The small heat shock protein of Neurospora crassa, Hsp30, when employed in affinity chromatography, bound two cellular proteins that were identified as Hsp70 and Hsp88. Both Hsp70 and Hsp88 bound to Hsp30 in preference to other proteins, but binding of Hsp88 was more selective for Hsp30, and a direct interaction was observed. Transcripts for Hsp88, a newly characterized protein, are present at normal temperature, but they are strongly induced by heat shock. Its cDNA sequence predicts a protein with homology to mammalian Hsp110 family proteins, which are distantly related to Hsp70. Hsp88 and its homologues show greater similarity to Hsp70 in its N-terminal ATPase domain than in the C-terminal peptide-binding domain, and its ATP-binding motifs are conserved. Nevertheless, the N-terminal domain of Hsp88 (and related proteins) is consistently more hydrophobic and more basic than that of Hsp70 proteins. Within the C-terminal domain, the sequence corresponding to the DnaK α subdomain is conserved in the Hsp88/Hsp110 family proteins, whereas the DnaK β subdomain sequence is not conserved. The interaction between Hsp70 and Hsp30 may reflect their cooperation as cochaperones for denatured proteins, whereas Hsp88 and Hsp30 may form a complex that interacts with potential substrates.

Exposure to high temperature induces all organisms to synthesize a distinct group of proteins, the heat shock proteins (hsp), many of which act as chaperones that assist in the folding or unfolding of other proteins (1). These activities are especially useful during cell exposure to high temperatures that denature proteins and can lead to protein aggregation, but they are also required under conditions of normal growth. The chaperone functions of prominent high molecular weight hsps, such as Hsp70, Hsp104, and Hsp60, depend on cycles of ATP binding and hydrolysis; and cochaperones are required for optimal activity.

We have been interested in understanding the roles of small hsps, which are strongly expressed in response to high temperature. These proteins were first characterized by their homology to eye lens α-crystallin (2), which is also a heat shock-induced protein in non-lens tissue (3). Multiple small hsps are produced by several organisms, including Drosophila melanogaster and Caenorhabditis elegans (4), and they are especially abundant in plants, which have organellar as well as cytosolic forms of these proteins. The small hsps of diverse organisms show only limited sequence conservation, and their activities appear to be dispensable at normal temperature. However, overexpression of these proteins increased the resistance of mammalian cells to high temperature and toxic chemicals (5). Furthermore, disruption of the single copy Hsp30 gene of Neurospora crassa showed that this small hsp dramatically improves cell survival at high temperature, under conditions of glucose limitation (6). Like other hsps, the small hsps appear to be chaperones that in vitro prevent thermal aggregation of substrate proteins (7, 8), but the targets of their chaperone activity in vivo and the nature of the requirements for this activity, including cooperation with cochaperones, are not yet known.

Hsp30 is the sole α-crystallin-related small hsp of N. crassa (9), and it is strongly induced by high temperature (45 °C). Under inducing conditions Hsp30 associates with membranes, chiefly with mitochondrial membranes, but it dissociates, becoming a soluble cytosolic protein at normal temperature (9). To understand better the cellular and molecular roles played by Hsp30 during heat shock, we have attempted to identify cellular proteins that interact with Hsp30. We report here that two proteins, identified as Hsp70 and Hsp88, which is a previously uncharacterized protein, bind specifically to Hsp30 linked to a matrix in affinity chromatography. Furthermore, purified Hsp30 binds to an immobilized Hsp88-containing fusion protein. We determined the cDNA sequence of Hsp88 and aligned its predicted amino acid sequence with that of five related proteins, mammalian Hsp110 (10) and Hsp70RY (11), Hsp87 of C. elegans (12), Hsp79 (Sse2) of Saccharomyces cerevisiae (13), and Arabidopsis thaliana Hsp91 (14). These proteins were also aligned with Hsp70, to which they are distantly related; and properties of these two classes of proteins were compared. This is the first report of a specific interaction between a small hsp and cellular proteins, whose identities suggest that they may be cochaperones.

**EXPERIMENTAL PROCEDURES**

*Isolation of Recombinant Hsp30—Hsp30 was synthesized as a fusion protein with glutathione S-transferase (GST) from which it was cut by thrombin after glutathione-agarose chromatography (6). For cloning Hsp30 cDNA into the pET-21c (+) vector (Novagen), an NdeI restriction site was inserted by PCR amplification at the 5′ end of Hsp30-coding sequences, and the 3′ end incorporated either the existing EcoRI site, for nontagged Hsp30, or an introduced XhoI site, for C-terminal hexahistidine-tagged Hsp30. A Perkin-Elmer DNA Thermal Cycler was used for this amplification. The sense strand primer was 5′-CTACCATGTCGCTCTCCCTCCTCGTGGGCTTCTA, and the antisense primers were, respectively, 5′-CTCGAATTCTGGCATCCGCGGAGTTAATAGCAATGGT and 5′-CTGGCTCAGGGTGAATAGCAATGGCAGATGT (Integrated DNA Technologies). After ligation into a pGEM-T vector (Promega) and propagation in...*
Escherichia coli, the insert DNA was cut at its restriction sites and ligated into the pET vector. Transformation of JM109 cells was followed by transformation of BL21(DE3) cells, which contain the T7 RNA polymerase gene. Expression of Hsp30 was induced at 30 °C for 2.5 h by isopropyl β-D-thiogalactoside.

Hsp30 Affinity Chromatography with Cell Extracts—Affi-Gel 15 (Bio-Rad) was used as the solid support for purified fusion protein-derived Hsp30, for GST, and for bacterial lysates enriched in untagged and histidine-tagged Hsp30. Covalent linkage was performed according to the manufacturer's instructions. Hexahistidine-Hsp30 and β-galactosidase were also selectively bound to separate nickel-chelating resins (Novagen).

Resin linked to either Hsp30 or a control protein, or simply blocked, was mixed with wall fractions of the Hsp30-disrupted 88-kDa strain of N. crassa (4). DNA from this strain was then ligated into the pET vector. Construction of the recombinant Hsp88 fusion protein was confirmed by sequencing with primers 9'-GCGGGATCCGCTCATCA and 3'-CGTTGATTAAGCTTCTGTT, respectively. Expression of GST-Hsp88 was induced by isopropyl β-D-thiogalactoside for 4 h at 37 °C. The fusion protein was isolated by glutathione-agarose chromatography (6) and elution with 10 mM glutathione.

Hsp30 Affinity Chromatography—Hsp88 fusion protein or GST was buffer-exchanged into 20 mM Tris (pH 7.5). Purified fusion protein-derived Hsp30 (300 μg) was brought to 20 mM NaCl, 1 mM Pefabloc (extract only), and 10 mM β-mercaptoethanol and 5% acetonitrile for buffer A and 60% acetonitrile, 130 A Separation System (Perkin-Elmer) using a Vydac C18 column (7). The extracted peptides were separated on an Applied Biosystems (ABI) Thermospray MS spectrometer. Peptide sequencing was performed on an ABI 492 Procise Protein Sequencer (8). Flanking universal primers (5′-CTCTCTGTCGTCGGTGTCGAT and 3′-CTCTCTGTCGTCGGTGTCGAT) were used for sequence analysis and comparisons and for analyzing properties of the predicted sequence of Hsp88 and related proteins, as well as Hsp70.

RESULTS Binding of Proteins to Recombinant Hsp30—To identify N. crassa proteins that bind to Hsp30, we reacted fractionated extracts from heat-shocked cells with a resin linked to Hsp30, which was derived from a recombinant fusion protein with GST. We employed the Hsp30-deficient 88-kDa strain of N. crassa (6) to avoid competition from endogenous Hsp30 for binding to interacting proteins. This also prevented the potentially strong binding of endogenous Hsp30 to the affinity resin, since Hsp30, like other small hsp's, readily forms homo-oligomers both in vivo and in vitro.

We found that two radiolabeled proteins of 70 and 88 kDa, from both the post-mitochondrial supernatant and a Triton X-100 mitochondrial extract, bound to the Hsp30 resin, in preference to ethanolamine-blocked Affi-Gel resin (Fig. 1, Table 1). The extent of binding of the 70- and 88-kDa proteins

1133
88-kDa hsp Interacts with Hsp30

40% water, 0.09% trifluoroacetic acid for buffer B, running a linear gradient from 5% B to 90% B in 70 min. The collected peptide peaks were spotted onto pieces of high density polyvinyliden fluoride (ABI ProBlott) and treated with 5 μl of a Biorene/methanol mixture (1:1). Peptide sequencing was performed on an ABI 492 Procise Protein Sequencer System, using the pulsed liquid mode, and the data were analyzed with the ABI model 610A data analysis software.

Nucleic Acid Sequencing—The double-stranded phosphodiester was isolated by QIAGEN-tip 20 and subjected to ABI automated fluorescent sequencing. Flanking universal primers (5′-CTCTCTGTCGTCGGTGTCGAT and 3′-CTCTCTGTCGTCGGTGTCGAT) were used for sequence analysis and comparisons and for analyzing properties of the predicted sequence of Hsp88 and related proteins, as well as Hsp70.

ATP Binding—Post-mitochondrial supernatant was prepared from heat-shocked cells as for affinity chromatography, but without EDTA in the buffer, and was passed through a 0.45-μm filter. The GST-Hsp88 fusion protein or GST was buffer-exchanged into 20 mM Tris (pH 7.5). Solutions containing the cell extract (2 ml) or the isolated proteins (0.18 mg) were brought to 20 mM NaCl, 1 mM Pefabloc (extract only), and 10 mM MgCl₂, MnCl₂, or CaCl₂. The samples were applied to a 0.2-ml (cell extract) or 0.12-ml ATP-agarose column (Sigma) equilibrated in 20 mM Tris (pH 7.5), 20 mM NaCl, and 10 mM MgCl₂, MnCl₂, or CaCl₂. The column was washed with 5 column volumes of the same buffer containing 0.1 or 0.5 mM NaCl. Following reequilibration of the column in 20 mM NaCl buffer, ATP-binding proteins were eluted 3–4 times with 0.25 or 0.5 ml equilibration buffer containing 10 mM ATP (pH 7.0). An aliquot of the eluate (50–100 μl) was ethanol-precipitated for electrophoresis.

Amino Acid Sequencing—An Affi-Gel column of purified fusion protein-derived Hsp30 was used to isolate binding proteins, which were separated by denaturing gel electrophoresis (12) and blotted to polyvinyliden fluoride (Millipore) for N-terminal sequencing. After the 88-kDa protein proved to be N-terminally blocked, it was digested in a gel slice (16) with sequencing grade trypsin (Boehringer Mannheim). The extracted peptides were separated on an Applied Biosystems (ABI) 130A Separation System (Perkin-Elmer) using a Vydac C18 column (The Separations Group). Separation conditions consisted of 0.1% trifluoroacetic acid and 5% acetonitrile for buffer A and 80% acetonitrile, 40% water, 0.09% trifluoroacetic acid for buffer B, running a linear gradient from 5% B to 90% B in 70 min. The collected peptide peaks were spotted onto pieces of high density polyvinyliden fluoride (ABI ProBlott) and treated with 5 μl of a Biorene/methanol mixture (1:1). Peptide sequencing was performed on an ABI 492 Procise Protein Sequencer System, using the pulsed liquid mode, and the data were analyzed with the ABI model 610A data analysis software.

Protein Sequence Analysis—GC Gel (Madison, WI) programs FastA, TFASTA, Gap, Map, PeptideStructure, PeptideSort, and Translate were used for sequence analysis and comparisons and for analyzing properties of the predicted sequence of Hsp88 and related proteins, as well as Hsp70.

Denatometric Analysis—Fluorograms or Coomassie Brilliant Blue-stained protein gels were scanned with a Molecular Dynamics Personal Densitometer SI at 100- and 200-kDa markers for bands of interest. The fluorograms were analyzed with the ABI model 610A data analysis software. Multiple fluorograms, representing different film exposures for any one gel, were compared to arrive at representative values.

RESULTS Binding of Proteins to Recombinant Hsp30—To identify N. crassa proteins that bind to Hsp30, we reacted fractionated extracts from heat-shocked cells with a resin linked to Hsp30, which was derived from a recombinant fusion protein with GST. We employed the Hsp30-deficient 88-kDa strain of N. crassa (6) to avoid competition from endogenous Hsp30 for binding to interacting proteins. This also prevented the potentially strong binding of endogenous Hsp30 to the affinity resin, since Hsp30, like other small hsp's, readily forms homo-oligomers both in vivo and in vitro.

We found that two radiolabeled proteins of 70 and 88 kDa, from both the post-mitochondrial supernatant and a Triton X-100 mitochondrial extract, bound to the Hsp30 resin, in preference to ethanolamine-blocked Affi-Gel resin (Fig. 1, Table 1). The extent of binding of the 70- and 88-kDa proteins

2 N. Plesofsky-Vig and R. Brambl, unpublished observations.

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applied to reflect their relative abundance in the two cell fractions, since the 88-kDa protein is predominantly located in the post-mitochondrial supernatant and the 70-kDa protein is enriched in the mitochondrial fraction. However, increased binding to Hsp30 was shown by both these proteins in the post-mitochondrial supernatant (Table I), due to higher background binding in the mitochondrial fraction. The 70- and 88-kDa proteins also bound to Hsp30 at higher levels than to recombinant GST alone, linked to Affi-Gel 15 (Fig. 2, Table I).

We also made a recombinant Hsp30 whose isolation did not depend on a proteolytic step, thus avoiding the proteolytic fragments produced during cleavage of Hsp30 from the GST fusion protein. This Hsp30 was either identical in sequence to Hsp30, divided by the same ratio for the lane of proteins that bind to control (resin, GST, or β-galactosidase).

![Fig. 1](image1.png)

**Fig. 1.** Radiolabeled proteins that bind to Hsp30 linked to Affi-Gel resin, compared with proteins that bind to resin alone. Proteins in mitochondria and post-mitochondrial supernatant of heat-shocked strain 88-4 were tested for binding.

| Table I |
| --- |
| **Binding of Hsp88 and Hsp70 to Hsp30 or control molecules** |
| The fluorograms that are presented as Figs. 1–4 in this report were scanned, and volumes of individual protein bands were determined. Increase was calculated by finding the ratio of the densitometric value of Hsp88 or Hsp70 relative to Hsp88 (as standard protein) in the lane of proteins that bind to Hsp30, divided by the same ratio for the lane of proteins that bind to control (resin, GST, or β-galactosidase). |
| Fig. 1 | Ratios Fold Increase |
| Hsp88 | Hsp70/88 | Hsp88 | Hsp70 |
| Hsp88 | 3.51 | 75.84 | 5.2 | 12.7× |
| Hsp30 | 0.17 | 2.06 | 136.3× | 83.0× |
| Fig. 2 | GST | Hsp30 | 0.41 | 9.53 |
| Hsp30 | 2.26 | 71.52 | 5.5 | 7.5× |
| Fig. 3 | β-Gal, | Hsp30 | 0.06 | 1.92 |
| Hsp30 | 0.19 | 1.92 | 3.2 | 1.9× |
| Fig. 4 | Percent change |
| Hsp30 | 0.78 | 8.37 |
| Hsp30 | 0.51 | 9.30 | −35% | +11% |
| Hsp30 | 0.66 | 18.36 | −15% | +119% |

![Fig. 2](image2.png)

**Fig. 2.** Radiolabeled proteins in post-mitochondrial supernatant of heat-shocked cells that bind to Hsp30-Affi-Gel, compared with proteins that bind to GST-Affi-Gel as control.

The fluorograms that are presented as Figs. 1–4 in this report were scanned, and volumes of individual protein bands were determined. Increase was calculated by finding the ratio of the densitometric value of Hsp88 or Hsp70 relative to Hsp88 (as standard protein) in the lane of proteins that bind to Hsp30, divided by the same ratio for the lane of proteins that bind to control (resin, GST, or β-galactosidase).
RNA Analysis—Antisense oligonucleotides were designed that conceptually encode two peptide sequences of the 88-kDa protein: KNELETYIYDLR and AKDDEMTDA. As probes in a Northern blot, they hybridized to one RNA species of approximately 2.7 kilobase pairs, which was present at 30 °C but was strongly induced after cells were exposed to a 45 °C heat shock (Fig. 5). This induction established that the 88-kDa protein is a heat shock protein, but it also indicated that its cDNA should be present in a representative 30 °C cDNA library.

Hsp88 Sequence—Since these oligonucleotides did not identify a cloned cDNA during library screening, we PCR-amplified a genomic DNA fragment, using a sense-antisense oligonucleotide pair as primers. cDNAs that hybridized to this probe were identified and isolated from a λZAP library. We sequenced one 2.6-kilobase pair cDNA, which apparently encodes the complete Hsp88 protein and includes 113-bp upstream and 185-bp downstream noncoding sequence.2 We verified the translation termination codon of the cloned Hsp88 cDNA by sequencing PCR-amplified regions of both genomic DNA and reverse-transcribed RNA that contain the putative translational stop codon and found that they were identical in sequence to the cloned cDNA.

The predicted 707 amino acid sequence of Hsp88 is shown in Fig. 6. Like homologous proteins from mammals, yeast, invertebrates, and plants, Hsp88 shows a distant relationship to Hsp70, with which it is 30% identical. Among homologous proteins, Hsp88 is 48% identical to S. cerevisiae Hsp79 (Sse2, Ref. 13) and 45% identical to human Hsp70RY (11) in a 500–600 amino acid overlap. The homologous regions of these proteins include the first 509 amino acids and residues 551 to 653 (Hsp88 numbering). The Hsp88-related proteins are not as well conserved as the Hsp70 family itself, whose N. crassa (24) and S. cerevisiae (25) representatives are 80% identical.

The N-terminal 385 amino acids of Hsp88 correspond to the ATPase domain of Hsp70, with which it is 38% identical. This domain is more conserved than the rest of the protein, being 56% identical between Hsp88 and Hsp79 and 49% identical between Hsp88 and Hsp70RY. The central region of unique sequence varies in length; where Hsp88 has 41 residues, Hsp110 (10) has 104, and A. thaliana Hsp91 (14) has 89. The C-terminal tails also differ in length and sequence, except for proteins from related organisms.

Binding of Hsp30 to an Hsp88 Fusion Protein—To test the possibility that Hsp30 and Hsp88 might interact directly, without requiring other cellular components, purified Hsp30 was allowed to bind to a GST-Hsp88 fusion protein or to GST, as a control protein, immobilized on glutathione-agarose. All bound proteins were eluted with glutathione, and antibody against Hsp30 was used to probe a Western blot of the electrophoretically separated proteins. This showed that Hsp30 had bound to GST-Hsp88 but not to GST alone (Fig. 7).

Sequence Characteristics of the Hsp110 Family—We aligned the sequence of Hsp88 with those of five related proteins2: Hsp110, Hsp70RY, C. elegans Hsp87 (12), yeast Hsp79, and A. thaliana Hsp91. These proteins were also aligned with Hsp70s of N. crassa, yeast, Drosophila (26), human (27), and chicken (28). From these alignments, we determined which sequences are shared (identical or similar) by all members of the Hsp110 family and which are common to this family and Hsp70 (Fig. 6). Of 56 residues that are identical among the Hsp88-related proteins and Hsp70, 49 fall within the ATPase domain (residues 1–390) and only seven within the rest of the protein. Of 59 residues that are identical only among the Hsp88-related proteins and Hsp70, 49 fall within the ATPase domain, with 551 to 653 (Hsp88 numbering). The Hsp88-related proteins are not as well conserved as the Hsp70 family itself, whose N. crassa (24) and S. cerevisiae (25) representatives are 80% identical.

The N-terminal 385 amino acids of Hsp88 correspond to the ATPase domain of Hsp70, with which it is 38% identical. This domain is more conserved than the rest of the protein, being 56% identical between Hsp88 and Hsp79 and 49% identical between Hsp88 and Hsp70RY. The central region of unique sequence varies in length; where Hsp88 has 41 residues, Hsp110 (10) has 104, and A. thaliana Hsp91 (14) has 89. The C-terminal tails also differ in length and sequence, except for proteins from related organisms.
residues. Twenty-two similar residues common only to Hsp110 family proteins are included in the N terminus and 19 similar residues are in the C terminus (Fig. 6).

Several Hsp110 family members contain more cysteine residues, chiefly in their N-terminal domains, than are found in Hsp70 (10). There are 18 cysteines in Hsp110, 16 in Hsp70RY, and 14 in Athsp91, whereas only four cysteines are in Hsp70. However, *N. crassa* Hsp88 has only seven and yeast Hsp79 only four cysteines. The cysteine at position 378 of Hsp88 is strictly conserved in the Hsp88-related proteins; it is near the end of the putative ATPase domain where Hsp70 has an alanine. Whereas Hsp70s have a higher amount of glycine in the C-terminal residues, which are likely involved in peptide binding, despite the high sequence divergence of this domain.

The N-terminal domains of these two groups of proteins are characterized also by contrasting isoelectric points (Table II). The pl values of this 400 amino acid domain are 7.96 and 7.50, respectively, for Hsp88 and Hsp110, whereas the pl of this region between residues 248 and 278 has a hydrophilicity of 0.147 in Hsp88 and 0.753 in Hsp110. The same region in Hsp70 (244–274) is 1.357 in hydrophilicity. Between amino acids 354 and 363 the hydrophilicity is 0.461 in Hsp88 and 0.403 in Hsp110, whereas the aligned N-terminal 40 amino acids of *N. crassa* Hsp70 have a hydrophilicity of 0.146. The region between residues 248 and 278 has a hydrophilicity of −0.442 and −0.403, respectively, whereas the aligned N-terminal 40 amino acids of *N. crassa* Hsp70 have a hydrophilicity of 0.146. The region between residues 248 and 278 has a hydrophilicity of −0.147 in Hsp88 and 0.753 in Hsp110. The same region in Hsp70 (244–274) is 1.357 in hydrophilicity. Between amino acids 354 and 363 the hydrophilicity is −0.461 in Hsp88 and 0.200 in Hsp110 (residues 356–365), whereas the hydrophilicity in Hsp70 (350–360) is 2.330. There are no dramatic differences in hydrophilicity within these proteins’ C-terminal domains, which are likely involved in peptide binding, despite the high sequence divergence of this domain.

The ATPase domains of Hsp70, actin, and hexokinase have a common three-dimensional structure (29). Five sequence motifs, involved in ATP binding and interdomain coupling (30), have been identified within these structurally similar domains: PHOSPHATE1, CONNECT1, PHOSPHATE2, ADENOSINE, and CONNECT2 (31). We find that these motifs are also present in the Hsp88-related proteins (Fig. 6), with some exceptions. The conserved DXG of PHOSPHATE1 is present in all six sequences that we aligned. The CONNECT1 sequence (E/D)VXXA is only partially conserved in most of the Hsp110 family proteins, but it is completely conserved in Hsp88 and yeast Hsp79. The DXG of PHOSPHATE2 is two-thirds conserved (DXG) in all the proteins we aligned except for Hsp110, which has GXG instead. At the ADENOSINE motif, the Ara-

![Fig. 6. Analysis of conserved sequences of Hsp88, based on alignment with related proteins: hamster Hsp110, human Hsp70RY, C. elegans Hsp87, yeast Hsp79, and A. thaliana Hsp91; and with Hsp70 of *N. crassa*, yeast, *Drosophila*, human, and chicken. Identical and similar amino acids that are shared with Hsp70 are indicated by + and * (boldface), respectively, whereas amino acids that are identical or similar only among the Hsp88-related proteins are indicated by + and *, respectively. + has precedence over *. Similar amino acids are: T, S, C; I, V, L, M; A, G, S; Y, F, W; Q, E; D, N; E, D; R, K, H. Sequence motifs of the ATPase domain of Hsp70 and structurally similar ATPases are underlined: F, phosphate; C, connect; A, adenosine. The peptides whose sequence was determined are in boldface.](image1)

![Fig. 7. Western blot showing immunodetected Hsp30 that bound to GST-Hsp88 but did not bind to GST.](image2)
**Table II**  
Properties of Hsp88 and related proteins, compared with Hsp70

| Protein     | Average hydrophilicity of amino acid regions | pi |
|-------------|---------------------------------------------|----|
|             | 1–39 | 248–278 | 354–363 | 1–400 | 401–end |
| Hsp88nc     | 0.442 | 0.147 | 0.461 | 7.96 | 4.48 |
| Hsp110cg    | 0.403 | 0.753 | 0.200 | 7.50 | 4.78 |
| Hsp70Ryu    | 0.413 | 0.603 | 0.245 | 8.06 | 4.41 |
| Hsp70sc     | 0.074 | 0.843 | 0.128 | 7.25 | 4.41 |
| Hsp91at     | 0.011 | 0.425 | 1.696 | 6.89 | 4.53 |
| Hsp70nc     | 0.146 | 1.357 | 2.330 | 5.32 | 4.54 |
| Hsp72sc     | 0.227 | 1.533 | 2.128 | 5.10 | 4.50 |

**bidopsis** protein has GS instead of GG, but the conserved G within the CONNECT2 motif is present in all the proteins. PHOSPHATE2 is the least conserved motif in the Hsp88-related proteins, but this motif also diverges in hexokinase and other sugar kinases (31).

**ATP Binding**—Sequence conservation of the Hsp70 ATPase domain and ATP-binding motifs by Hsp88-related proteins led us to ask whether Hsp88 also bound ATP. By using the standard assay for ATP binding by Hsp70 (32), we applied soluble cell extract to ATP-agarose and eluted with ATP. When Mg²⁺ was the divalent cation, Hsp70 alone bound strongly, although binding of extremely low amounts of Hsp83 and Hsp98, the N. crassa ClpB homologue (33), was also detected. ATP binding by Hsp98, Hsp83, and other proteins was moderately enhanced when Ca²⁺ was substituted for Mg²⁺ and was strongly enhanced when Mn²⁺ was substituted; furthermore, binding of Hsp88 to Mn²⁺-ATP-agarose was detected (Fig. 8). The purified GST-Hsp88 fusion protein also bound to Mn²⁺-ATP-agarose, but not to Mg²⁺-ATP-agarose, at a higher ratio to applied sample than GST alone, according to densitometry (data not shown).

**DISCUSSION**

To identify cellular proteins that interact with Hsp30, the small hsp of N. crassa, we analyzed proteins in a cellular extract that were retained by an Hsp30 affinity resin. We found that Hsp70 and Hsp88 specifically bound to immobilized Hsp30. We confirmed the identity of Hsp70 by N-terminal amino acid sequencing. Hsp88 is a previously uncharacterized protein, whose cDNA we isolated and sequenced. Hsp88 appears to be a normal cellular constituent, whose expression increases severalfold in response to high temperature stress. Distantly related to Hsp70, Hsp88 is homologous to several recently characterized proteins, which are also reported to be induced by heat shock. Mammalian Hsp110 is one of the most strongly induced hsps in heat-stressed Chinese hamster ovary cells (10). Murine OspF, in addition to being induced by heat shock, is strongly synthesized in response to hyperosmotic stress (35). These proteins localize chiefly to the cytosol, although Hsp110 is also peripherally associated with nucleoli (10) and Hsp105 with nuclei (36). N. crassa Hsp88 is also predominantly cytosolic with a minor portion being membrane-associated. Unlike Hsp70 and small hsps, these proteins do not appear to change their location in response to high temperature (10).

There are consistent differences between the Hsp110 family proteins and Hsp70. The two Neurospora proteins have only 18% identity in their C-terminal putative peptide-binding domains, indicating extensive sequence divergence. In contrast their putative ATPase domains have 38% identity, and the recognized ATP-binding motifs are conserved in Hsp88. In our alignment of Hsp88-related and Hsp70 proteins, we found 49 identical and 67 similar residues conserved within the N-terminal domain and only 7 and 9, respectively, within the C-terminal domain. Nevertheless, properties of the N-terminal ATPase domains display surprising contrasts. This domain is more hydrophobic in Hsp88-related proteins than in Hsp70, due particularly to regions within three separate subdomains of the Hsp70 crystal structure, IA, IIA, and IIB (29). Furthermore, the Hsp88/Hsp110 ATPase domain is slightly basic, whereas this domain in Hsp70 is acidic. These properties would be expected to affect binding and hydrolysis of ATP by Hsp88 and related proteins. We found that binding of Hsp88 to ATP-agarose does not occur in the presence of Mg²⁺ but does occur when Mn²⁺ is the divalent cation, as has also been reported for purified Hsp90/Hsp88 (34). Hsp105 was also reported not to bind Mg²⁺-ATP-agarose (36). Except for Hsp70, other N. crassa proteins in addition to Hsp88, such as Hsp83 and Hsp98, also bind more strongly to Mn²⁺-ATP-agarose than to Mg²⁺-ATP-agarose, with binding to Ca²⁺-ATP-agarose being intermediate. The distinctive properties of the ATPase domain of Hsp88-related proteins are likely to influence the function of these proteins. In S. cerevisiae, the Ssb subgroup of Hsp70 is required for growth at low temperature, and domain swapping experiments showed that the N-terminal ATPase domain of Ssb1 is responsible for conferring this function (37).

The C-terminal domains of the Hsp88-related proteins and Hsp70 include a region of conserved sequence (Hsp88: 351–363) that corresponds to the α-helical region within the peptide-binding domain of E. coli DnaK, an Hsp70 homologue (38). The preceding region of β structure in the peptide-binding domain is not conserved in Hsp110 family proteins, although it is more strongly conserved than the α-helical region among Hsp70s (38). The β strands comprise the actual peptide-binding subdomain, and the α helices apparently form a lid over the peptide bound within the β sandwich. Movement of the longest α helix relative to the β sandwich, seen in an alternate crystal structure, may reflect nucleotide-induced conformational changes within DnaK (38). Sequence conservation of the α subdomain suggests that the Hsp88-related proteins retain the Hsp70 mechanism of nucleotide-regulated peptide-binding and release. The lack of conservation of the β subdomain suggests that the actual peptides bound by these proteins differ.

Several interactions between small hsps and purified protein substrates have been reported. For example, the small hsp of avian cells was found to inhibit actin polymerization in vitro (39). Murine Hsp25 (8) and pea Hsp18.1 (40) form complexes
with unfolded citrate synthase and other model substrates, and α-crystallin binds to denatured proteins, including β- and γ-crystallin (41). The interaction that we have detected between Hsp30 and Hsp88 (and Hsp70) is the first report of complex formation between a small hsp and a protein selected from a cellular extract, and it implies that this association likely occurs in vitro. Hsp30 and Hsp88 appear to interact directly, since the purified proteins bind to one another in the absence of other components. This is also the first report that a member of the Hsp110 protein family associates with a specific protein.

Hsp70 was the first hsp proposed to act as a chaperone (42), and its effect on protein unfolding is well established (43). The chaperone activity of small hsps is only beginning to be understood; they appear to bind to unfolded proteins and prevent their aggregation (8, 40). Under moderate conditions of heat stress, small hsps may be sufficient to reactivate substrate enzymes. We found that α-crystallin protected hexokinase activity from thermal inactivation,3 and pea small hsps were reported to protect citrate synthase from being inactivated (7). Under more stringent conditions, however, in addition to murine Hsp25, reactivation of citrate synthase required Hsp70 and ATP, which apparently dissociated the Hsp25-citrate synthase complex (8). This suggests cooperation between Hsp25 and Hsp70. Small hsps were proposed to complex with unfolded proteins early in heat shock and to be released from substrate proteins by ATP-dependent chaperones, such as Hsp70, when ATP-generating processes recover in stressed cells (8). The activity of most chaperones either requires or increases in cooperation with cochaperones. E. coli DnaK, for example, is aided by DnaJ, which enhances its ATPase activity, and by the nucleotide exchange factor GrpE (43). The multi-protein complexes formed by Hsp90 with Hsp70, p60, immunophilins, and p23 are required for Hsp90 to bind to and activate unfolded steroid receptors (44).

The affinity binding we report between Hsp70 and Hsp30 may reflect the cooperation between these two classes of chaperone in reactivating a substrate enzyme in vitro (8). In contrast to Hsp70, the binding of Hsp88 to Hsp30 is more selective and is not increased by a reaction temperature which denatures proteins in vitro (Table I). Furthermore, the interaction between purified Hsp88 and Hsp30 occurs in the absence of substrate proteins, implying that Hsp88 and Hsp30 may together form a chaperone complex before interacting with substrate proteins. An additional possibility is that the temperature-dependent dissociation of Hsp30 from membranes depends on Hsp88, which is located predominantly in the cytosol.

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