was instead dramatically reduced in PGA_1-treated samples (Fig. 4). Since replication of the SV genome takes place only in the presence of an adequate level of intracellular unassembled NP protein, these results also suggested that a decrease in protein synthesis after PGA_1 treatment could be responsible for this effect. As previously shown (42), a similar block of SV genome replication was obtained if protein synthesis was inhibited by cycloheximide (10 μg/ml).

We then investigated the possibility that the reduction of SV protein synthesis in PGA_1-treated cells is due to a preferential inhibition in the binding of viral mRNA to ribosomes. 37RC cells were infected with SV and treated with PGA_1 starting 6 h p.i. to avoid the drop in RNA level occurring after early treatment with the drug (see Fig. 3). Under these conditions, NP mRNA levels were not affected by PGA_1 (Fig. 4). Five hours after PGA_1 administration, cells were lysed and ribosome-bound RNAs were isolated by sucrose gradient centrifugation. Northern blot hybridization of the ribosome-bound mRNA fraction from PGA_1-treated and untreated cells, using the SN11 probe, indicated that PGA_1 treatment, while it did not reduce the level of the ribosome-bound β-actin mRNA, caused a decrease of about twofold in the amount of NP mRNA bound to cell ribosomes (Fig. 5). At the same time, a large amount of HSP70 mRNA was found to be bound to ribosomes in PGA_1-treated cells. In the same experiment, synthesis of virus proteins in vivo was inhibited by approximately 50% in PGA_1-treated cells (data not shown).

Effect of PGA_1 on in vitro SV mRNA translation. PGAs have been shown to be transported inside the cell and to be able to form Michael's adducts with cellular nucleophiles and to directly bind to proteins or nucleic acids (15, 19). To investigate the possibility that PGA_1 acts by binding to mRNA or to ribosomes, directly interfering with the cell translational machinery, cytoplasmic RNAs from SV-infected 37RC cells untreated or treated with PGA_1 soon after infection were isolated 8 h p.i. and translated in vitro in a reticulocyte lysate in the

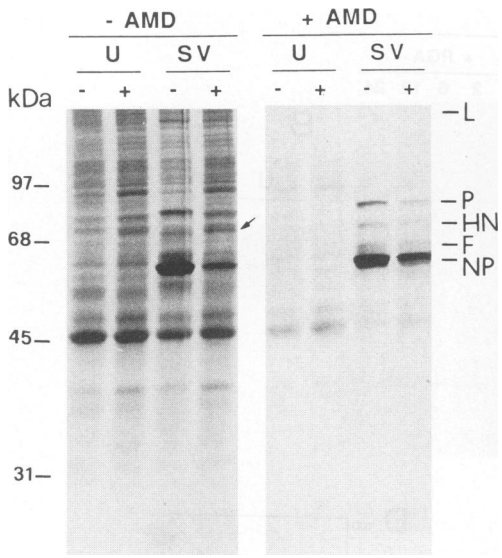

FIG. 2. Effect of AMD on the PGA_1-mediated block of SV protein synthesis. Confluent monolayers of 37RC cells were treated with AMD (1 μg/ml) 12 h before virus infection. Uninfected (U) or SV-infected (SV) 37RC cells were treated with PGA_1 (+) or control diluent (−) soon after infection and labeled with [^{35}S]methionine 12 h p.i. (1-h pulse). Samples containing equal amounts of radioactivity were separated by SDS-PAGE and processed for autoradiography. HSP70 is indicated by an arrow.

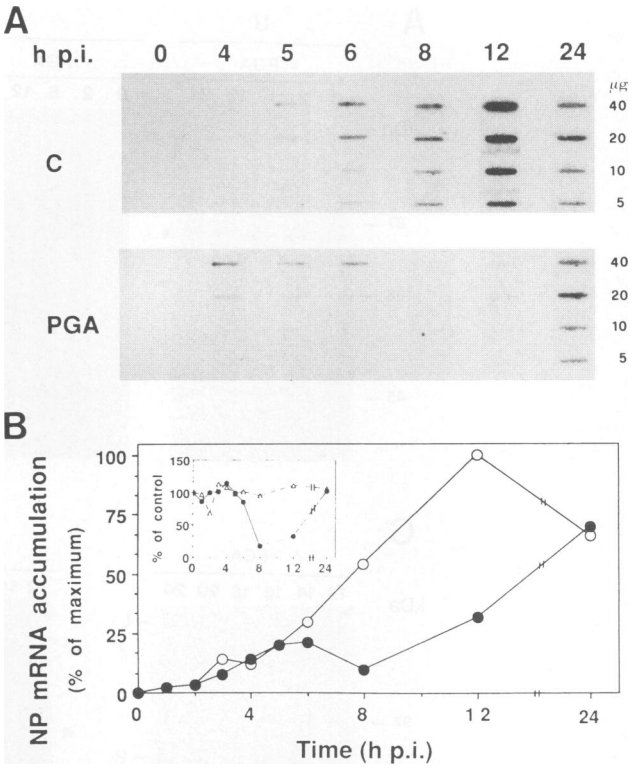

FIG. 3. Effect of PGA_1 on SV RNA transcription. Uninfected or SV-infected 37RC cells were treated with PGA_1 or control diluent (C); at the indicated times, SV mRNAs were isolated and analyzed by dot blot hybridization as described in the text (A). (B) Quantitative determination of NP mRNA levels in untreated (○) or PGA_1-treated (●) cells at different times after SV infection. Levels of β-actin mRNA were also determined by dot blot hybridization in the same samples as a control. Levels of β-actin mRNA (△) and NP mRNA (●) in PGA_1-treated cells, expressed as percentages of untreated control levels, are shown in the inset. Levels of NP mRNA were dramatically reduced in PGA_1-treated cells between 8 and 12 h p.i., while no difference in β-actin mRNA levels was found. Levels of β-actin and NP mRNA were quantitated by scanning laser densitometry and plotted as percentages of maximal accumulation. Data represent results from a representative experiment. Three experiments were carried out with the same results.

presence of PGA_1 (4 μg/ml) or control diluent. The translational products were then analyzed by SDS-PAGE separation. As shown in Fig. 6, addition of PGA_1 in vitro had no effect on SV mRNA translation, excluding the possibility of a direct effect of PGA_1 on RNA structure or binding to ribosomes. As expected, no SV protein synthesis was detected in samples which had been treated with PGA_1 in vivo, as a result of the dramatic reduction of SV mRNA levels 8 to 12 h after virus infection (see Fig. 3). Synthesis of HSP70 was detected in both uninfected and SV-infected cells treated with PGA_1 in vivo (Fig. 6).

Role of HSP70 in PGA_1-induced block of SV protein synthesis. The results described in the previous paragraph, together with the observation that AMD prevents both the induction of HSP70 synthesis and the block of SV mRNA translation in PGA_1-treated cells, suggested that HSP70 itself could interfere with SV protein synthesis. To investigate whether HSP70 could be actively involved in the shutoff of virus protein synthesis or was merely an associated event, we studied the effects of different inducers of HSP70 on SV protein synthesis in the same model.

37RC cells were mock infected or infected with SV and treated with sodium arsenite ($NaAsO_2$; 50 μM) or control diluent soon after the 1-h infection period. Cells were pulse-labeled with [^{35}S]methionine (5 μCi/2 × 10^5 cells, 1-h pulse) at different times during the next 24 h. Samples containing equal amounts of radioactivity were processed for SDS-PAGE analysis and autoradiography. Sodium arsenite treatment induced the synthesis of HSP70 and other stress proteins starting 2 h after treatment for a period of 24 h (Fig. 7A). The kinetics of HSP70 synthesis in $NaAsO_2$-treated SV-infected cells was similar to that in uninfected cells. Also in this case, SV protein synthesis was dramatically inhibited as long as HSP synthesis occurred. Only 24 h p.i., when the level of HSP70 synthesis was reduced, could the NP protein begin to be detected (Fig. 7A and C).

A dramatic block of SV protein synthesis was obtained also with other inducers of HSP70 synthesis. In a separate experiment, 37RC cells infected with SV either were treated with 50 μM $NaAsO_2$, 10^{-5} M cadmium chloride ($CdCl_2$), and 4 μg of PGJ_2 per ml after the 1-h adsorption period for the following 10 h or were subjected to heat shock at 42°C starting 3 h p.i. for a period of 5 h. Cells were labeled with [^{35}S]methionine 10 h p.i. All treatments induced the synthesis of HSP70, as well as HSP90 and other stress proteins, in both uninfected and SV-infected cells, and in every case, SV protein synthesis was selectively and dramatically suppressed (Fig. 8). Similar results were obtained when HSP70 synthesis was induced by 2 mM L-azetidine (data not shown). In a parallel experiment, SV yields were determined in the supernatant of infected cells 24 and 48 h after infection. At 24 h p.i., a 10-h treatment with $NaAsO_2$, cadmium, PGJ_2, and heat shock resulted in decreases of virus yield of 94, 97, 97, and 50%, respectively, of the control level. After 48 h, if treatment was not repeated, virus yields

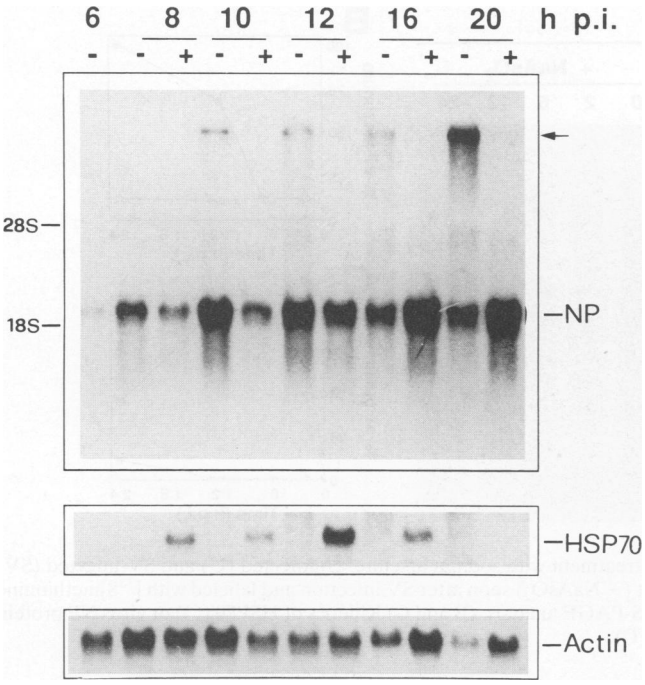

FIG. 4. Effect of late PGA_1 treatment on SV- and HSP70 mRNA transcription. SV-infected 37RC cells were treated with PGA_1 (+) or control diluent (−) at 6 h p.i. At the indicated times, total cytoplasmic RNA was extracted and analyzed by Northern blotting using clone SN11 for identification of NP mRNA and clone pH 2.3 for identification of HSP70 mRNA. The SV 15-kb full-length genomic RNA band is indicated by the arrow. Levels of β-actin mRNA in the same samples are shown as controls.

were still suppressed by 50, 75, and 80% of the control level by $NaAsO_2$, cadmium, and PGJ_2, respectively; no difference in virus titers was found between heat-shocked and control cells, indicating that the antiviral effect was reversible and not due to

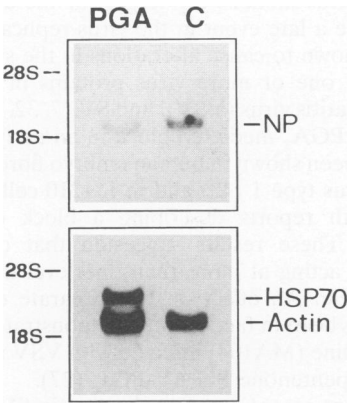

FIG. 5. Analysis of ribosome-bound RNAs. SV-infected 37RC cells were treated with PGA_1 or diluent control (C) 6 h p.i. Five hours after PGA_1 treatment, cells were lysed and ribosome-bound RNAs were isolated and analyzed by Northern blot hybridization using the SN11 probe (top) or the pH 2.3 clone and β-actin probe (bottom) as a control. Levels of ribosome-bound RNAs were quantitated by scanning laser densitometry. Northern blot analysis from a representative experiment is shown. Three experiments were carried out with the same results.

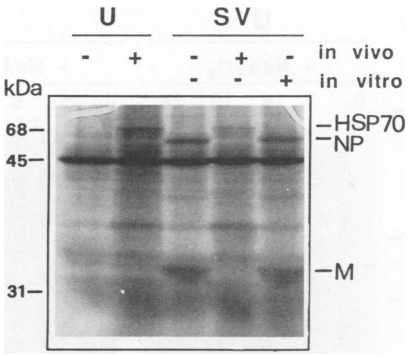

FIG. 6. In vitro mRNA translation. Uninfected (U) or SV-infected (SV) 37RC cells were treated with PGA_1 (+) or control diluent (−) after the 1-h adsorption period. Eight hours after PGA_1 addition, cytoplasmic RNAs were isolated and translated in vitro in a reticulocyte lysate in the presence of 4 μg of PGA_1 per ml (+) or control diluent (−). The translational products were analyzed by SDS-PAGE and autoradiography. Treatment with PGA_1 in vitro had no effect on SV mRNA translation, while no SV protein synthesis was detected in samples treated with PGA_1 in vivo.

a cytotoxic effect. It should be pointed out that virus replication can be affected at different levels by heat, depending on the temperature used, the length of treatment, and especially the time of virus cycle at which hyperthermic treatment is applied (11). Then, in the case of heat shock, which induces HSP70 synthesis for a shorter (2 to 4-h) period compared with the other inducers described, HSP induction has to be carefully timed in relation to the virus replication cycle.

In an attempt to evaluate the effect of PGA_1 on SV protein synthesis in vivo, in a situation in which HSP70 was not induced, we used as a cellular model a murine leukemic cell line, FLC. These cells lack the ability to induce HSP70 expression in response to heat (17). FLC (10^7/ml) were infected with SV (20 HAU/10^6 cells) for 1 h at 37°C. After the 1-h adsorption period, virus inocula were removed and cells were treated with PGA_1 (5 μg/ml) or ethanol diluent. Cells were labeled with [^{35}S]methionine (7 μCi/10^6 cells, 1-h pulse) 12 h after virus infection. Radiolabeled samples were processed for SDS-PAGE analysis and autoradiography (Fig. 9A), immunoprecipitation with anti-SV polyclonal antibodies (Fig. 9B), or immunoblot analysis using anti-HSP70 monoclonal antibody 3A3, which recognizes both the 73-kDa constitutive HSC70 and the 72-kDa inducible HSP70 (Fig. 9C). As previously reported for heat shock (17), PGA_1 did not induce HSP70 synthesis in FLC either uninfected or infected with SV. This was shown by analyzing both the level of HSP70 synthesis and accumulation 12 h after treatment by autoradiography and immunoblot analysis, respectively (Fig. 9A and C). In this situation, PGA_1 had no effect on SV protein synthesis, a result comparable to that for the control (Fig. 9A and B).

Since PGA_1 has been shown to induce FLC differentiation at concentrations equal to or lower than those used in our experiments (34), the lack of effect of PGA_1 treatment on HSP70 induction and SV protein synthesis cannot be due to a lack of PG uptake by these cells.

DISCUSSION

Compared with other chemotherapeutic agents, the antiviral action of cyclopentenone PGs is characterized by the following features: (i) a wide spectrum of action, since it affects both naked or enveloped DNA and RNA viruses and has been

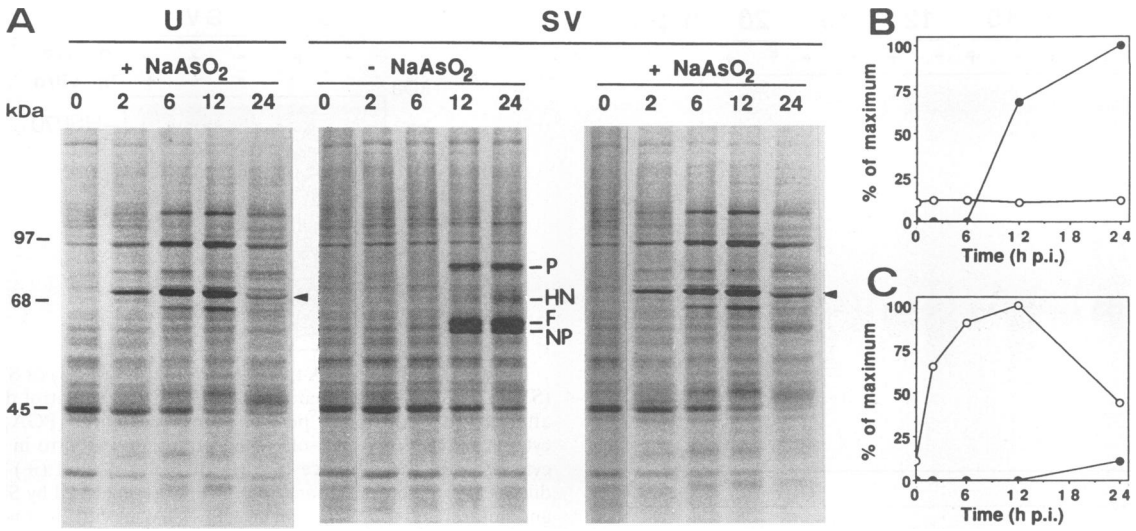

FIG. 7. Kinetics of HSP70 and SV protein synthesis in 37RC cells after treatment with sodium arsenite. Uninfected (U) and SV-infected (SV) 37RC cells were treated with 50 μM $NaAsO_2$ (+ $NaAsO_2$) or control diluent (− $NaAsO_2$) soon after SV infection and labeled with [^{35}S]methionine (1-h pulse) soon after virus adsorption (0) or at 2, 6, 12, and 24 h p.i. (A) SDS-PAGE analysis. (B and C) Kinetics of HSP70 (○) or virus NP protein (●) synthesis in SV-infected cells untreated (B) or treated with $NaAsO_2$ (C).

demonstrated in mouse, monkey, dog, or human fibroblastic or epithelial cell types; (ii) activity at concentrations which are nontoxic to the host cell and generally do not significantly affect DNA, RNA, or protein synthesis in uninfected cells; and (iii) ability to suppress virus replication even when administered in relatively late stages of the virus replication cycle (reviewed in reference 37). These characteristics make cyclopentenone PGs an interesting new class of antiviral agents which could be readily available, since they can be synthesized chemically and since a large variety of prostanoids analogous to PGA can be obtained from natural sources, especially marine organisms (8).

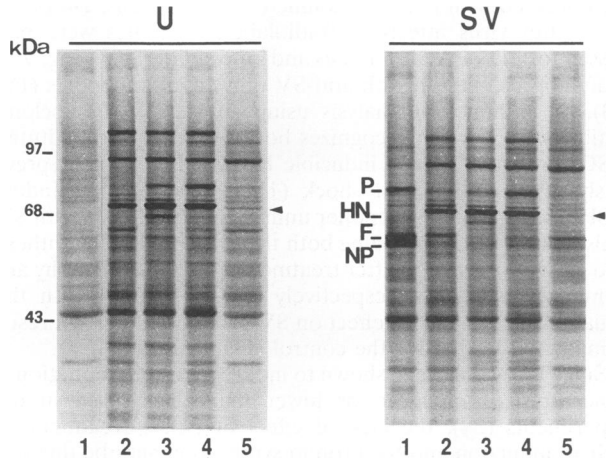

FIG. 8. Effects of different HSP70 inducers on SV protein synthesis in 37RC cells. Uninfected (U) and SV-infected (SV) 37RC cells were treated with 50 μM $NaAsO_2$ (lane 2), 10^{-5} M $CdCl_2$ (lane 3), 4 μg of PGJ_2 per ml, (lane 4), or ethanol diluent (lane 1) soon after infection or were subjected to heat shock at 42°C starting 3 h p.i. for a period of 5 h. Cells were labeled with [^{35}S]methionine (5 μCi/2 × 10^5 cells, 1-h pulse) 9 h p.i. All treatments resulted in the induction of HSP70 (indicated by arrowheads) and in the suppression of SV protein synthesis.

Even though in the last decade major advances have taken place in the identification of the cellular and viral targets of PGs, the mechanism of the antiviral activity is not completely elucidated. The antiviral activity has been recently associated with the ability of cyclopentenone PGs to function as signals for the induction of HSP synthesis (1, 37). In fact, in different types of mammalian cells, PGA_1 induces the transcription of classical heat shock genes through activation of heat shock transcription factor (HSF), which binds to the heat shock element, composed of multiple adjacent inverted repeats of the pentamer nGAAn (2, 26, 36). Activation of HSF1, but not HSF2, by PGA_1 has been recently shown in human cells (29a).

In several virus models, the target for the antiviral activity appeared to be a late event in the virus replication cycle, and PGAs were shown to cause alterations in the synthesis and/or maturation of one or more virus proteins in vaccinia virus, vesicular stomatitis virus (VSV), and SV (7, 32, 38, 39). On the other hand, a PGA_1-mediated block in an early stage of virus infection has been shown in human embryo fibroblasts infected with herpesvirus type 1 (43) and in L-1210 cells infected with VSV (5), both reports describing a block of virus RNA transcription. These results suggested that cyclopentenone PGs could be acting at more than one level during the virus replication cycle. An effect on two separate events of virus replication has been in fact recently demonstrated in a monkey epithelial cell line (MA104) infected with VSV after treatment with the cyclopentenone PG Δ^{12}-PGJ_2 (27).

In the present report, we describe that in SV-infected cells, PGA_1, apart from the alteration of HN and F protein glycosylation previously reported (32), also causes a selective, complete, and temporary block of virus protein synthesis, indicating that PGA_1 can affect at least two separate events in SV replication cycle.

No differences in SV mRNA levels were found in PGA_1-treated versus untreated cells up to 6 h p.i., suggesting that PGA_1 does not inhibit SV primary transcription during early

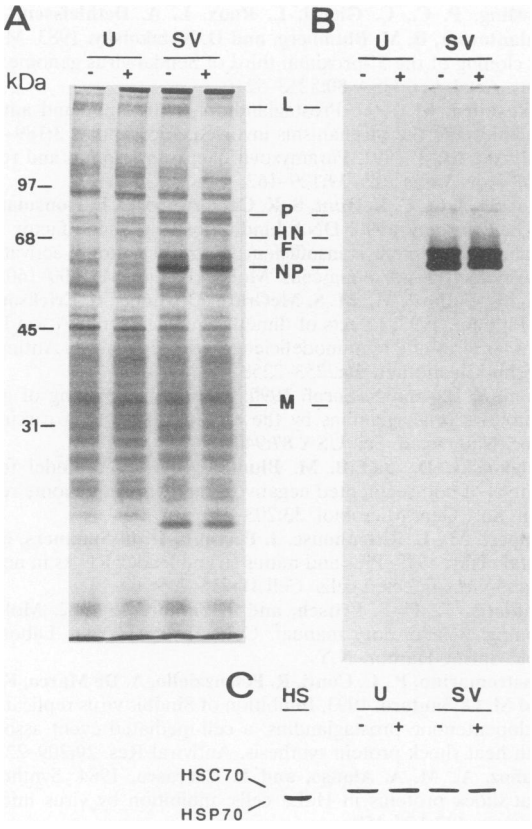

FIG. 9. Effect of PGA_1 treatment on HSP70 and SV protein synthesis in FLC. Uninfected (U) or SV-infected (SV) FLC were treated with 5 μg of PGA_1 per ml (+) or control diluent (−) after the 1-h adsorption period and labeled with [^{35}S]methionine (1-h pulse) 12 h p.i. PGA_1 only slightly (~25%) inhibited total protein synthesis in uninfected cells and had no effect in SV-infected FLC. (A) SDS-PAGE analysis of radiolabeled samples. (B) Identification of SV proteins in the same samples by immunoprecipitation with anti-SV polyclonal antibodies. (C) Samples containing equal amounts of protein were separated by SDS-PAGE and processed for immunoblot analysis using anti-HSP70 monoclonal antibody 3A3. PGA_1 treatment did not induce HSP70 synthesis in these cells. As a positive control, an equal amount of protein from 37RC cells subjected to a 20-min 45°C heat shock was processed identically (HS).

infection. That primary transcription is not the target for PGA_1 antiviral activity was also suggested by the fact that addition of the compound 6 h after virus infection (at which time NP mRNA had already accumulated in infected cells) was as effective in inhibiting SV replication as when PGA_1 was added immediately after virus adsorption. In cells treated with PGA_1 soon after SV infection, no accumulation of NP mRNA was found from 8 to 12 h p.i., while at 24 h p.i., NP mRNA levels were similar to control levels. Since virus protein synthesis after primary transcription is essential for the progression of the virus replication cycle and for the amplification of virus mRNAs, these results suggest that a block of the translation of the primary transcription products could consequently impair the accumulation of the message. On the other hand, treatment with PGA_1 started at 6 h p.i. did not suppress the levels of NP mRNA up to 20 h p.i., but it reduced the amount of ribosome-bound NP mRNA and caused a dramatic decrease in the level of SV 15-kb genomic RNA. Since the replication of the SV genome takes place only in the presence of an adequate level of intracellular unassembled NP protein, these results also suggest that PGA_1-induced inhibition of virus protein synthesis causes a disregulation of the SV replication cycle, consequently blocking SV genome transcription.

Inhibition of RNA translation appears not to be a direct effect of PGA_1, as shown by in vitro translation of SV mRNA. The fact that AMD was able to revert the block of SV protein synthesis in PGA_1-treated cells also indicates a cell-mediated effect.

SV infection did not affect the cell stress response after PGA_1 treatment, and the kinetics of PGA_1-induced HSP70 synthesis were similar in infected and uninfected cells. Interestingly, SV protein synthesis was blocked as long as HSP70 protein was synthesized by the host cell and was turned on only after HSP70 synthesis had returned to control levels. These results, together with the observation that AMD prevents both the induction of HSP70 synthesis and the block of SV mRNA translation in PGA_1-treated cells, suggest that HSP70 protein itself could interfere with SV protein synthesis. This possibility is reinforced by the finding that treatment of 37RC cells with different inducers of HSP70, including sodium arsenite, cadmium, and heat shock (5 h at 42°C, starting 3 h p.i.), also caused a selective block of SV protein synthesis as long as HSPs continued to be synthesized by the host cell. Moreover, studies in a murine erythroleukemic cell line (FLC) in which HSP70 is not induced by heat shock (17) or, as we have now shown, by PGA_1 confirmed that in the absence of HSP70 induction, PGA_1 treatment had no effect on SV protein synthesis. Similar results were recently obtained in a different virus model, VSV, after infection of monkey kidney MA104 cells or C2C12 mouse myoblasts. Also in this case, treatment of VSV-infected MA104 cells with PGA_1, sodium arsenite, azetidine, and prolonged heat shock at 42°C resulted in the induction of HSP70 and in a dramatic inhibition of VSV protein synthesis. In VSV-infected C2C12 murine myoblasts, however, treatment with PGA_1 or with sodium arsenite or heat shock had different effects on virus replication. In these cells, PGA_1 does not induce HSP70, while it increases the synthesis of the constitutive HSC70 protein and dramatically induces the synthesis of the 32-kDa stress protein heme oxygenase (29). HSP70 is instead normally induced by sodium arsenite or heat shock. Treatment of C2C12 cells with PGA_1 even at high concentrations (up to 25 μg/ml) had no effect on VSV protein synthesis, while in sodium arsenite-treated or heat-shocked cells, VSV protein synthesis was shut off as long as HSP70 was synthesized by the host cell (29a).

Together, these results indicate that high levels of HSP70 synthesis block the expression of virus proteins. The mechanism by which HSPs can interfere with viral protein synthesis remains to be determined. In eukaryotic cells, constitutive HSP70 has been shown to reversibly bind to nascent polypeptide chains before they are properly folded and to be needed to chaperone unfolded proteins to and from specific cellular organelles (6). It has been suggested that large amounts of inducible HSP70 can irreversibly bind to newly synthesized proteins, causing a translational block (6). Schlesinger et al. (40) have also shown that the presence of HSP70 protein during in vitro translation of mRNA encoding for the Sindbis virus capsid protein interfered with normal polypeptide synthesis.

Our results suggest that HSP70 can interfere with SV mRNA translation only during its synthesis by the host cell. In fact, 24 h p.i., when HSP70 synthesis is turned off but high levels of HSP70 are still detected in infected cells, SV proteins are translated at the rate of the control. It could be hypothesized that HSP and virus messages, both of which can be

translated in conditions in which cellular protein synthesis is impaired, possess similar mechanisms of preferential translation and can then compete with each other. For example, both HSP70 mRNA (24) and negative-strand RNA virus mRNA (25) are preferentially translated at increased cytoplasmatic ionic concentrations. While in the presence of large amounts of SV mRNA, induction of HSP70 causes only a partial inhibition of virus protein synthesis, in the early stage of SV infection, the translational block of even a minimal amount of virus proteins could have dramatic effects by hindering the virus amplification process.

The possibility that HSPs can interfere with virus replication is particularly interesting in the view of the fact that HSP synthesis is induced during increases in body temperature in mammalian brain and other organs (10). A better understanding of the role of HSPs in virus replication could be useful in the comprehension of the beneficial effect of fever in virus diseases.

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