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Heat shock proteins and virus replication: hsp70s as mediators of the antiviral effects of prostaglandins

M. G. Santoro

Institute of Experimental Medicine, CNR, Via. le K. Marx 15, I-00137 Rome, and Department of Experimental Medicine, University of L'Aquila, I-67100 L'Aquila (Italy)

Abstract. Acute infection of mammalian cells with several types of RNA and DNA viruses often results in induction of heat-shock gene expression. The presence of hsp70 in intact virions, as well as the transient association of HSP with viral proteins and assembly intermediates during virus replication, has also been reported in several experimental models. Moreover, a possible role of heat shock proteins in the beneficial effect of fever and local hyperthermia during acute virus infection has been hypothesized. However, the role of HSP in virus replication remains to be defined.

At the beginning of the 1980s, the use of virus models to investigate the molecular events that follow the exposure of mammalian cells to prostaglandins led to the serendipitous discovery that specific arachidonic acid derivatives are potent inhibitors of virus replication. This finding was rapidly followed by the observation that treatment of virus-infected cells with the antiviral prostaglandin A_1 (PGA_1) resulted in the accumulation of a 70 KDa cellular protein, which was identified as hsp70. It is now well established that cyclopentenone prostaglandins, which exert potent antiviral activity in several DNA and RNA virus models, induce hsp70 synthesis through cycloheximide-sensitive activation of heat shock transcription factor.

This chapter discusses the role of heat shock proteins in the control of virus replication and summarizes the results of our recent work, which indicate that hsp70 is actively involved in the antiviral activity of prostaglandins.

Key words. Antiviral; heat shock proteins; hyperthermia; prostaglandins; Sendai virus (SV); vesicular stomatitis virus (VSV).

Introduction

One of the most intriguing aspects of the study of the heat shock response is the relationship between heat shock proteins (HSP) and virus replication.

In prokaryotic cells, a role for HSP in bacteriophage growth has been shown at different levels. In *Escherichia coli* the DnaK protein, a product of the *DnaK* gene, presents a 50% homology with the Drosophila hsp70[35] and possesses ATPase and autophosphorylating activity[84]. DnaK interacts, together with other *E. coli* HSP (DnaJ/GrpE) with λ phage O and P replication proteins, and is essential for bacteriophage λ replication[21,85]. Two other *E. coli* HSP, GroES and GroEL, the products of the *GroES* and *GroEL* genes, are involved in the assembly of the head proteins of bacteriophage λ and T4, and the tail proteins of bacteriophage T5 (reviewed in 10, 13, 35).

In eukaryotic cells a growing body of literature focuses on the relationship between virus replication and heat shock proteins. Different types of interactions have been described. These could be summarized as:

1) modulation of the stress response by virus infection;
2) evidence for the presence of HSP in intact virions or association of HSP with virus proteins during infection;
and 3) effect of induction of HSP, by hyperthermia or other agents, on virus replication in cells acutely or persistently infected by viruses.

Modulation of the stress response by virus infection

Several RNA viruses induce HSP synthesis in the host cell during the infectious cycle. Peluso et al. reported that infection of cultured chicken embryo cells with two paramyxoviruses, SV5 and Sendai virus, induced the expression of glucose-regulated proteins (GRP)[48,49], and the transcriptional induction of the GRP78 gene following SV5 infection of monkey CV1 cells has been further characterized[77]. A different paramyxovirus, Newcastle disease virus, was also shown to cause accumulation of heat shock proteins in infected chick embryo cells[16]; avirulent strains were stronger inducers and also stimulated the synthesis of glucose-regulated proteins. Induction of stress proteins by Togaviruses and Rhabdoviruses (Sindbis virus and vesicular stomatitis virus, respectively) in chick embryo cells was also reported by Garry et al.[24]. In this case, it was shown that the capsid protein (C) of Sindbis virus and nucleocapsid protein (N) of VSV were physically associated with an 89 KDa HSP. Finally, intracellular accumulation of GRP78/immunoglobulin heavy chain binding protein (BiP) has

been shown after infection with picornaviruses. In fact translation of GRP78/BiP mRNA was found to be increased in poliovirus-infected HeLa cells at a time when cap-dependent translation of cellular mRNA is inhibited[70].

Different types of DNA viruses can influence the cellular stress response. During the lytic infection of monkey and mouse cells with SV40 or polyoma virus, there is a marked increase in the synthesis of two host heat-inducible proteins of 92 and 72 KDa mol. wt[31], while increased levels of hsp70 mRNA are detected in human monocyte-macrophages after infection with vaccinia virus[28]. Infection of human cells with adenovirus has also been found to increase the expression of HSP genes, particularly of hsp70 genes, and the earliest virus protein formed during infection, the E1A 13S gene product, has been shown to activate hsp70 gene transcription in human cells[29,30,42,79,80].

Numerous reports have shown the accumulation of heat shock proteins in several cell lines during herpes simplex virus (HSV) infection. Mutants of HSV type 1 (HSV1) and 2 (HSV2) induce HSP during infection of chick embryo fibroblasts[44] and human neuroblastoma cells[82] respectively. The presence of abnormal forms of the HSV1 immediate early polypeptide Vmw175 was found to be the signal for induction of the stress response in chick embryo fibroblasts infected with the HSV mutant tsK[54]. Lytic infection of BHK cells with several strains of HSV2 was found to cause intracellular accumulation, as well as translocation to the cell surface, of a 90 KDa protein (p90) related to the hsp90 family[34], while increased levels of 57 to 61 KDa proteins, whose identity is unclear, were reported in BHK cells infected with both HSV1 and HSV2[33]. Another member of the herpes virus family, the human cytomegalovirus, has been reported to transiently induce hsp70 expression in human diploid fibroblasts[57], while infection of human B lymphocytes with Epstein-Barr virus (EBV) was recently found to induce the synthesis of both hsp70 and hsp90 proteins[14]. In the latter case, induction was dependent on EBV-induced trans-membrane Ca^{2+} fluxes and was independent of viral gene expression. It was suggested that hsp70 and hsp90 induction is one of the earliest changes in gene expression induced by this virus in a newly-infected host cell.

These results taken together clearly indicate that the entry of a virus into a eukaryotic cell initiates a cascade of events which can result in switching on HSP or GRP gene expression. Is the increased synthesis of HSP during viral infection simply part of a non-specific induction of host genes?

Studies on the transcriptional regulation of the 70 KDa heat shock gene family in human and monkey cells infected with different DNA viruses, adenovirus type 5 (Ad5), HSV1, SV40 and vaccinia virus, indicated that induction of hsp70 genes is not a general response to the stress following viral infection[50]. The fact that in these cells only Ad5 and HSV1 viruses were able to induce hsp70 expression, and that of three 70 KDa heat shock genes examined only hsp70 was induced, indicated in fact a highly specific cellular response. It remains to be established whether induction of HSP by viruses reflects a stress situation of the host cell with no effect on virus replication, or whether HSP could be actively involved in the control of virus replication.

Association of HSP to virus proteins

The presence of heat shock proteins in intact virions as well as the binding of HSP to virus components has been shown in different types of virus infections. The interactions of HSP with the C capsid protein of Sindbis virus and with the N nucleocapsid protein of VSV were described above[24]. The presence of a 72 KDa HSP and the major nucleocapsid protein N was recently described in intranuclear inclusion bodies of astrocytes infected with canine distemper virus[46]. Moreover, the constitutively expressed hsc70, but not the inducible hsp70, was found to be selectively incorporated into rabies virions as well as into other negative-strand RNA viruses, including vesicular stomatitis virus, Newcastle disease virus and influenza A virus[55]. Hsp70 is also associated with the capside precursor P1 of poliovirus and coxsackievirus B1 in infected HeLa cells[39]. The hsp70-P1 complex was found to be part of an assembly intermediate of picornavirus, rather than of the mature virion, suggesting a role for hsp70 in viral assembly.

In DNA virus models, a 60 KDa protein related to the chaperonin t-complex polypeptide 1 (TCP-1) was found to be associated with hepatitis B virus core polypeptides in two different assembly intermediates (but not with the unassembled proteins or with the final mature capsid product) in a cell-free system, suggesting that eukaryotic cytosolic chaperonins may play a distinctive role in virus multimer assembly, apart from their involvement in assisting monomer folding[36]. Association of cellular hsp70 with viral components was also detected in vaccinia virus-infected human monocyte-macrophages[28]. Colocalization of cellular hsp70 with adenovirus E1A proteins was shown in the nucleus of adenovirus type 2 infected HeLa cells[78], while hsp70 was found to be associated with the adenovirus type 5 fiber protein in infected HEp-2 cells[38]. Finally, transient association of GRP78/BiP to virus proteins during maturation and intracellular transport has been shown in different virus models[11,17,25,77].

The observed interactions of HSP with virus proteins may simply reflect a 'housekeeping' function of HSP in recognizing and facilitating the clearing of foreign or abnormal proteins in the cell. Conversely, different types of viruses may utilize different HSP components of the cellular machinery which folds, translocates and

assembles proteins, to complete their morphogenesis. What happens, however, if under the influence of external stimuli the amount of different cellular stress gene products is altered?

Effect of hyperthermia on virus replication

A beneficial effect of fever and local hyperthermia during virus infections has been hypothesized from the time of the ancient Greek physicians to the present[32].

A differential effect of hyperthermia in acute and chronic virus infections has been reported. Heat shock induces the appearance of Epstein-Barr virus (EBV) early antigen in EBV-infected lymphoblastoma cell lines[83], and rapid in vivo reactivation of HSV in latently infected murine ganglionic neurons has been detected after transient hyperthermia[71]. Moreover, human cells with stably integrated human immunodefficiency virus can be activated by heat shock to produce virus through an increase in viral gene transcription, suggesting a negative role of hyperthermia in chronic virus infection[73].

On the other hand, it is known that continuous hyperthermia at 3 to 4 °C above the physiological range can block the replication of several DNA and RNA viruses during primary infection in cultured cells[9,37,47]. A 12 h hyperthermic treatment (HT) at 43 °C inhibits simian virus 40 protein synthesis in CV1 cells[3], while a 90 min HT at 9 °C above the physiological temperature strongly suppresses togavirus replication in *Aedes albopictus* cells[12]. It has recently been shown that brief hyperthermic treatment (45 °C for 20 min), if applied during specific stages of the virus cycle, is extremely effective in blocking the replication of vesicular stomatitis virus (VSV) during primary infection[18]. In this model a brief exposure to high temperature did not damage uninfected cells, and only moderately (25 to 35%) inhibited host cell protein synthesis for a period of approximately 4 hours. No effect on virus replication was found when HT was applied soon after virus entry into the cells, or at later times of infection (8–10 h), when the virus had taken control of the cellular protein synthesis machinery and HT-induced synthesis of HSP was impaired, indicating that the antiviral effect is not due to a general change in membrane fluidity or cell metabolism. If hyperthermic treatment was applied between 2 and 4 h after virus infection, at the time of genomic amplification when virus proteins start to be synthesized, virus yield was found to be reduced to less than 5% of control[18]. Depending on the temperature used and on the length of treatment, virus replication appeared to be affected at different levels. These results suggested that synthesis of heat shock proteins at specific stages of the virus cycle could interfere with virus replication. Possibly, in order to achieve an antiviral effect during acute infections, HSP induction has to be carefully timed in relation to virus replication. It is interesting to point out that local hyperthermia (20 to 30 min at 43 °C) was found to improve the course of the disease in patients with natural and experimental common colds[74].

Induction of hsp70 by antiviral prostaglandins

Prostaglandins (PGs) are a class of naturally occurring cyclic 20-carbon fatty acids, synthesized by eukaryotic cells from polyunsaturated fatty acid precursors, in response to external stimuli, through the action of phospholipase A and a phosphatidylinositol-specific phospholipase C (ref. 56). Since their discovery, they have been shown to function as microenvironmental hormones and intracellular signal mediators and to participate in the regulation of a large variety of physiological and pathological processes of eukaryotes, including inflammation[75] and the febrile response[19], cell proliferation and differentiation[23], the immune response[43], cytoprotection[52], and virus replication[58].

The ability of prostaglandins of the A type to inhibit virus replication and prevent the establishment of persistent infections was first reported in 1980[59]. It is now well established that prostaglandins 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 viruses, including poxviruses[8,61] and herpesviruses[27,81], and RNA viruses, including orthomyxoviruses[64], paramyxoviruses[1,59], picornaviruses[4], rhabdoviruses[6,62], togaviruses[40] and retroviruses[5,20,27]. The antiviral activity of a long-acting synthetic analogue of PGA_2, 16,16-dimethyl-PGA_2 methyl ester (di-M-PGA_2) has also been shown in vivo, in a mouse model infected with influenza A virus[64]. The antiviral action of cyclopentenone prostaglandins is characterized by the following aspects: 1) a wide spectrum of action, since it affects both naked or enveloped DNA and RNA viruses in different types of mammalian cells; 2) it is active at concentrations which are non-toxic to the host cell, and generally do not inhibit DNA, RNA or protein synthesis in uninfected cells; and 3) it can suppress virus replication even when it is administered at relatively late stages of the virus replication cycle (reviewed in 68). These characteristics make cyclopentenone prostaglandins a new interesting class of antiviral agents, which could be at hand since several prostanoids analogous to PGA are available from natural sources, especially from marine organisms[15].

A major characteristic common to prostaglandins with antiviral activity is the ability to function as a signal for the induction of heat shock protein synthesis[60,63,65,67]. Induction of hsp70 gene transcription by prostaglandin A is mediated by cycloheximide-sensitive activation of heat shock transcription factor (HSF)[2]. Activation of HSF1, but not HSF2, by prostaglandin A has been recently shown in human cells (Rossi and Santoro, unpubl. observ). Induction of hsp70 gene expression has

now been described in a large variety of monkey, canine, porcine and human cell lines, as well as in human peripheral blood lymphocytes and macrophages, and primary cells derived from cord blood (reviewed in 67, 68). We have recently described an interesting exception. Mouse cells behave differently from other mammalian species in their response to PGA, which is not able to induce hsp70 synthesis, while it increases the synthesis of the constitutive hsc70 in several murine cell lines, including L929 fibroblasts and C2C12 myoblasts[53].

It has to be pointed out that synthesis of large amounts of hsp70 or hsc70 proteins can be achieved with concentrations of prostaglandins that do not inhibit nucleic acid or protein synthesis in all cell types tested. Moreover, in contrast to synthesis after brief heat shock, the synthesis of hsp70 continues at high level for several hours after induction with PGAs, and up to 24 h after induction with PGJ_2 (fig. 1).

The possibility that hsp70 could be involved in the control of virus replication was first suggested by the fact that only prostaglandins with antiviral activity were able to induce hsp70 synthesis, and inhibition of virus replication was obtained only at concentrations of PGs at which hsp70 was actively synthesized[1,67,68].

Role of hsp70 in the antiviral activity of cyclopentenone prostaglandins

Even though in the last decade major advances have taken place in the identification of the cellular and viral targets of prostaglandins, the mechanism of the antiviral activity is complex and has not been yet completely elucidated.

In several virus models the target for the antiviral activity is a late event in the virus replication cycle. PGAs were in fact shown to cause alterations in the synthesis and/or maturation of one or more virus proteins in vaccinia, vesicular stomatitis virus (VSV) and Sendai virus[8,61,62,66]. On the other hand, a PGA_1-mediated block at an early stage of virus replication has been shown in human embryo fibroblasts infected with HSV1 (ref. 81) and in L-1210 cells infected with VSV[6], both 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.

We have now demonstrated that, at least in the case of negative strand RNA viruses, cyclopentenone PGs affect two separate events of virus replication, respectively at an early and a late phase of the virus cycle.

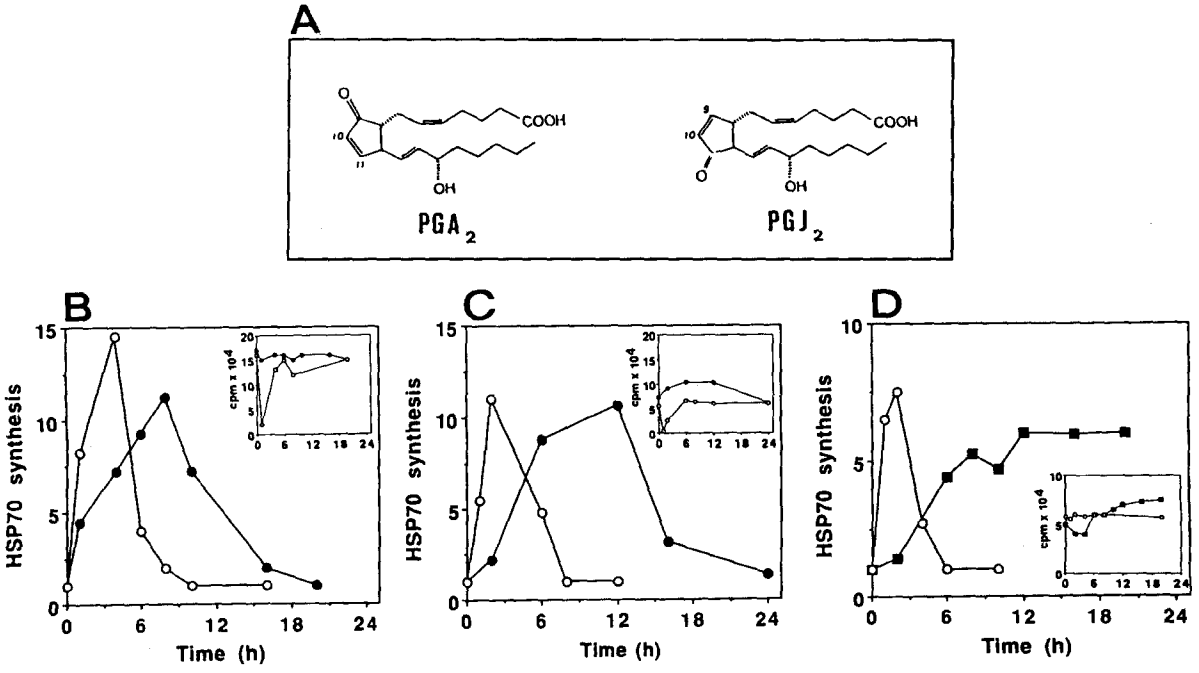

Figure 1. Kinetics of hsp70 synthesis after induction by cyclopentenone prostaglandins or heat shock in different types of mammalian cells. Chemical structures of cyclopentenone prostaglandin A_2 (PGA_2) and J_2 (PGJ_2) are shown in *A*. Human erythroleukemic K562 cells (*B*), African monkey kidney 37RC cells (*C*) or epithelial monkey kidney MA104 cells (*D*) were pulse-labelled for 1 h with ^{35}S-methionine at various times after the addition of PGA_1 (4 μg/ml, ●), PGJ_2 (8 μg/ml, ■), or after heat shock (45 °C, 20 min, ○). Samples containing equal amounts of ^{35}S-methionine labelled-proteins were processed for SDS-PAGE separation and quantitative immunoblot analysis, using anti-hsp70 monoclonal antibodies, as described[18]. Hsp70 synthesis is expressed as cpm × $10^3/10^6$ cells in panel *B*, and cpm × $10^3/2 \times 10^5$ cells in panels *C* and *D*. Total protein synthesis, determined as ^{35}S-methionine incorporation into TCA-insoluble material, is shown in insets of panel *B*, *C* and *D* respectively.

When epithelial monkey MA104 cells are infected with a rhabdovirus, vesicular stomatitis virus, virus protein synthesis starts approximately 3–4 hours after infection (p.i.), and by six hours p.i. cellular protein synthesis is shut off and only virus proteins are synthesized (fig. 2A). VSV cytopathic effect, which causes cell rounding and detachment from substrata, is evident 8–10 h after infection, and 100% cell death is observed after 24 h. If cells are treated with the cyclopentenone Δ^{12}-PGJ_2 soon after the 1 h infection period, the virus-induced cytopathic effect is delayed and the virus yield is reduced to 1–5% of the control[51]. Δ^{12}-PGJ_2 treatment at this time dramatically inhibits VSV protein synthesis, and prevents the virus-induced shut-off of cellular protein synthesis (fig. 2A, B). The antiviral effect is accompanied by induction of hsp70 synthesis, which persists for several hours (fig. 2C). Hsp90 and hsp110 are also induced.

Treatment with cyclopentenone PGs in a late phase of the VSV replication cycle, 6–8 h p.i., when virus proteins have been already synthesized, still causes a dramatic block of infectious virus production, which is mediated by alteration in virus G protein maturation and intracellular translocation[51,62,69].

Are heat shock proteins involved in the antiviral activity of prostaglandins, and if so, at which level? While there is no information at the moment about a possible involvement of hsp70 in alterations of virus protein maturation or intracellular translocation, more and more experimental evidence is accumulating which indicates that high levels of hsp70 synthesis in infected cells antagonize virus protein synthesis in the early phase of acute infections. This has been recently studied in detail in a model of paramyxovirus infection, Sendai virus in the African green monkey kidney 37RC cell line.

Sendai virus (SV), a non-segmented ssRNA paramyxovirus of negative polarity, consists of an inner nucleocapsid containing the genomic 15 Kb RNA tightly associated with the nucleocapsid protein (NP), surrounded by an envelope composed by the internal non-glycosylated polypeptide M and the two viral HN and F glycoproteins. The viral polymerase responsible for RNA synthesis is a complex formed by L and P proteins, associated with the nucleocapsid. SV genomes and antigenomes are found only encapsidated with the NP protein and their synthesis is dependent on on-going protein synthesis, while the switch from transcription to replication appears to be determined by the level of intracellular unassembled NP protein[22,76].

SV-infection does not affect the cell stress response after PGA_1-treatment, and the kinetics of PGA_1-induced hsp70 synthesis are similar in infected and uninfected cells. As previously shown for MA104 cells infected with VSV, also in Sendai virus-infected cells, cyclopentenone PGs cause a selective and complete block of virus protein synthesis, which lasts as long as hsp70 is synthesized by the host cell. The block appears to be at the translational level, as indicated by the fact that PGA_1 had no effect on SV primary transcription; moreover, treatment with PGA_1 started at 6 h p.i., at which time SV mRNA was already accumulated in infected cells, still inhibited virus replication and reduced the amount of ribosome-bound NP mRNA[1]. When cytoplasmic RNAs isolated from SV-infected cells were translated in vitro, addition of PGA_1 to the reticulocyte lysate had no effect on SV mRNA translation, excluding the possibility that PGA_1 could be directly interfering with the cell translational machinery by binding to mRNA or to ribosomes. Moreover, the fact that actinomycin D (AMD, which blocks cellular but not SV RNA transcription) was able to reverse the block of SV protein synthesis in PGA_1-treated cells, also indicated a cell-mediated effect. The fact that SV protein synthesis was blocked as long as hsp70 protein was synthesized by the host cell, together with the observation that AMD prevented both the induction of hsp70 synthesis and the block of SV mRNA translation in PGA_1-treated cells, suggested that hsp70 protein itself could interfere with SV protein synthesis. To investigate this possibility 37RC cells infected with Sendai virus were treated with different inducers of hsp70 synthesis, including sodium arsenite, cadmium, azetidine and heat shock. Virus protein synthesis was dramatically and selectively inhibited as long as heat shock protein synthesis was occurring in infected cells[1]. Similar results were obtained in the model of rhabdovirus infection previously described. Also in this case, induction of hsp70 synthesis by either PGA_1, sodium arsenite, azetidine or heat shock was associated with a dramatic decrease of virus protein synthesis after hsp70 induction (fig. 2D). Moreover, studies in a murine erythroleukemic cell line (FLC) in which hsp70 is not induced by heat shock[26] or, as we have recently shown, by PGA_1, confirmed that in the absence of hsp70 induction, PGA_1 treatment had no effect on SV protein synthesis[1]. We had previously shown that in L929 murine fibroblasts, which do not respond to PGA-treatment by synthesizing hsp70, PGA_1-treatment did not inhibit VSV protein synthesis, while it caused an alteration of G glycoprotein maturation[62].

Conclusions

Altogether, these results indicate that high levels of hsp70 synthesis inhibit the expression of virus proteins. The mechanism by which HSP can interfere with viral protein synthesis remains to be determined. In eukaryotic cells, constitutive hsp70 has been shown to bind reversibly to nascent polypeptide chains before they are properly folded, and to be needed to chaperone unfolded proteins to and from specific cellular organelles[7].

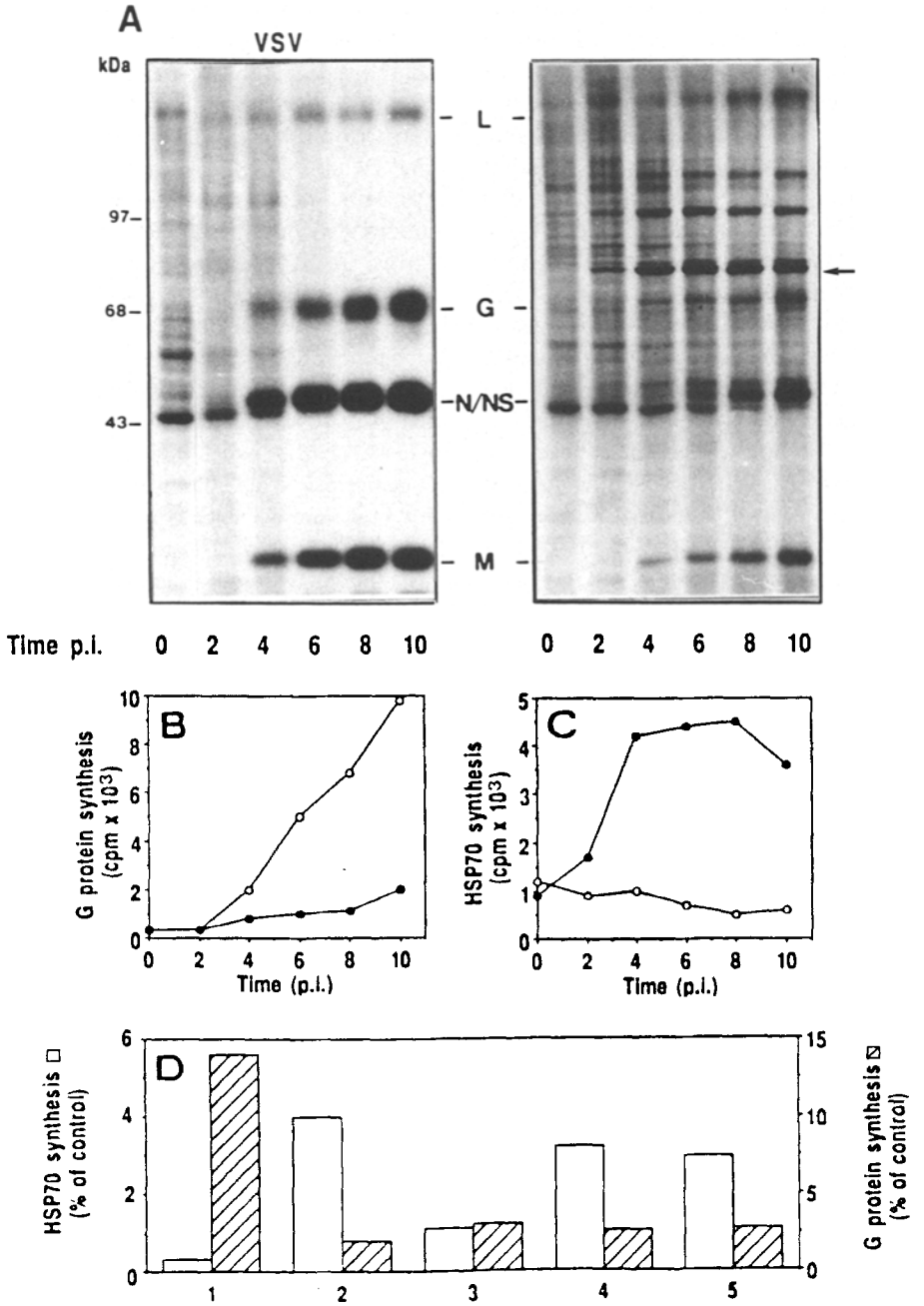

Figure 2. Effect of Δ^{12}-PGJ_2 and other inducers of hsp70 synthesis on VSV protein synthesis. MA104 cells infected with VSV (3 PFU/cell) were treated with Δ^{12}-PGJ_2 (8 μg/ml) or control diluent after the 1 h adsorption period, and labelled with ^{35}S-methionine (1 h pulse) at different time intervals (hours) after infection (p.i.).

A SDS-PAGE analysis and autoradiography of samples containing equal amounts of radioactivity. Virus proteins L, G, N, NS and M are indicated. Hsp70 is indicated by arrow.

B, *C* VSV G glycoprotein synthesis (*B*) and hsp70 synthesis (*C*) were quantified in control (○) or Δ^{12}-PGJ_2-treated (●) VSV-infected MA104 cells, after immunodetection as described[18].

D MA104 cells infected with VSV (3 PFU/cell) were treated with control diluent (lane 1), sodium arsenite (50 μM, lane 2), L-azetidine 2-carboxylic acid (2 mM, lane 3), PGA_1 (10 μg/ml, lane 4) or were subjected to heat shock (42 °C, 2 h) 1 h after infection. Cells were labelled with ^{35}S-methionine (2 h pulse) 4 h after infection, and samples containing equal amount of radioactivity were processed for SDS-PAGE analysis and autoradiography. Hsp70 synthesis (□) and G glycoprotein synthesis (▨) were determined by densitometric analysis[65], and expressed as percent of total protein synthesis.

It has been suggested that large amounts of inducible hsp70 could irreversibly bind to newly synthesized proteins, causing a translational block[7]. Schlesinger et al.[72] 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 recent results suggest that hsp70 could interfere with SV mRNA translation dur-

ing its synthesis by the host cell. It could be hypothesized that HSP and virus messages, both of which can be translated in conditions where cellular protein synthesis is impaired, could possess similar mechanisms for preferential translation, and could then compete with each other. For example, both hsp70 mRNA and negative strand RNA virus mRNA are preferentially translated at increased cytoplasmatic ionic concentrations[41,45].

Due to the many cellular functions of hsp70 and to the many levels of interactions described above, the relationship between viruses and heat shock proteins appears to be a multifaceted and complex phenomenon, which depends on the type of infection, acute or persistent, and on the environmental conditions.

The possibility that HSP and hsp70 in particular could be part of an intracellular defense strategy against viruses is fascinating. However, many questions remain to be answered. A better understanding of the role of HSP in virus replication could be useful in the comprehension of the beneficial effect of fever during virus infections, and, as suggested by the pioneer studies of Tyrrell et al.[74], could indicate new strategies in the treatment of virus diseases.

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* Address for correspondence.

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