Modulation of cellular AP-1 DNA binding activity by heat shock proteins

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Abstract Recent studies have indicated that ubiquitously expressed molecular chaperones of the heat shock protein (Hsp) class may have an additional, nuclear, role in the regulation of gene expression. Experiments on cellular transcription factors derived from the rat adrenal gland have now shown that Hsps modulate in vitro DNA binding activity of the AP-1 factor. Both Hsc70 (p73) and Hsp70 (p72) were demonstrated to exert this effect through a mechanism that appears to be independent of both redox, and phosphorylation state. Further studies on the effect of Hsps on recombinant Fos/Jun protein binding activity indicated that the mechanism of action involves a selective attenuation of high affinity c-Fos:c-Jun binding as compared with c-Jun homodimer binding activity. Because cellular and physiological stress are associated with the induction of both AP-1 and Hsps it is apparent that Hsps may play a modulatory role in the regulation of AP-1 responsive genes.

Key words: Hsp70; Hsc70; AP-1; c-Fos; c-Jun; Rat adrenal

1. Introduction

A role for heat shock proteins (Hsp) as molecular chaperones in various extra-nuclear cellular compartments is now well established [1,2]. The localization of Hsp in the nucleus [3,4], and the demonstration that Hsps control the assembly of protein complexes [1] has led to the suggestion that Hsps may affect gene expression at the level of transcription through modulation of transcription factor (and transcription factor/DNA) complex assembly [5]. Indeed, Hsp70 has been implicated in the oligomerization mechanism of the heat shock transcription factor [6]. Hsps are known to associate with other nuclear proteins such as p53 [7], and both DnaK, an *E. coli* homologue of Hsp70, and human Hsps have been shown to control the stability of p53 DNA binding activity in vitro [8].

Our laboratory has been studying the regulation of AP-1 transcription factor activity in the adrenal gland [9]; unusually there is a high constitutive level of AP-1 DNA binding activity in the rat adrenal [10] but this activity is also modulated by various pharmacological and physiological stimuli including stress [11]. A corresponding induction of Hsp expression in the rat adrenal gland during stress [12] is interesting in the context of the potential nuclear effects of Hsp because adrenal AP-1 may represent a physiologically regulated transcription factor complex that is influenced by Hsps. Accordingly, we have now investigated the basis of such potential interactions through an investigation of the effects of Hsp on AP-1 activity in vitro. It has been demonstrated that Hsps modulate both adrenal gland-derived, and recombinant AP-1 DNA binding activity in vitro, through a mechanism that is selective for particular binding complexes.

2. Materials and methods

Purified and recombinant Hsps were obtained from StressGen Biotechnologies (Victoria, BC, Canada). Recombinant c-Fos and c-Jun were synthesized using the TNT coupled reticulocyte lysate system (Promega, Madison, WI) using, as templates, rat c-fos (see [13]) and rat c-jun [13]. Hsp antibodies were from StressGen (anti-Hsc70 monoclonal, 1B5), Affinity Bioreagents (Golden, CO; anti-Hsp70 monoclonal, 3a3) and Amersham International (Arlington Heights, IL; anti-Hsp72, RPN 1197). A c-Jun antiserum (N; sc-45X) and a glucocorticoid receptor (GR) antiserum (P-20; sc1002) were obtained from Santa Cruz Biotechnologies Inc, Santa Cruz, CA. Reagents used in electrophoretic mobility shift assays (EMSA) were from Promega: oligonucleotides and T4 polynucleotide kinase, Boehringer Mannheim GmbH (Mannheim, Germany): poly [d(I-C)] and Amersham: [γ-^{32}P]ATP. Reagents used in the Western blotting procedure including blocking solution (ProtoBlock), and chemiluminescence detection reagents (HPRL kit) were from National Diagnostics (Atlanta, GA). Secondary antisera (sheep, anti-mouse Ig), molecular weight markers (Rainbow) and nitrocellulose membrane (Hybond-N, nitrocellulose) were from Amersham. All other reagents were from Sigma (St. Louis, MO).

2.1. Electrophoretic mobility shift analysis

Whole-cell extracts of rat (Sprague-Dawley) adrenal tissue were prepared as described [9,14]. The protein content of the extracts was determined using the method of Bradford ([15], Bio-Rad, Hercules, CA). Recombinant AP-1 proteins were synthesized in vitro as described above. EMSAs of DNA binding activity were performed using established protocols [9,14]. EMSA probes were ^{32}P-labelled, double-stranded oligonucleotides; either an AP-1 consensus element-containing 21mer 5′-CGCTTGATGAGTCAGCCGGAA-3′ (Promega), or a SP-1 consensus element-containing 22mer 5′-ATTCGATCGGGGCGGGGCGAGC-3′ (Promega). The oligonucleotides were labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase and purified on a Sephadex G50 column. The specificity of adrenal AP-1 binding activity has been determined previously [9], and in the present study the specificity of both recombinant AP-1 protein, and adrenal SP-1 binding activity was determined using a molar excess of either unlabelled AP-1 or SP-1 oligonucleotide respectively as competitor. Binding reactions were performed with 10 μg of whole-cell extract or an aliquot of TNT lysate, 2 μg poly [d(I-C)], 2 μl of buffer (50 mM HEPES, pH 7.9, 0.125 mM DTT (except where indicated), 1% NP-40, 5 mM spermidine, 1.5 mM $MgCl_2$, 36% glycerol), and 20 000 cpm of labelled oligonucleotide. The reactions were incubated on ice for 15 min before adding the ^{32}P-labelled oligonucleotide, and then for an additional 15 min at room temperature (22°C). Except where indicated, Hsps and antibodies were incubated with adrenal cell extracts for 1 h at 4°C, and 1 h at 22°C, prior to the above-described binding reactions. Adrenal protein extracts were de-phosphorylated by treating with calf intestinal phosphatase (CIP, 0.05 U, 30°C, 20 min) and then inactivating the phosphatase with NaH_2PO_4 (40 μM). DNA-protein complexes were resolved on 4% non-denaturing polyacrylamide gels at 10 V/cm in 0.25×TBE buffer prior to autoradiography. Relative amounts of DNA binding activity were estimated by densitometric

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scanning of appropriately exposed autoradiographs (ImageMaster, Pharmacia, Sweden). Statistical comparisons were performed using Student's *t*-test, and significance was accepted for $P < 0.05$.

2.2. Hsp depletion and immunoblotting

Protein samples were depleted of Hsp using a standard ATP-agarose (A2767, Sigma) binding procedure [16] either in the presence or absence of an Hsp binding competitor (free ATP, 20 mM). Equal aliquots of either depleted or non-depleted protein samples were taken for both EMSA analysis and immunoblotting. For immunoblotting, protein samples were denatured (boiled for 3 min, 1:1 with electrophoresis sample buffer [17]) and separated on 10% denaturing polyacrylamide gels at 10 V/cm. The resolved proteins were then transferred (mini Trans-Blot cell (Bio-Rad) running at 100 V for 1 h at room temperature, in 25 mM Tris-base, 192 mM glycine, 20% methanol) to a nitrocellulose membrane. Immunoblotting was then performed using standard techniques according to the HRPL kit protocol. Protein bands were visualized using the chemiluminescence reaction (HPRL kit) and compared with protein molecular weight standards.

3. Results

In accordance with previous observations [9,10], AP-1 DNA binding activity in whole-cell adrenal gland extracts was found to be readily demonstrated using an EMSA (Fig. 1A). The specificity of AP-1 binding activity in the adrenal has been determined in previous studies using both unlabelled oligonucleotiodes as competitors, and also various antibodies in super-shift experiments that have indicated the presence of c-Fos-, c-Jun- and JunD-like immunoreactivities in the binding complex [9].

Hsps (either purified bovine Hsc70 (p73) or recombinant human Hsp70 (p72)) were shown to attenuate the level of adrenal AP-1 DNA binding activity following incubation with the adrenal extract (Fig. 1A). The inhibitory action of the Hsps was consistently observed in multiple experiments (Fig. 1B, $P < 0.05$ for both Hsc70 and Hsp70, Student's *t*-test); the purified Hsc70 (approximately 10% Hsp70; StressGen data sheet, and see Fig. 3B) was observed to exert a consistently greater effect than the recombinant Hsp70 (Fig. 1B). The effect of the Hsps was also shown to be concentration-dependent (Fig. 1C). With regard to the concentration of Hsp required to elicit effects on in vitro AP-1 binding activity (0.7 and 1.4 μM, Fig. 1A and C), it should be noted that this concentration is comparable to that used for both glucocorticoid receptor heterocomplex assembly in vitro (3.1 μM) [19], and modulation of p53 DNA binding in vitro (3.4 μM) [8]. AP-1 binding activity is known to be determined by a variety of factors [19] including protein content of binding buffer, and control experiments were performed using a protein of similar molecular weight (BSA) which indicated that the observed effect was selective for Hsp (Fig. 1A and B). Similarly, it was also demonstrated that addition of the Hsp buffer constituents (StressGen data sheets) alone did not significantly modify AP-1 binding (data not shown). The effect of Hsp on adrenal DNA binding activity was shown to be at least partially selective for AP-1 in a further experiment in which adrenal SP-1 binding activity was found to be reduced by only 17% in the presence of a similar concentration of Hsc70 (Fig. 2). Selectivity in the action of Hsps on DNA binding activity was further indicated by the finding that a non-specific AP-1-associated band that is observed following prolonged autoradiograph exposure (eg. see Fig. 4B), is also not attenuated in the presence of Hsc70.

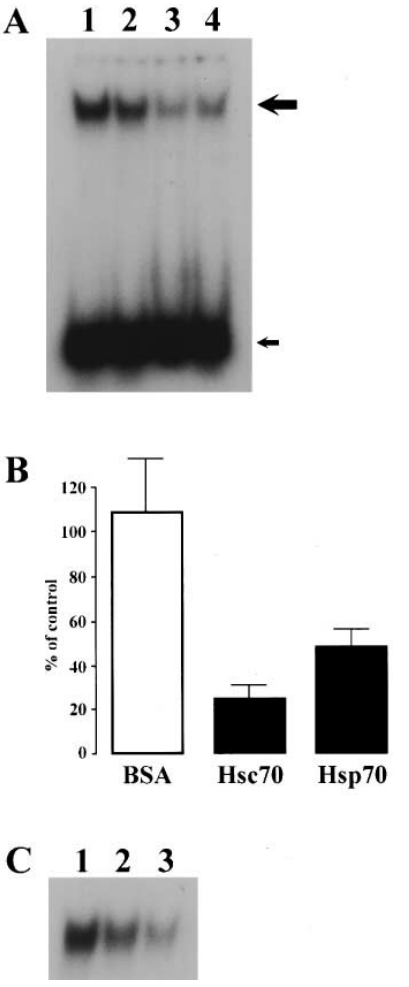

Fig. 1. Effect of Hsps on AP-1 DNA binding activity. Electrophoretic mobility shift analysis of rat adrenal whole-cell extract binding to a ^{32}P-labelled AP-1 element oligonucleotide following incubation with Hsps. A: Lane 1, no addition; lane 2, BSA (1.5 μM); lane 3, Hsc70 (p73) (1.4 μM); lane 4, Hsp70 (p72) (1.4 μM). Large arrow indicates the AP-1 binding complex, and small arrow indicates free probe. B: Quantitative analysis of the effects of Hsps and a control protein (BSA) on AP-1 DNA binding activity expressed as a percentage of AP-1 binding in the absence of added protein. $n = 4$ experiments (BSA and Hsp70), $n = 6$ experiments (Hsc70). C: Dose-dependency of the effect of Hsps on AP-1 DNA binding activity. Lane 1, no addition; lane 2, Hsc70 (0.7 μM); lane 3, Hsc70 (1.4 μM). Free probe is not shown.

In order to confirm Hsps as the active inhibitory component of the exogenous protein preparations, the effect of Hsp antibodies was examined in further experiments. However, this approach proved to be inappropriate for blocking the effect of Hsps on AP-1 activity because it was found that the 3a3 monoclonal antibody, which interacts with both Hsp70 and Hsc70, also caused a concentration-dependent attenuation of AP-1 binding activity when used alone (data not shown). Similar effects were observed with two other commercially available Hsp antibodies (1B5, and RPN 1197), but because a similar effect was also observed using similar dilutions of a GR antiserum (data not shown), it is apparent that the effect of Hsp antisera on AP-1 activity may be indirect (see Section 4). An alternative strategy was therefore employed in which the purified Hsc70 preparation was depleted using ATP-agarose [16,18]. Following the ATP-agarose procedure,

it was found that the efficacy of Hsc70 in attenuating AP-1 binding was markedly reduced (although not eliminated), whereas a similar procedure performed in the presence of a competitor (free ATP) was associated with near normal inhibitory activity (Fig. 3A). Depletion of Hsp was confirmed by immunoblotting analysis, and it was shown that the extent of depletion accorded with the EMSA results in that Hsp depletion was marked, but not complete, and depletion was also not fully competed in the presence of free ATP (Fig. 3B). The mechanism by which Hsps modulate in vitro binding of adrenal proteins to a DNA element was investigated in further experiments. Firstly, it was shown that Hsp alone did not exhibit AP-1 element binding activity (data not shown), indicating that Hsps do not directly compete with AP-1 proteins. Secondly, because it is well established that AP-1 binding activity is subject to redox regulation [19], and it has been suggested that Hsps may affect protein structure through a redox mechanism [20], we investigated whether the effect of Hsps in vitro could be attenuated in the presence of a optimal concentration of the reducing agent (5 mM DTT) [19] that is included in the EMSA binding buffer. However, although it was confirmed that AP-1 activity is upregulated in the presence of a higher concentration of DTT (see [19]), the inhibitory action of Hsc70 was unaffected (Fig. 4A). Thirdly, it was shown that in vitro dephosporylation of the adrenal protein extract, which caused a marked reduction in DNA binding activity (Fig. 4B), also did not affect the inhibitory action of Hsc70 (Fig. 4B).

The adrenal AP-1 binding activity used in the experiments described above consists of a complex of Fos and Jun proteins [9,10] in which a variety of different Jun:Jun homodimers and Fos:Jun hererodimers may be formed, and therefore the mechanism of action of Hsps was investigated further using specific recombinant proteins (Fig. 5). Initially, we confirmed that the total AP-1 binding activity of recombinant c-Jun alone was relatively low compared with the binding observed in the presence of both c-Jun and c-Fos (Fig. 5A), and because c-Fos alone did not exhibit detectable binding activity (data not shown), it is apparent that the increased activity is due to the formation of higher affinity c-Fos:c-Jun heterodimers as demonstrated in previous studies [21,22]. It should be noted that the binding complex formed in the presence of c-Jun alone migrates relatively slowly compared with the c-Fos:c-Jun complex (as observed previously) [21], and that using shorter EMSA autoradiograph exposures of c-Fos:c-Jun mixtures, the two complexes can be resolved (see Fig. 5C, lane 3). In confirmation of Fos:Jun heterodimer formation, we also showed that the enhanced binding activity of c-Fos:c-Jun mixtures was fully super-shifted in the presence of an antiserum to c-Jun (Fig. 4B). The specificity of recombinant protein binding was further confirmed by competition with unlabelled AP-1 oligonucleotide which markedly reduced the binding activity (Fig. 5A) whereas a similar molar excess of the SP-1 oligonucleotide exhibited only a minor effect (data not shown). Following addition of Hsc70 to the recombinant protein binding assays, it was found that AP-1 binding activity was attenuated but that the effect was selective for Fos:Jun heterodimers as compared with the binding associated with Jun alone (Fig. 5C). Thus, using short exposures of the EMSA autoradiographs (Fig. 5C) it was shown that the faster migrating c-Fos-associated band was reduced by 50% in the presence of Hsc70 whereas the binding of c-Jun alone was not significantly inhibited.

Fig. 2. Effect of Hsps on SP-1 DNA binding activity. Electrophoretic mobility shift analysis of rat adrenal whole-cell extract binding to a ^{32}P-labelled SP-1 element oligonucleotide following incubation with Hsc70 (p73). Lane 1, no addition; lane 2, binding reaction performed in presence of a 50 M excess of unlabelled SP-1 oligonucleotide; lane 3, Hsc70 (1.4 μM). Large arrow indicates the major SP-1 binding complex. Small arrow indicates free probe.

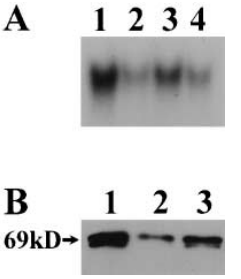

Fig. 3. Effect of Hsp on AP-1 DNA binding activity is attenuated following depletion. Electrophoretic mobility shift analysis (EMSA) of rat adrenal whole-cell extract binding to a ^{32}P-labelled AP-1 element oligonucleotide. A: EMSA analysis of the effect of Hsp on AP-1 activity following depletion with ATP-agarose (see text). Lane 1, no addition; lane 2, Hsc70 (p73) (1.4 μM), no depletion; lane 3, Hsc70 (1.4 μM), depleted; lane 4 Hsc70 (1.4 μM), depleted in presence of excess ATP. B: Western blot of equal aliquots of Hsc70 derived from the treatments in B. Lane 1, no depletion; lane 2, depleted; lane 3, depleted in presence of excess ATP. Arrow indicates the position of protein molecular weight marker.

4. Discussion

AP-1, a variable complex of Fos and Jun family proteins that exhibits cell-type and activation-dependent specificity, is an important transcription factor that has been implicated in numerous developmental, physiological and pathological processes [23–27]. While it is clear that de novo induction of AP-1 is an important mechanism by which AP-1 activity is controlled [23], recent studies have increasingly shown that AP-1 is subject to other modes of regulation that include inhibitory interactions with other transcription factors (see below), and chemical modifications such as reduction by cellular redox factors [19] and phosphorylation [28]. We have now provided evidence to show that AP-1 DNA binding ac-

tivity, which is one determinant of transcriptional activation potential, is modulated by the ubiquitously expressed Hsps. Although Hsps are known to be active in extra-nuclear compartments, there is substantial evidence to show that Hsps are also active in the nucleus [3–8]. In addition Hsps are known to be induced following stimuli that also induce AP-1. For example, Hsp70 is markedly induced in the rat brain following epileptic seizures [29], a stimulus that is also associated with induction of AP-1 [23]. In relation to the present study of adrenal AP-1, expression of both Hsp and AP-1 is increased in the adrenal cortex following restraint stress [11,12]. When considered in the light of the present findings, these studies indicate that one of the roles of Hsp may be to modulate the functional consequences of a change in AP-1 activity through regulated attenuation of DNA binding activity. Following seizure-related Hsp70 (p72) induction in the brain such a role would appear to be non-acute given the delayed temporal profile of Hsp induction [29], but the rapid onset of stress-induced Hsp induction in the adrenal [12] may indicate a more acute modulatory interaction of induced Hsps and AP-1 transcription factors. Alternatively, the constitutively expressed Hsc70 is known to be rapidly relocated into the nucleus following cellular stress [4], and the potent modulatory effects of Hsc70 demonstrated in the present study indicate that induction of the 'stress-specific' Hsp70 (p72) is not necessarily required for a potentially rapid modulation of AP-1 activity via Hsps. Following the in vitro studies described here, in vivo approaches are now required to investigate the physiological relevance of these possibilities. In the case of the adrenal gland, it is apparent that AP-1 responsive genes such as pro-enkephalin [30] may be subject to regulation by Hsps.

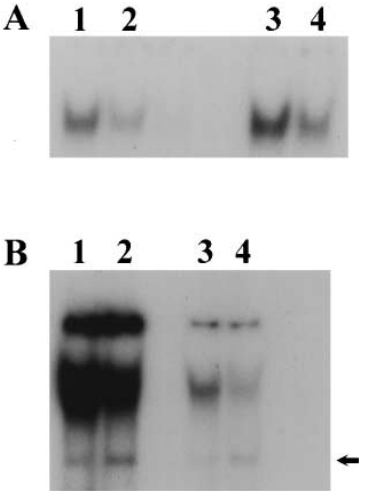

Fig. 4. Effect of Hsp on AP-1 DNA binding activity is not modified following either redox modification or dephosphorylation of AP-1 activity. A: Electrophoretic mobility shift analysis (EMSA) of rat adrenal whole-cell extract binding to a ^{32}P-labelled AP-1 element oligonucleotide in the presence of either 0.125 mM DTT (lanes 1 and 2) or 5 mM DTT (lanes 3 and 4). Lane 1, no addition; lane 2, Hsc70 (p73) (1.4 μM); lane 3, no addition; lane 4, Hsc70 (p73) (1.4 μM). Free probe is not shown. B: EMSA analysis of either control-treated (lanes 1 and 2) or dephosphorylated (lanes 3 and 4) rat adrenal whole-cell extract binding to a ^{32}P-labelled AP-1 element oligonucleotide in either the presence or absence of Hsp. Lane 1, no addition; lane 2, Hsc70 (p73) (1.4 μM); lane 3, no addition; lane 4, Hsc70 (p73) (1.4 μM). Free probe is not shown. Note that the autoradiograph has been over-exposed with respect to the binding activity in lanes 1 and 2. Arrow indicates a non-specific AP-1-associated band.

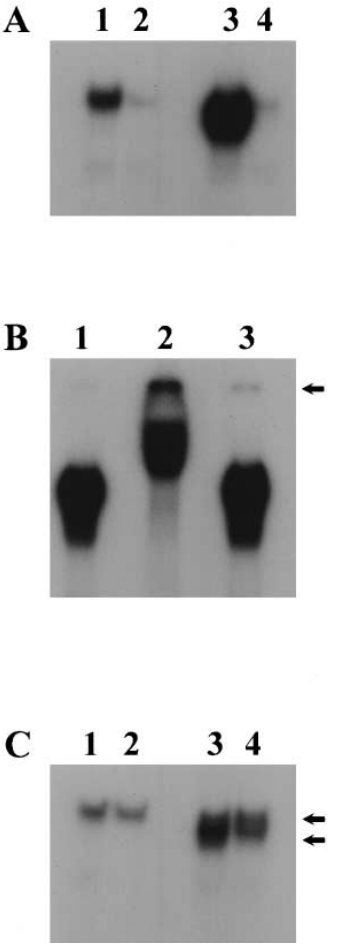

Fig. 5. Hsps exhibit selective inhibition of c-Fos:c-Jun heterodimer binding activity. Electrophoretic mobility shift analysis (EMSA) of recombinant c-Jun (1 ul of rat c-jun-primed reticulocyte lysate) and c-Fos (1 ul rat c-fos-primed reticulocyte lysate) binding to a ^{32}P-labelled AP-1 element oligonucleotide. A: EMSA analysis of c-Jun (lanes 1 and 2) or c-Jun plus c-Fos (lanes 3 and 4) binding activity. The binding reactions of lanes 2 and 4 contained a 50 M excess of unlabelled AP-1 oligonucleotide. Free probe is not shown. (Note that lysate primed with c-fos alone exhibited no detectable AP-1 binding activity; not shown.) B: EMSA analysis of c-Jun plus c-Fos binding activity following incubation with either no antibody (lane 1), a c-Jun antibody (lane 2; 2 ul IgG) or a control antibody (lane 3; sheep, anti-mouse Ig; 2 ul). Arrow indicates the origin. C: EMSA analysis of either c-Jun (lanes 1 and 2) or c-Jun plus c-Fos (lanes 3 and 4) binding activity following incubation with Hsp. Lane 1, no addition; lane 2, Hsc70 (p73) (1.4 μM); lane 3, no addition; lane 4, Hsc70 (p73) (1.4 μM). Free probe is not shown. Arrows indicate the position of the two resolved EMSA bands in lanes 3 and 4.

The modulation of AP-1 binding activity by Hsp does not involve a direct competition for DNA binding, and also does not appear to involve a redox [18,20] or de-phosphorylation (see [28]) mechanism. It is reasonable to speculate that the modulatory activity demonstrated here is related to the characterized function of Hsps as chaperones that influence the assembly of protein complexes [2]. Thus, the integrity of cellular AP-1 factors, which are composed of a variable complex of Fos:Jun heterodimers, and Jun:Jun homodimers that exhibit different DNA binding affinities [31] may be dependent

on a local influence of Hsps. Our finding that Hsps appear to selectively attenuate the binding of high affinity Fos:Jun heterodimers as compared with Jun:Jun homodimers has provided a mechanistic basis for the demonstrated reduction in AP-1 binding activity by Hsps. A selective attenuation of Fos:Jun heterodimer formation would be biologically significant in that the transcriptional activity of c-Fos and c-Jun together is markedly greater than either factor alone [32,33]. That the attenuation of c-Jun:c-Fos binding activity by Hsp was found to be somewhat less marked than the attenuation of adrenal AP-1 activity, may be explained by the presence of alternative heterodimeric binding complexes in the adrenal extract, for example JunD:c-Fos [9]. With respect to interacting protein complexes other than AP-1, it has been shown that over-expression of Hsp70 can negatively influence the stability of heat shock transcription factor (HSF) trimers (see [6]). Hsps might affect transcription factor binding at the level of protein:DNA interaction because it has been suggested that local folding transitions which are coupled to DNA binding [34] may be influenced by Hsps [5]. A similar mode of regulation may underlie the effect of Hsp70 on estrogen receptor (ER)/estrogen response element (ERE) stability that appears to be due to an increase in the association rate of ER with ERE [35], and also the effect of Hsps on p53/DNA complex stability [8]. An interesting corollary of the present findings relates to the interaction of Hsp70 with the glucocorticoid receptor (GR) complex (see [15]) because recent studies have revealed complex regulatory interactions between GR and AP-1 [36,37]. In addition to the implication of a functional role in stress-related gene expression (see above), the present findings may also relate to other cellular mechanisms that involve AP-1; for example, a post-transcriptional attenuation of AP-1 activity is associated with some forms of neuronal cell death [38].

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