SHORT COMMUNICATION

Interaction between Arabidopsis heat shock transcription factor 1 and 70 kDa heat shock proteins

Byung-Hoon Kim1 and Fritz Schoeffl2

Universität Tübingen, ZMBP–Zentrum für Molekularbiologie der Pflanzen, Allgemeine Genetik, Auf der Morgenstelle 28, D-72076 Tübingen, Germany

Received 30 July 2001; Accepted 4 September 2001

Abstract
The activity of the Arabidopsis heat shock transcription factor (HSF) is repressed at normal conditions but activated by cellular stresses. Circumstantial evidence suggests that HSP70 may function as a negative feedback regulator of HSF activity. Here the interaction between HSF and HSP70 is reported using electrophoretic mobility shift and yeast two-hybrid assays. Subdomain mapping indicates an interaction of the activation domain and DNA-binding domain of HSF1 with HSP70.

Key words: Arabidopsis HSF, HSP70, yeast two-hybrid, electrophoretic mobility shift.

Introduction
Heat shock transcription factors (HSF) are important regulators of cellular functions in eukaryotes. HSFs specifically bind to heat shock promoter elements (HSE) and activate transcription of heat shock genes. HSF orthologues are found in a number of different organisms including plants (for a review see Schoeffl et al., 1998). In higher eukaryotes HSF1 controls the expression of heat shock proteins (HSP) and is a major positive regulator of the heat shock response. HSF1 is expressed in unstressed cells and its activity is negatively regulated at multiple levels. In plants, members of class A-HSF, including Arabidopsis HSF1 and HSF3, play a fundamental role in regulating the heat shock response (for reviews see Nover et al., 1996; Schoeffl et al., 1998).

Several lines of evidence from various organisms led to the model of feedback regulation of HSF activity by HSP70. Over-expression of HSP70 in Drosophila, rat and human cells results in a more rapid attenuation of the heat shock response than the wild-type control (Rabindran et al., 1994; Mosser et al., 1993), and interaction between HSP70 and HSF has been demonstrated in vitro (Abravaya et al., 1992; Rabindran et al., 1994; Baler et al., 1996). Based on the HSP70 antisense gene expression which leads to a prolonged shut-off time, i.e. the time required for the inactivation of Arabidopsis HSF1 activity after heat stress, HSP70/HSC70 proteins were implicated in the regulation of HSF activity in plants as well (Lee and Schoeffl, 1996).

In order to verify the physical basis for negative feedback regulation and the proposed repressing effect of HSP70 on HSF, the interaction of recombinant HSP70 proteins with cellular HSF:HSE binding complexes in vitro and, using the yeast two hybrid system, the in vivo interaction between HSP70/HSC70 and HSF1, including truncated forms for subdomain mapping was investigated.

Materials and methods

Construction of recombinant plasmids
DNA constructs were generated by cloning PCR amplified (Pfu pol.) fragments into vector plasmids. The integrity of the new constructs was verified by DNA sequencing. For yeast two-hybrid assays, all Athsf1 constructs were cloned in the SalI site of the bait plasmid, pAS2-1 (trp1, Clontech). Full-length Athsp70 cDNA (GenBank Accession No. AJ002551) was cloned into NeoI/BamHI sites and, respectively, Athsc70 (GenBank Accession No. X74604) into the BamHI site of the prey plasmid, pACT2 (leu2, Clontech). For expression of AtHSP70 the full length hsp70 cDNA with stop codon was amplified by PCR and cloned into the EcoRI and ApaI sites of pPICZa (Invitrogen), so that the translation stop signals were before the C-terminal His- and myc-tag sequences in the plasmid. Instead, a 6× histidine-tag sequence, constructed by annealing of a pair of complementary synthetic oligonucleotides, was inserted in the N-terminal EcoRI site of the pPICZa.

1 Present address: Department of Botany, University of Tennessee, Knoxville, TN 37996-1100, USA.
2 To whom correspondence should be addressed. Fax: +49 7071 29 5042. E-mail: friedrich.schoeffl@zmbp.uni-tuebingen.de

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Expression and purification of recombinant AtHSP70

pPICZa carrying the 6×His-tagged AtHSP70 expression cassette was transformed into the yeast _Pichia pastoris_ KM71 (Invitrogen). Forty-eight hours after induction by 0.5% meßithanol, the cells were harvested and lysed using glass beads in lysis buffer (50 mM phosphate buffer pH 8.0, 300 mM NaCl, and 10 mM histidine). Recombinant proteins were affinity purified using Ni-NTA agarose (Qiagen) and then dialysed and 10 mM histidine. Recombinant proteins were affinity purified using Ni-NTA agarose (Qiagen) and then dialysed and 10 mM histidine. Recombinant proteins were affinity purified using Ni-NTA agarose (Qiagen) and then dialysed and 10 mM histidine. Recombinant proteins were affinity purified using Ni-NTA agarose (Qiagen) and then dialysed and 10 mM histidine. Recombinant proteins were affinity purified using Ni-NTA agarose (Qiagen) and then dialysed and 10 mM histidine.

Electrophoretic mobility shift assay

Whole cell extracts were prepared by grinding 0.2 g of leaves from 3-week-old _Arabidopsis thaliana_ C24 plants in 400 μl of protein extraction buffer (10 mM TRIS pH 8, 1 mM EDTA, 10 mM boric acid, and 0.1 mM PMSF). Extracts were cleared by centrifugation at 14 000 × g for 10 min. If indicated, heat stress was administered prior to protein extraction by incubation of cut leaves submerged in a buffer (1% sucrose, 1 mM KH2PO4, pH 6) in a shaking water bath at 37 °C for 1 h. For the standard binding reaction 30 μg of whole cell extract was mixed with 1 ng of [32P]-labelled (3′-end filling) HSE (5′-TCGGCCGAAGCTTCAGAAAGCC-3′; conserved consensus sequences are underlined) and 1 μg of poly(dI·dC). The final volume was adjusted to 20 μl with protein extraction buffer. The binding reactions were incubated for 20 min at room temperature. Glycerol was added to a final concentration of 5% prior to electrophoresis which was carried out overnight at 100 V in 5% polyacrylamide in 1×TBE buffer (10 mM TRIS pH 8, 1 mM EDTA, and 10 mM boric acid). For the supershift assay 1 μg of purified _At_HSP70 or BSA was added to the binding reaction. Antibodies were diluted (Abravaya et al., 1992) 1 : 1 in PBS containing 10% BSA and 1 μl was added to the binding reaction.

Yeast two-hybrid assay

All procedures concerning the yeast two-hybrid system were carried out according to the manufacturer’s instructions (Clontech). Briefly, bait (pAS2-1 carrying the GAL4 DBD fusion construct) and prey (pACT2 carrying the GAL4 AD fusion construct) were co-transformed into _Saccharomyces cerevisiae_ Y190, and then the ability to grow on minimal media lacking leucine, tryptophan and histidine was examined. Qualitative protein interaction was detected by selectable histidine autotrophy and a β-galactosidase filter assay. To measure the interaction quantitatively, β-galactosidase liquid assays were carried out using three independent colonies. Chlorophenol red-β-D-galactopyranoside (CPRG) was used as substrate.

Results and discussion

Binding of recombinant _At_HSP70 to HSE:HSF complexes

To determine whether HSP70 binds to HSF∶HSE binding complexes, gel mobility shift assays were performed using crude protein extracts from _Arabidopsis_ leaves with or without purified recombinant _At_HSP70_ (Fig. 1). In non-stressed cells, constitutive HSE-binding by components in the extracts was indicated by the formation of a number of shifted bands; however, changes in the banding pattern occurred when extracts from heat-stressed plants were used. The heat-inducible increase in the binding of HSF to HSE resulted in four new major bands (see asterisks in Fig. 1A, B).

The addition of purified 6×His-tagged HSP70 to the binding reaction caused the formation of an additional high molecular weight HSE-binding complex that migrated more slowly than the other complexes (see arrow marked band in Fig. 1A). HSP70 alone was unable to bind to HSE. It is known from the human system that interaction of HSF with HSP70 is insufficient to suppress DNA-binding activity of HSF (Rabindran et al., 1994). It is obvious from Fig. 1 that the intensity of the 373

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HSP70-supershifted bands was decreased when extracts of heat-stressed cells were used in the binding assay. In this case, compared to control extracts, the lower tendency of the complex formation between DNA-bound HSF and HSP70 may reflect the higher demand of HSP70 for the binding of heat-denatured proteins in the cellular extract.

To verify that HSP70 is indeed driving the formation of the supershifted complex, specific antibodies against recombinant HSP70 were added to the binding reactions. Anti-polyhistidine antibody, but not antibodies against mouse IgG or bovine brain HSP70 (clone BRM-22) interfered with the formation or stability of the supershift-complex (Fig. 1A). These data suggest that the binding of anti-polyhistidine antibodies to recombinant HSP70 cause depletion of free 6×His-tagged HSP70 and/or dissociation from the supershift complex. This conclusion is further supported by the finding that the supershift was also reversed by the addition of ATP to the binding reaction (Fig. 1B), a known effector for the dissociation of HSP70 from its substrate proteins (Lewis and Pelham, 1985).

Although the heterologous anti-HSP70 antibody was able to recognize the Arabidopsis HSP70 in Western or in immunoprecipitation analysis (Fig. 1C), it failed to revert the HSP70-dependent supershift. This may be due to a masking of the antibody-binding site on HSP70 in the supershift complex. Owing to the multiplicity of HSF in Arabidopsis (Schoffl and Prandl, 1999) and the unavailability of suitable antisera against individual HSF, it is not known whether HSP70 interacts only with one individual or also with other HSFs in HSE binding complexes. HSF are the known major HSE-binding factors, but it cannot entirely be excluded that other cellular proteins recognize HSE and are involved in the formation of band shift-complexes.

Two-hybrid interaction betweenATHSF1 and ATHSP70 at HSC70

In order to test whether HSF1, the major A-class HSF in Arabidopsis, and specifically its putative activation domain is involved in the binding of HSP70, the truncated HSF1 deletion mutants, HSFΔ1, HSFΔ2 and HSFΔDBD were generated (Fig. 2A), for testing protein:protein interaction in the yeast two-hybrid system. In addition to the interaction with HSP70,
HSC70 constructs were also tested, since the constitutively expressed HSC70 protein, highly homologous to HSP70, is likely to act as a negative regulator of HSF under non-stress conditions. HSF1-constructs were expressed in the ‘bait’ version (N-terminal Gal4-DNA-binding domain fused to HSF), and HSP70 or HSC70 were expressed in the ‘prey’ version (HSF binding domain fused to HSF) in yeast. Different combinations of bait and prey constructs were co-expressed in yeast auxotrophic for histidine, a selectable marker for the two-hybrid interaction. The expression levels of co-expressed proteins were examined by Western blot analysis (data not shown). The interaction of proteins in the two-hybrid system was further verified by $\beta$-galactosidase filter assays (Fig. 2B), a second marker of the two-hybrid interaction system. Differences in the strength of interaction were quantified by enzymatic assays using three independent colonies from each positive combination (Fig. 2C). In the absence of any prey protein constructs, yeast strains expressing full length HSF1 (HSF) or HSF1 lacking its DNA-binding domain (HSF$\Delta$DBD) were able to grow slowly on selection media and also stimulated the $\beta$-galactosidase reporter to low levels (Fig. 2B, and columns 1 and 4 of 2C), which is due to the transcriptional activation by HSF1 (see below). However, the interaction between full-length HSF and HSP70/HSC70 resulted in much higher levels of $\beta$-galactosidase activity (Fig. 2C, columns 2 and 3). Western blot analysis (data not shown) indicated that this increment of reporter gene activity was not due to an increased expression level of full-length HSF, but was dependent on the expression of HSP70/HSC70. Interaction between C-terminally truncated HSF (HSF$\Delta$2), lacking the activation domain, and HSP70/HSC70 resulted in only a few slow-growing colonies which lacked $\beta$-galactosidase activity (Fig. 2B). This indicates that there is only a very weak interaction between the proteins of these combinations. The expression levels of HSF$\Delta$2 and HSP70/HSC70 were not lower than the protein levels in strongly interacting combinations (data not shown). Co-expression of HSP70 with HSF$\Delta$1, a truncated form of HSF lacking a large part of the C-terminal region including the putative transcriptional activation domain, resulted in no growth of colonies. Only a few slow-growing colonies were obtained after co-expression of HSF$\Delta$1 with HSC70 (Fig. 2B), but these colonies failed to stain positive in $\beta$-galactosidase activity assays. Co-expression of HSP70 or HSC70 with truncated HSF lacking its DNA-binding domain (HSF$\Delta$DBD) failed to show any detectable increment of $\beta$-galactosidase activity in comparison with the basal level of reporter gene activity (Fig. 2C, columns 4, 5, 6).

These results demonstrate that interaction between $At$HSF1 and $At$HSP70/HSC70 is possible in vivo and depends on the presence of the C-terminal activation and the DNA-binding domains of HSF1. In Kluyveromyces lactis and Saccharomyces cerevisiae, the DNA-binding domain, oligomerization domain and CE2 region (directly upstream of the activation domain) of HSF are involved in the repression of transcriptional activation at non-stress temperatures (Jacobsen and Pelham, 1991; Bonner et al., 1992). Furthermore, point mutations in the DNA-binding domain of Saccharomyces cerevisiae HSF lead to constitutive transcriptional activation (Hardy et al., 2000). In Drosophila, the C-terminal region of the DNA-binding domain and the activation domain are essential for the repression of HSF trimerization, which is a prerequisite for DNA-binding of this factor (Orosz et al., 1996). In human cells as well, a region directly downstream of the DNA-binding domain appears to have the same function (Liu and Thiele, 1999). Those results were discussed in relation to changes in the intramolecular conformation of HSF. However, it cannot be excluded that such domains are potential binding sites for negative regulators like HSP70/HSC70, since a conformational change may be induced by trans-interacting factors. It seems plausible that the DNA-binding domain is not a binding site for HSP70/HSC70, but its presence may be necessary for maintaining HSF1 conformation in a state that is accessible for effective binding to HSP70/HSC70. Owing to the lack of direct evidence, the possible function of the DBD in the interaction with HSP70/HSC70 remains unclear. On the other hand, the human HSF1 activation domain alone could interact directly with HSP70 in vitro and the HSP70-overexpression in human cells neither affected the activation of HSF DNA-binding activity nor inducible phosphorylation, but had strong negative effects on the expression of a heat shock reporter gene (Shi et al., 1998). Although detailed mapping of the functional domains of $At$HSF1 has not been performed, its structural conservation, compared with other plant and animal HSF parts (Nover et al., 1996), and the transcriptional activation by the ‘bait’ constructs in the two-hybrid assay (this paper) suggest that the potential for transcriptional activation is also encoded in the C-terminal region of $At$HSF1. Interaction of HSP70 with this part of HSF would suggest an interference with transcriptional stimulation, since exactly this region of HSF1 has also been mapped as the target site for the interaction with the Arabidopsis TATA-box binding protein (Reindl and Schöffl, 1998; Sarhan and Schöffl, unpublished results).

It is still unknown at exactly which step in the life or reactivation cycle of HSF the interaction with HSP70 is required. These findings, together with the previous data from HSP70 antisense plants which show a prolonged inactivation time of HSF after heat shock (Lee and Schöffl, 1996), suggest that HSP70/HSC70 may have a function in the attenuation of the transcriptional activity late in the heat shock response when excess HSP70 is
available and has the potential to bind to the activation domain of DNA bound HSF.

Acknowledgements

This work was supported by grants to FS from DFG (Deutsche Forschungsgemeinschaft, SFB446). B-HK was supported by a DAAD (Deutscher Akademischer Austausch Dienst) fellowship.

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