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Intranuclear targeted delivery of functional NF-κB by 70 kDa heat shock protein

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The 70 kDa heat shock protein (Hsp70) is a highly conserved, ubiquitous protein involved in chaperoning proteins to various cellular organelles. Here we show that when added exogenously to cells, Hsp70 is readily imported into both cytoplasmic and nuclear compartments in a cell-type-specific fashion. We exploited this ability of Hsp70 to deliver NF-κB, a key transcriptional regulator of inflammatory responses. We demonstrate that a fusion protein composed of a C-terminal Hsp70 peptide and the p50 subunit of NF-κB was directed into the nucleus of cells, could bind DNA specifically, and activated Igκ expression and TNFα production. We therefore propose that Hsp70 can be used as a vehicle for intracytoplasmic and intranuclear delivery of proteins or DNA to modulate gene expression and thereby control immune responses.

Keywords: Hsp70/heat shock protein/NF-κB/nuclear transport/protein delivery

Introduction

Heat shock proteins (Hsps) are a family of molecular chaperone proteins that have long been known to play essential roles in a multitude of intra- and intercellular processes, including protein synthesis and folding, vesicular trafficking, and antigen processing and presentation. Hsps are among the most highly conserved proteins known and carry out many of their regulatory activities via protein–protein interactions. Since one of the most well-characterized functions of Hsp70 is to assist in the translocation of proteins across intracellular membranes into different compartments of the cell, we were interested to find out whether this property of Hsp70 could be utilized to deliver proteins across the plasma and nuclear membranes. Intracellular transport activity has been reported for viral proteins such as the HSV-1 structural protein VP22 (Elliott and O'Hare, 1997) and the HIV Tat protein (Vivès *et al*., 1997), as well as peptide sequences derived from Antennapedia homeodomain, fibroblast growth factor (Hawiger, 1997) and most recently the neuropeptide galanin (Pooga *et al*., 1998). Delivery of protein substrates has been demonstrated by some of these transport peptides as well (Fawell *et al*., 1994; Phelan *et al*., 1998; Rojas *et al*., 1998).

Data suggesting that Hsp70 is able to cross the plasma membrane have come from a number of studies over the past few years, which characterize the nature of Hsps in generating protective immunity. A series of recent studies (Román and Moreno, 1996; Suzue and Young, 1996) demonstrated that bacterial Hsp70 could be used as an adjuvant-free carrier protein to enable its bound peptide or protein substrate to enter the endosomal compartment and subsequently access the MHC class II processing pathway for exogenous antigens. In these experiments it was shown that immunization of mice with Hsp70–peptide complexes or Hsp70 fusion proteins could elicit cargo-specific proliferative T cell responses in the spleen. However, other experiments in which cancer-cell-derived Hsp70 used to immunize mice resulted in specific anti-tumor cytotoxic T lymphocyte (CTL) responses (Udono and Srivastava, 1993) suggested that the Hsps were using the endogenous MHC class I processing pathway. These studies indicate that Hsps can transit not only through endosomes, but into the cytoplasm as well. Early studies by Hightower and Guidon (1989), which reported the release of Hsps from cultured newborn rat cells by a non-heat shock-dependent mechanism, support the observation that some Hsp family members can cross the plasma membranes of certain cells. Although these studies imply a plasma membrane translocation capacity for Hsp70, such an activity has not been demonstrated directly.

In this report we present direct evidence that the Hsp70 protein, Hsp70-derived peptides and chimeric proteins composed of Hsp70 peptides fused to the p50 subunit of NF-κB can all translocate across cell membranes to rapidly gain cytoplasmic and nuclear entry. Though cellular import activity has been reported for various diverse peptides, intranuclear transport generally requires the presence of specific nuclear localization sequences (NLS). While heat shock is known to induce increased synthesis and nuclear translocation of endogenous heat shock protein following heat shock factor (HSF) activation, we show that nuclear localization of exogenous heat shock protein can result without prior heat shock. Since the majority of the peptide binding and regulatory functionality of Hsp70 resides in the C-terminus, we focused on this region for our studies. We found that the transport and nuclear localization properties of Hsp70 were retained within a 90 amino acid C-terminal fragment, and our subsequent experiments utilized this fragment as well as one encompassing a larger portion of the peptide-binding domain. Our data suggest that this C-terminal domain of Hsp70 contains a novel NLS. To our knowledge, this is the first report to establish the ability of exogenous Hsp70 fusion proteins to cross the cell membrane, gain nuclear entry and exert a biological function. These data indicate that Hsp70 would present an ideal candidate for delivery of molecules with biological function into both cellular and nuclear compartments.

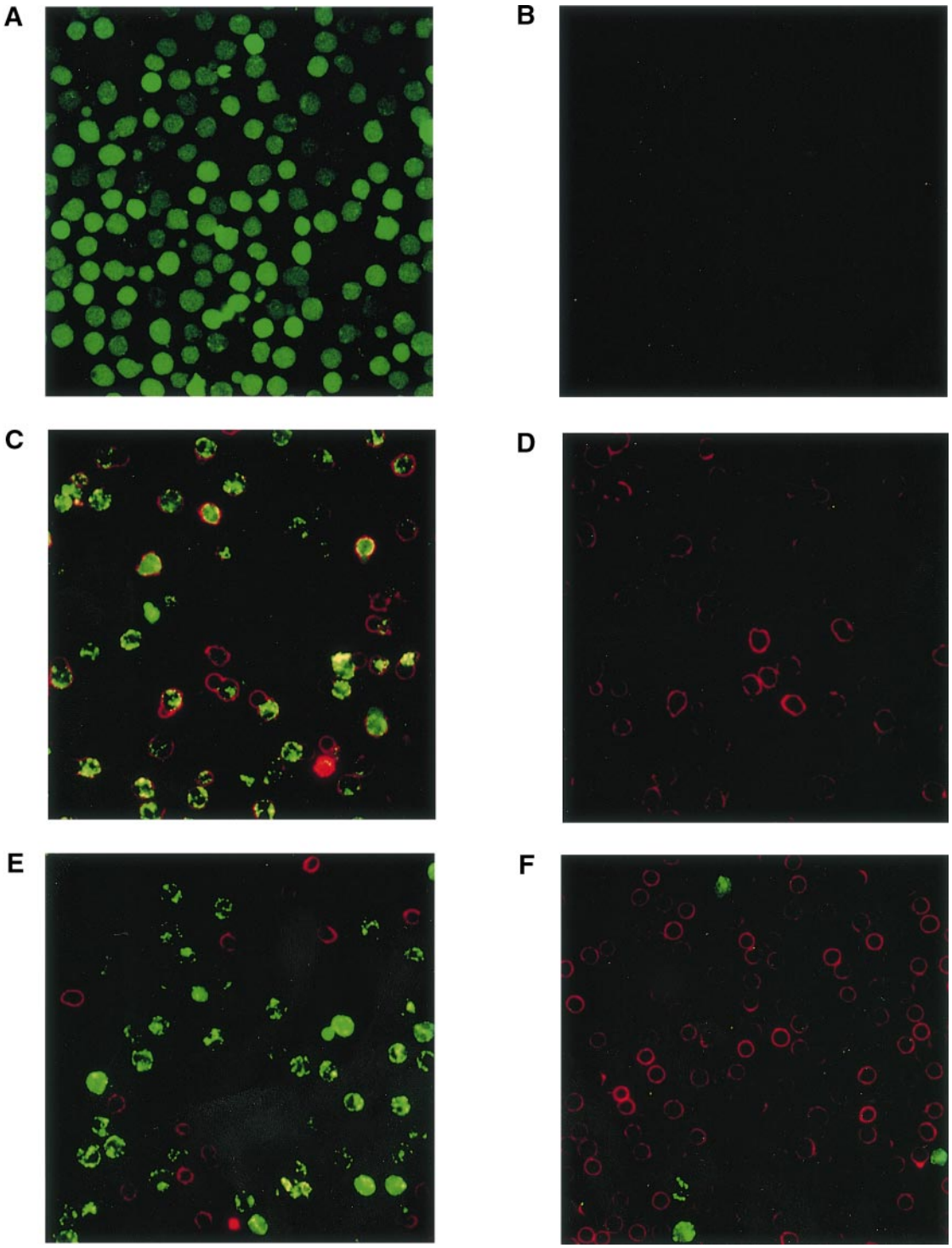

Fig. 1. Hsp70 is transported in a cell-type-specific manner. Cells were treated with 10 μg/ml Hsp70-FITC (green) added to the culture media. Human peripheral blood lymphocyte populations were stained with anti-CD14-PE (red) as a marker for monocytic cells, anti-CD19-PE for B cells, or anti-CD3-PE for T cells. After 1 h of incubation at 37°C, cells were washed, fixed and visualized by confocal laser scanning microscopy. Equimolar amounts of BSA-FITC were used in parallel experiments as a control. (**A**) 70Z/3 cells + Hsp70-FITC, (**B**) 70Z/3 cells + BSA-FITC, (**C**) E-rosette-negative (containing monocytes and B cells) PBLs treated with anti-CD14-PE + Hsp70-FITC, (**D**) E-rosette-negative PBLs treated with anti-CD14-PE + BSA-FITC, (**E**) E-rosette-negative PBLs treated with anti-CD19-PE + Hsp70-FITC, (**F**) E-rosette-positive (containing T cells only) PBLs treated with anti-CD3-PE + Hsp70-FITC.

Results

Hsp70-mediated transport is cell-type specific

Recombinant human Hsp70 protein was generated for use in the following experiments. To investigate the intracellular localization of exogenously added Hsp70, we fluorescently labeled the full-length Hsp70 protein and tested its ability to become internalized by various cell types. Cells were typically treated with a final concentration of 10 μg/ml Hsp70-fluorescein isothiocyanate (FITC)

[or bovine serum albumin (BSA)-FITC as a control] for 1 h, then washed and fixed prior to confocal laser scanning microscopy. The bulk of the intracellular Hsp70-FITC localized uniformly to the nucleus and cytoplasm in murine pre-B 70Z/3 cells, whereas import of BSA-FITC was negligible (Figure 1A and B). We also observed significant uptake of Hsp70-FITC but not BSA-FITC by human peripheral blood monocytes (Figure 1C and D). The uptake by peripheral blood lymphocytes (PBLs) appeared to be specific for monocytes as opposed to B cells, as we observed intracellular staining only in cells which were $CD14^+$ (Figure 1C), and not in $CD19^+$ (Figure 1E). In addition, we did not observe uptake by peripheral blood T cells (Figure 1F). Interestingly, while the staining pattern in peripheral blood monocytes was often uniform throughout the cytoplasm and nucleus, we noted that it was sometimes characterized by punctate staining, implying vesicular localization within the cytoplasm. We found that the specific staining pattern of the PBLs varied by donor, suggesting that the state of cell activation may play a role in uptake efficiency or intracellular localization of exogenous Hsp70. Consistent with this hypothesis, we found that while resting peripheral blood B cells were resistant to Hsp70 uptake, they could be induced to transport the protein after 48 h of activation *in vitro* with anti-CD40 plus anti-immunoglobulin (Ig) antibodies (data not shown). This method of B cell activation is known to result in the expression of various differentiation- and proliferation-associated genes. In contrast, activation of peripheral blood T cells by anti-CD3 and anti-CD28 antibodies did not affect Hsp70 transport (data not shown). No intracellular uptake was observed by Jurkat T cell or HeLa cell lines, but we did observe uptake by several mature B cell lines, including Ramos, RAJI and BJAB (data not shown). In addition, we saw limited cytoplasmic uptake by two monocytic cell lines, THP-1 and U937, but only after extended (6–24 h) incubation with high concentrations (100 μg/ml) of Hsp70-FITC (data not shown). The B cell- and monocyte-specific Hsp70 transport activity we describe is consistent with reports which propose a role for Hsp70 heat shock family members in antigen presentation (Vanbuskirk *et al*., 1989; Manara *et al*., 1993), as these cells are generally considered to function as antigen-presenting cells. The cell-type specificity and inducibility of cellular Hsp70 uptake may reflect differential expression of a required surface or nuclear receptor for the Hsp70 protein. Studies are now in progress to investigate the surface proteins which may be involved in the binding and internalization of extracellular Hsp70.

Kinetics of Hsp70 cellular uptake

We next investigated the time course and dose–effect of Hsp70 uptake by 70Z/3 cells. From these studies we determined that the internalization process appears to be slow, since maximal internalization is not achieved until between 6 and 8 h after addition of Hsp70 (Figure 2A), and it remains constant for up to 2 days of incubation. For the dose range study, varying amounts of Hsp70-FITC were incubated with the cells for 1 h. Our analysis showed that the intensity of intracellular fluorescence increased with increasing concentrations of extracellular Hsp70, up to 100 μg/ml (1.4 μM), and was detectable even when

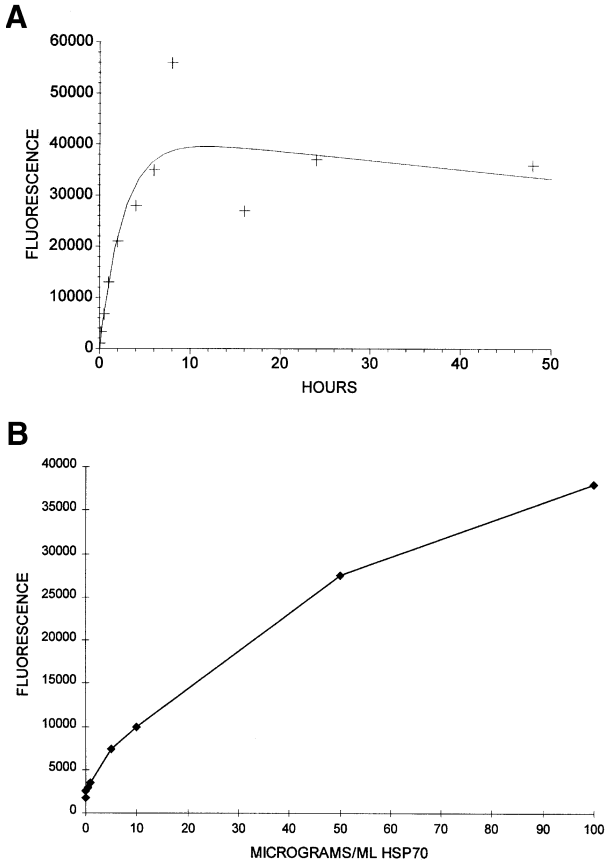

Fig. 2. Uptake of Hsp70 is time- and concentration-dependent. (**A**) Kinetics of uptake of Hsp70-FITC by 70Z/3 cells. Cells (5×10^5) were incubated at 37°C for increasing periods of time with 1 μM Hsp70-FITC, washed, and analyzed in a fluorimeter. The data curve was fitted by a modified regression program (XLfit). (**B**) Dose–effect of Hsp70 on uptake by 70Z/3 cells. The cells were incubated at 37°C for 1 h with various concentrations of Hsp70-FITC, then washed and analyzed as in (A).

cells were treated with levels as low as 0.1 μg/ml (1.4 nM; Figure 2B). We determined that treatment of cells with an extracellular concentration of 1 μM Hsp70-FITC resulted after 1 h of incubation in an intracellular concentration of 700 nM, assuming a volume of 1 pl/cell. This uptake efficiency is comparable to that reported for other intracellular targeting peptide sequences (Phelan *et al*., 1998). The uptake of Hsp70-FITC was not saturable in the concentration range we used (35 nM–1 μM), consistent with a high capacity receptor-mediated or non-receptor-mediated uptake mechanism. In addition, internalization by 70Z/3 cells could not be blocked by preincubation with a 10-fold excess of unlabeled Hsp70. However, we did observe inhibition of internalization using excess Hsp70 with cells that are less efficient at uptake, such as Ramos and U937 (data not shown).

Mechanism of Hsp70-mediated intracellular transport

To investigate the mechanism of transport, we examined the import of Hsp70 under various conditions. Full-length Hsp70 protein was transported by PBLs in the presence of 0.05% sodium azide, an inhibitor of oxidative phosphorylation, to nearly the same extent as control cells (Figure 3, compare A with B). However, this transport activity

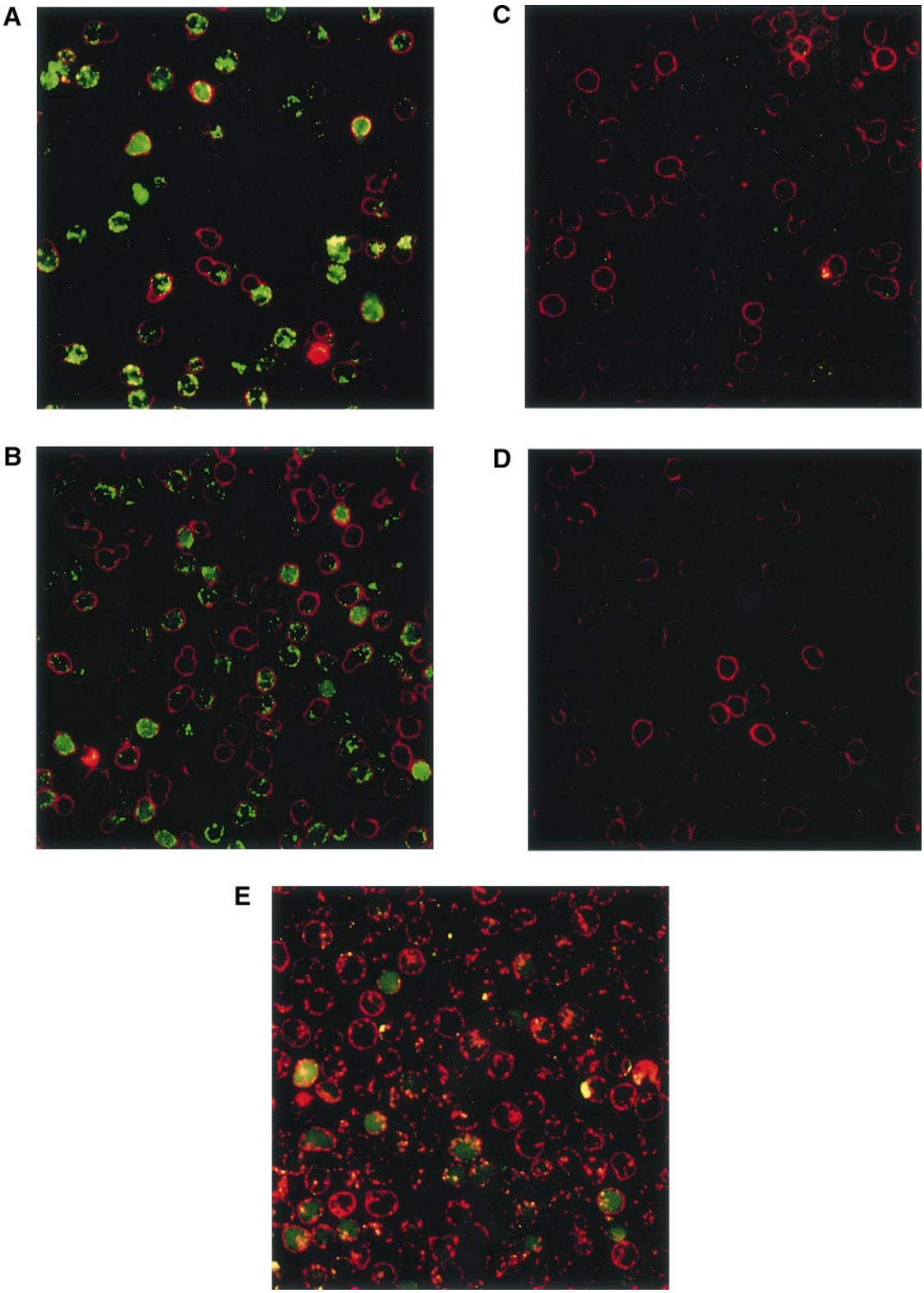

Fig. 3. Intracellular uptake of Hsp70-FITC was minimally affected by azide but was inhibited at 4°C. Peripheral blood lymphocyte cells in (A–D) were stained with anti-CD14-PE as a monocyte marker. Cells were either untreated (**A**) or pretreated for 30 min with 0.05% sodium azide (**B**) before incubation with 10 μg/ml Hsp70-FITC for 1 h at 37°C, or were pre-incubated at 4°C for 30 min prior to addition of Hsp70-FITC and an additional 1 h of incubation at 4°C (**C**). (**D**) BSA-FITC added to cells for 1 h at 37°C as a control. Internalized Hsp70-FITC did not colocalize with transferrin. 70Z/3 cells were treated with both Hsp70-FITC and Texas Red-conjugated transferrin, for 1 h at 37°C (**E**).

was completely eradicated when cells were incubated at 4°C (Figure 3C, compare with BSA-FITC treatment in D), suggesting that there is a temperature-dependent component to the Hsp70-mediated transport. These data, coupled with co-internalization studies using transferrin which revealed that accumulation of transferrin and Hsp70

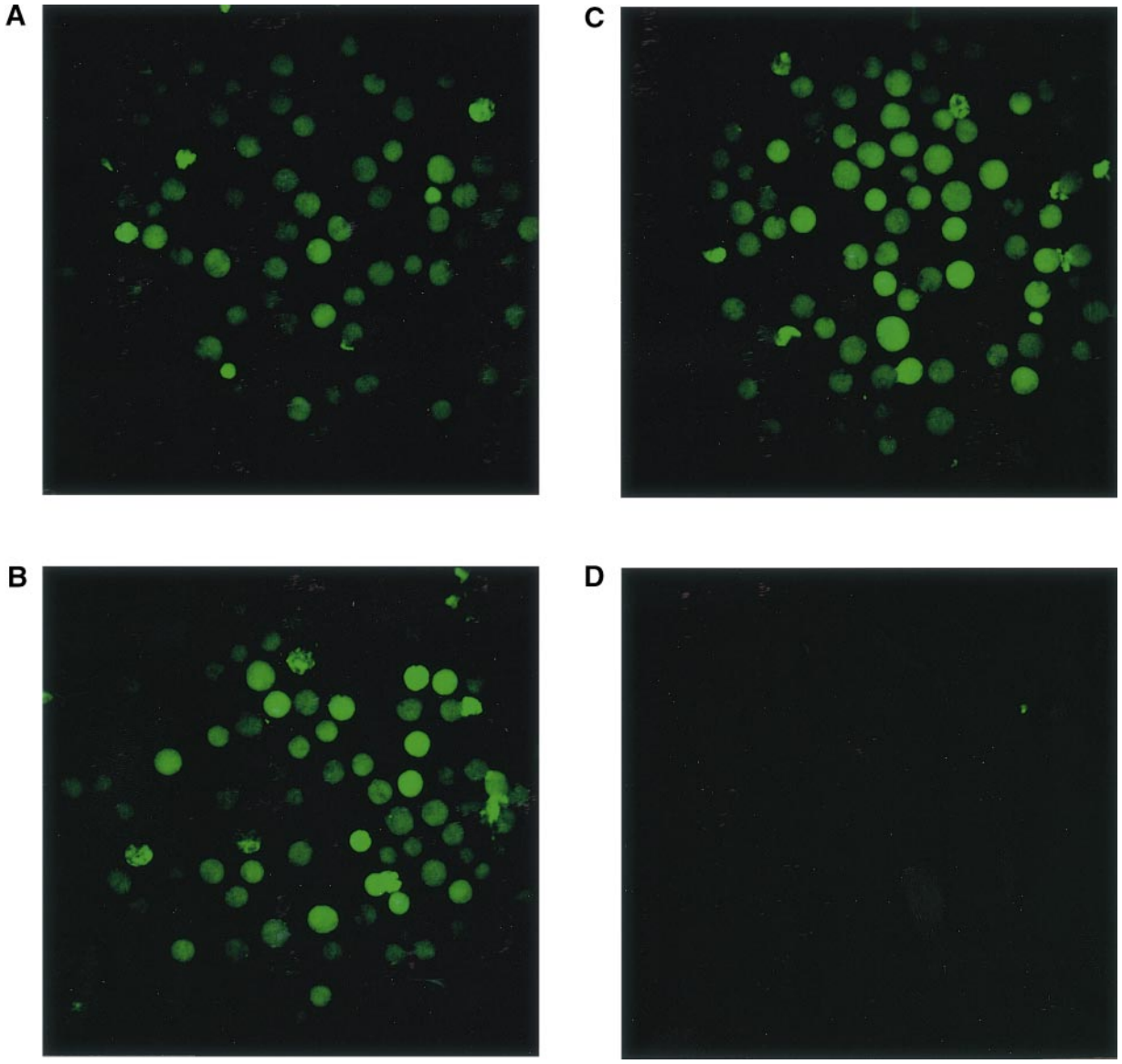

Fig. 4. FITC-labeled fusion proteins consisting of either the C-terminal 245 (Hsp70/28–p50) or 92 (Hsp70/10–p50) amino acids of Hsp70 fused to p50 were transported into 70Z/3 cells. 70Z/3 cells were treated with 10 μg/ml full-length Hsp70-FITC (**A**), Hsp70/28–p50-FITC (**B**), Hsp70/10–p50-FITC (**C**), or BSA-FITC as a control (**D**) for 1 h at 37°C as described in the Materials and methods. Intracellular localization of fusion proteins was visualized by confocal laser scanning microscopy.

occurred in separate intracellular compartments in both 70Z/3 cells (Figure 3E) and in human PBLs (data not shown), suggested that this mechanism does not involve classical endocytosis. However, we cannot rule out the possibility that Hsps are transported via a novel endocytic mechanism.

An exogenous NF-κB C-terminal Hsp70 fusion protein can be directed to the nucleus

Two different fusion proteins composed of either a 245 or a 92 amino acid Hsp70 C-terminal polypeptide fused to the p50 subunit of the transcription factor NF-κB were generated to examine the ability of the Hsp70 peptide sequence to direct other protein substrates into the cell. When added exogenously to cells, both FITC-conjugated fusion proteins entered the cytoplasm and nuclei of 70Z/3 cells (Figure 4) and PBLs (data not shown) with kinetics and specificity similar to the Hsp70 peptide alone. Internalized FITC-Hsp70 remained stable for 24 h after washout of unincorporated protein and incubation at 37°C, as determined by confocal microscopy and SDS–PAGE analysis. To assess protein stability, cells were pulsed with either 10 μg/ml FITC-conjugated full-length Hsp70 or Hsp70/28–p50 for 1 h prior to washing and a chase at 37°C for up to 96 h. Gel electrophoresis showed the presence of both the ~75 kDa fusion protein as well as full-length Hsp70 itself (distinguishable from endogenous Hsp70 by its fluorescent tag) in whole-cell extracts after up to 24 h of incubation without measurable change in size or appearance of smaller molecular weight degradation products (Figure 5). These data suggest that intracellular targeted Hsp70 and Hsp70–p50 fusion protein was stable, and remained within the cell without significant degradation with a half-life of $>$24 h.

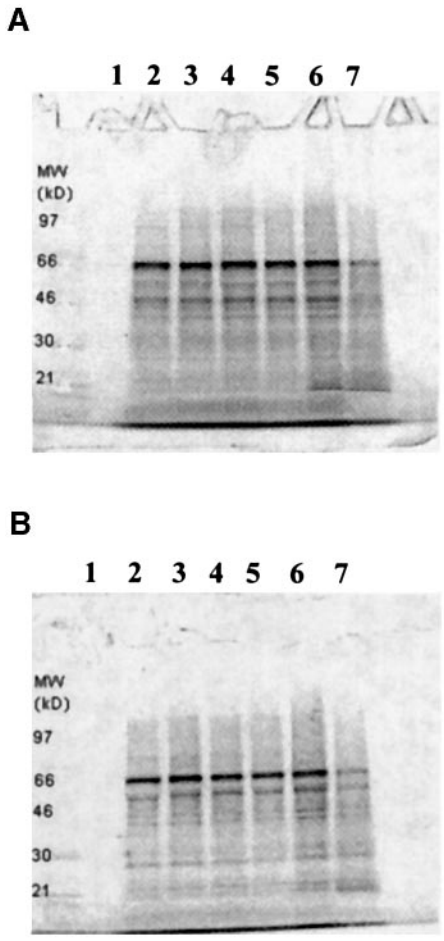

Fig. 5. Internalized intracellular Hsp70 or Hsp70–p50 remained stable for up to 24 h. 70Z/3 cells were treated with 10 μg/ml of either full-length Hsp70-FITC (**A**) or Hsp70/28–p50-FITC (**B**) for 1 h prior to washing and additional incubation at 37°C for increasing times. Cells were harvested at the indicated time points, lysed in Laemmli sample buffer and whole-cell lysate proteins were separated by SDS–PAGE. Gels were subjected to fluorimager analysis. Lane 1, cells untreated; lane 2, no chase; lane 3, 1 h of chase; lane 4, 2 h of chase; lane 5, 6 h of chase; lane 6, 24 h of chase; lane 7, 4 days of chase.

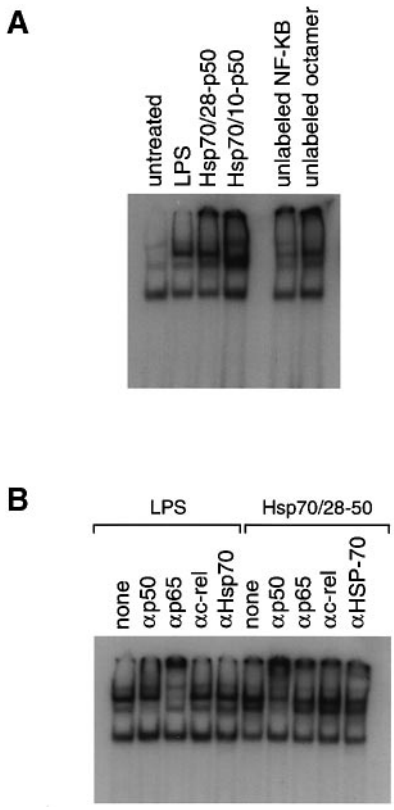

Fig. 6. Internalized Hsp70–p50 fusion proteins exhibited DNA-binding activity. 70Z/3 cells were treated as indicated for 1 h prior to lysis and preparation of nuclear extracts. Electrophoretic mobility shift assays (EMSA) were performed and specific DNA-binding complexes were identified by supershift assay with the indicated antibodies. (**A**) Lane 1, unstimulated cells control; lane 2, LPS treated; lane 3, Hsp70/28–p50 treated; lane 4, Hsp70/10–p50 treated; lane 5, Hsp70/28–p50-treated extracts competed with unlabeled NF-κB oligonucleotide; lane 6, same as lane 5 but competed with unlabeled octamer oligonucleotide. (**B**) Lane 1, LPS treated; lane 2, LPS-treated and supershifted with anti-p50; lanes 3–5, same as lane 2 but using the indicated antibodies for supershifting: lane 3, anti-p65; lane 4, anti-c-Rel; lane 5, anti-Hsp70. Lanes 6–10, same as lanes 1–5 but using Hsp70/28–p50 treated extracts.

Transported NF-κB p50 exhibits DNA-binding activity

We could show that purified fusion proteins were able to bind κ DNA (data not shown), suggesting that the p50 subunits were not conformationally impaired by the presence of the Hsp70 sequences. To address whether the internalized fusion proteins retained functional activity, we tested nuclear extracts of Hsp70–p50 fusion protein-treated cells for their ability to bind a specific κ DNA sequence. After 70Z/3 cells were treated with LPS, Hsp70/10–p50 or Hsp70/28–p50 for 1 h, nuclear extracts were prepared and gel-shift assays were performed. We found that DNA-binding activity was retained by the fusion protein after nuclear uptake by cells, indicating that the import process did not result in significant degradation or loss of activity (Figure 6A). This DNA-binding activity was specific, as the complex was largely competed with an excess of unlabeled NF-κB sequence but not with the consensus sequence for octamer-binding protein (Figure 6A). We saw distinct complexes formed by nuclear extracts from cells treated with different fusion proteins; furthermore, they differed from the complex formed by LPS-induced endogenous NF-κB. In supershift experiments we observed that anti-p50 antibodies were able to shift nearly the entire DNA-binding complex from fusion protein-treated cells, as expected, confirming that the fusion protein was probably binding to DNA mainly as a homodimer (Figure 6B). We also observed a detectable decrease in the specific complex upon incubation with both anti-p65 and anti-Hsp70 (directed against the C-terminal four amino acids EEVD) antibodies, indicating the presence of endogenous p65 subunits in addition to recombinant Hsp70–p50 subunits. In contrast, nuclear extracts from control LPS-treated cells formed a complex containing both p50 and p65 subunits, and the supershifted patterns differed from the cells treated with the fusion protein. These data indicate that distinct protein–DNA complexes were formed and suggest that the fusion proteins were binding DNA directly and not simply activating endogenous NF-κB.

Hsp70–p50 fusion protein activates surface Igκ expression and TNFα production

Since treatment of cells with Hsp70–p50 fusion proteins could potentially result in the formation of complexes

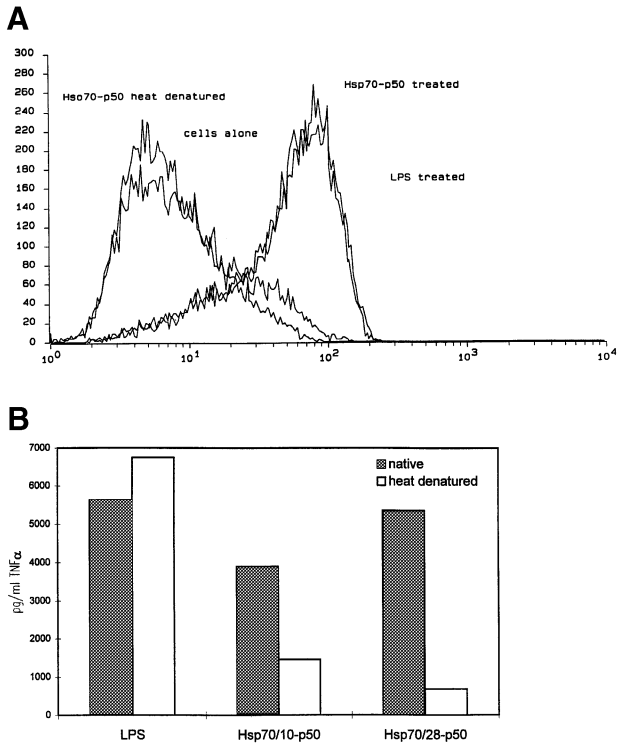

Fig. 7. Hsp70–p50-activated surface Igκ expression and TNFα production. (**A**) 70Z/3 cells were treated with 10 ng/ml LPS or 30 μg/ml Hsp70/10–p50 overnight prior to washing and staining with anti-κ-FITC and FACS analysis. (**B**) Human PBLs were treated with 5 ng/ml LPS, or 40 μg/ml of Hsp70/10–p50 or Hsp70/28–p50 for 6 h. Supernatants were harvested and analyzed for TNFα levels by ELISA.

which interact with NF-κB DNA binding sites in cells, we decided to evaluate downstream biological events in the NF-κB pathway. We observed that treatment of various cells with the Hsp70–p50 fusion proteins resulted in activation of several inflammatory and immunological responses normally regulated by NF-κB. Treatment of mouse 70Z/3 pre-B cells with Hsp70/10–p50 fusion protein was shown to be as effective as lipopolysaccharide (LPS) in inducing high levels of kappa Ig (Igκ) light chain on the surface (Figure 7A). In addition, in contrast to LPS-induced activation, the fusion protein-induced surface κ expression was abolished by 30 min of 65°C heat denaturation of the protein prior to treatment of cells, confirming that native protein was required for the activation. Similar results were obtained with Hsp70/28–p50 (data not shown).

TNFα production is another example of an inflammatory response also predominantly regulated by NF-κB. We observed that the internalized fusion protein was also able to induce TNFα production by human PBLs (Figure 7B). Freshly isolated PBLs were incubated with LPS or Hsp70–p50 for 6 h, after which time supernatants were collected and tested for cytokine levels by ELISA. Again, we found the fusion proteins to be as effective as LPS in inducing TNFα production, and established that intact protein was responsible for activation by showing that heat denaturation of the fusion protein abolished the effect.

Discussion

In this report we have shown that exogenously added heat shock protein Hsp70 is internalized by cells into both the cytoplasm and nucleus in a cell-type-specific manner. The intracellular transport appears to be temperature dependent, and occurs over a time course extending to 6–8 h. The transport-mediating domain can be localized to the C-terminus of Hsp70, and can be used to target an extracellular NF-κB protein to the nucleus where it can bind DNA and activate gene expression. Although the mechanism of uptake is unknown, the data suggest that the binding and internalization of Hsp70 may involve a high capacity receptor. Hsp70 does not appear to be internalized via the classical endocytic route since it does not co-localize with transferrin and is quite stable intracellularly; therefore, it is probably not transiting through lysosomes. We did observe differences in internalization of Hsp70 by different cell lines, exemplified by the distinct patterns of staining in 70Z/3 pre-B cells as opposed to the monocytic U937 cells. Guzhova *et al*. (1998) previously reported Hsp70 internalization by U937 cells, which they suggest utilize a mechanism involving patching and capping. While we occasionally observed internalization of Hsp70 by U937 that could be described as patching and capping, we also noted a more uniform fluorescence indicative of intracytoplasmic and intranuclear accumulation. On 70Z/3 cells we saw no evidence of patching, suggesting that the uptake mechanism in these cells is different from that in U937 cells. Identification of an Hsp70 receptor on the cell surface will yield more information on the mechanism of Hsp70-mediated transport.

To our surprise, we found that treatment of cells with the Hsp70–p50 fusion protein induced transactivation, as evidenced by the increase in surface κ expression and TNFα production. NF-κB p50 homodimers are commonly thought to act as transcriptional repressors in most cell types due to the absence of a C-terminal activation domain. There are a number of possible explanations for the lack of an observed dominant negative effect of the Hsp70–p50 fusion protein on transcriptional activation. First, the heat shock protein sequence, specifically the EEVD domain, may itself possess transactivation activity. Hsp70 is known to bind HSF in the nucleus and interfere with its transactivation activity via the EEVD domain. Since the Hsp70 sequence in our study was fused C-terminal to the p50 sequence, the EEVD domain is potentially available to provide transactivation, or even to interact with other cellular or nuclear cofactors. Closer analysis of the nuclear complex may yield clues as to other possible components with transcriptional activities. Secondly, p50 homodimers may simply exhibit transactivation activity in certain circumstances. Fujita *et al*. (1992) tested the various homo- and heterodimers of NF-κB subunits for transcriptional activation *in vitro* and determined that addition of p50 alone to some transcription mixtures resulted in significant transcriptional stimulation. They attributed this activation to differences in the fine structure of the nucleotide sequence within the κB motifs. Interestingly, this group observed a 4-fold stimulation of transcription by p50 homodimers over control using the Igκ sequence motif. These data would correspond to the activation we observed in studies analyzing surface Igκ expression. Thirdly, the Hsp70–p50 subunits may be recruiting other transactivating factors into the DNA-binding complex that we have not yet detected. We are

currently investigating the mechanism by which the fusion protein is activating transcription.

In addition to its ability to deliver NF-κB, the intracellular and intranuclear uptake of Hsp70 itself may have *in vivo* relevance. The observation that cell surface-associated and secreted forms of Hsp70 exist (Multhoff and Hightower, 1996) suggests that this protein may function in cell–cell communication, perhaps as a means of transferring cellular protection from environmental stressors by regulating transcription. In fact, several recent reports have described a physical interaction of Hsp70 with the transactivation domains of several transcription factors within the nucleus. For example, Hsp70 can bind directly to the transactivation domains of both HSF (Shi *et al*., 1998), resulting in inhibition of gene transcription, and Wilms tumor suppressor (Maheswaran *et al*., 1998), resulting in suppression of cellular proliferation. These data and others implicate a role for Hsp70 in the regulation of transcription factors and possibly other nuclear proteins. These data also complement others which have shown that Hsp70, as a cytoplasmic chaperone, can interact with transcription factors such as NF-κB itself, as well as a myriad of cofactors such as Hip, Hop, Hsp40, Hsp90, BAG-1 and others (Demand *et al*., 1998). Indeed, release and intercellular transfer of exported Hsp70 has been reported in cultured newborn rat cells (Hightower and Guidon, 1989), and accumulation of Hsp70 in a variety of human cell lines either by heat shock or by liposomal transfer has been shown to increase cell survival and protect from apoptotic cell death (Lasunskaia *et al*., 1997). Release of heat shock proteins from cells under harsh or damaging conditions may be a homeostatic mechanism for transfer of a protective stress response to neighboring cells that are unable to mount such a response. In addition, recent reports describing the ability of peptide-bound Hsp70 molecules to induce antitumor or antiviral immunity as well as the development of memory CTLs support the notion that these proteins might function to convey a protective immune response by providing an antigen-presentation function (Blachere *et al*., 1997; Ciupitu *et al*., 1998). We envision a scheme whereby endogenous Hsp70 (and associated peptides or proteins) are released into the environment by infected or apoptosing cells. These Hsp70 protein complexes would subsequently become available to neighboring cells, which may be compromised in their immune capacity, and act as a stimulus to boost or strengthen the immune response.

We demonstrate the effective cellular and nuclear uptake as well as long-term intracellular stability of exogenously supplied Hsp70 fusion proteins by several cell types. This rapid and stable transport activity has important implications for the utility of Hsp70-derived peptides as a vehicle for delivering therapeutic agents to the cytoplasm and nucleus, where they remain localized for long periods of time. As manipulation of the nuclear import process in particular becomes an increasingly interesting target for regulated control of gene expression (Fujihara and Nadler, 1998), we believe that future emphasis will be placed on developing more potent means of intracellular targeted delivery. The use of Hsp70 as a delivery system has a number of advantages over other previously described protein candidates, including the fact that the protein is of human origin and therefore does not contain foreign (i.e. viral, bacterial or insect) and potentially immunogenic material. As Hsp70 is a highly expressed and abundant protein, it would probably be well-tolerated in humans. In addition, the cell-type specificity we observed and the demonstration by others that it plays a role in immune responses might allow the targeting of compounds to specific cells of the immune system for more effective regulatory control. Finally, the preferential and long-lived nuclear-directed delivery of protein substrates may provide protection from cytoplasmic proteolysis. Our data support the potential use of Hsp70 sequences as a novel tool for delivery of molecules such as proteins or DNA that modulate gene expression.

Materials and methods

Expression and purification of the Hsp70–p50 fusion proteins

Two p50 fusion proteins were constructed using the nucleotide sequence corresponding to amino acids 1–406 of the NF-κB p105 subunit protein. This sequence includes the DNA-binding domain as well as the Rel homology domain. The two fusion proteins varied in the length of Hsp70 fragment used. The two Hsp70 sequences (DDBJ/EMBL/GenBank accession No. M59828) were both derived from the C-terminus, including either the terminal 276 or 735 nucleotides, which correspond to a 10 and a 28 kDa protein fragment. Either the 10 or the 28 kDa Hsp70 protein was fused C-terminal to the p50 protein, and the resulting fusion proteins were denoted Hsp70/10–p50 or Hsp70/28–p50, respectively. The prokaryotic expression vector ProEX HT (Life Technologies) was used for cloning, expression in *E.coli* DH59 cells and purification according to the manufacturer's recommendations. All FITC conjugations were performed using fluorescein isothiocyanate. Proteins to be conjugated were incubated with FITC for 30 min at room temperature, and unconjugated FITC was separated from labeled protein by gel filtration.

Cells and antibodies

Pre-B 70Z/3 cells were maintained in RPMI supplemented with 10% fetal bovine serum and β-mercaptoethanol. PBLs were prepared from healthy donors by Ficoll gradient centrifugation, and T cells were separated from monocytes and B cells in this fraction by rosetting with sheep red blood cells. Anti-CD14 and anti-CD19 antibodies were purchased from Becton Dickenson, and anti-κ antibodies were purchased from Southern Biotechnology Associates, Inc.

Confocal laser scanning microscopy

Cells were typically treated with 10 μg/ml FITC-conjugated proteins or as indicated in the text for 1 h at 37°C followed by a wash in phosphate-buffered saline (PBS), fixation in 2% paraformaldehyde, and an additional wash in PBS. Samples were examined with a Bio-Rad MRC1024 confocal microscope using Molecular Dynamics LaserSharp and Adobe Photoshop software.

Fluorescence analysis of imported Hsp70–p50 fusion proteins

Cells were treated with 10 μg/ml FITC-conjugated proteins for 1 h at 37°C, washed in PBS and used for preparation of nuclear extracts. Equal protein amounts were separated by SDS–PAGE. The gel was fixed in acetic acid and subject to fluorescence analysis by Fluorimager SI and ImageQuant software (Molecular Dynamics).

Electrophoretic mobility shift assay

Nuclear extracts from 70Z/3 cells were prepared using a modification of established protocols (Tepper *et al*., 1995). Protein concentrations were determined using the Bradford assay, and an NF-κB (5′-GATCCGAGGGGACTTTCCGCTGGGGACTTTCCAGG-3′) oligonucleotide (Promega, Madison, WI) was end labeled with [γ-^{32}P]ATP and T4 kinase. The conditions for binding reactions with oligonucleotide probes were as described previously. Supershift assays were performed with NF-κB p50, p65 and c-Rel polyclonal antibodies (Santa Cruz Biotechnology) and Hsp70 antibody (a polyclonal antibody generated at BMS, Seattle, WA, and known to be directed against the Hsp70 C-terminal EEVD amino acid sequence) by pre-incubating the nuclear extracts with 3 μl of the antibody in the reaction buffer for 30 min followed by EMSA

analysis. Competition experiments were performed using unlabeled NF-κB and octamer (5′-TGTCGAATGCAAATCACTAGAA-3′) oligonucleotides. Samples were analyzed on native 6% polyacrylamide gels and autoradiographed.

Immunofluorescence assay (FACS)

70Z/3 cells were treated with either 30 μg/ml Hsp70/10–p50 fusion protein or 10 ng/ml LPS and incubated overnight at 37°C. Cells were then washed in PBS and fixed in 2% paraformaldehyde prior to staining with FITC-conjugated anti-κ antibody. After an additional PBS wash, cells were subjected to imaging and analysis on the FACStar (Becton Dickenson).

TNFα assay

Human PBLs were isolated as previously described and treated with 40 μg/ml Hsp70 fusion protein or 5 ng/ml LPS for 6 h. Supernatants were collected and analyzed for TNFα by ELISA (Genzyme).

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