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NOTES

Members of the 70-Kilodalton Heat Shock Protein Family Contain a Highly Conserved Calmodulin-Binding Domain

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The 70-kilodalton heat shock protein (hsp70) family members appear to be essential components in a number cellular protein-protein interactions. We report here on the characterization of a new functional region in hsp70, a calmodulin-binding site. We have identified a 21-amino-acid sequence within the hsp70 protein that contains a calmodulin-binding domain. The peptide formed a potential amphipathic alpha helix and bound calmodulin with high affinity. Comparison of amino acid homology of this calmodulin-binding sequence with analogous hsp70 sequences from other species showed a high degree of conservation.

The members of the 70-kilodalton (kDa) heat shock protein (hsp70) family are a highly conserved group of proteins whose expression appears to be universal (1, 17–19, 25, 28, 34). Under nonstress conditions, the proteins are located in the cytoplasm as well as within organelles such as the endoplasmic reticulum and mitochondria. After heat shock, the cytoplasmic component appears to be rapidly translocated into the nucleus, resulting in hsp70 accumulation in both the nucleus and nucleolus (21, 24). Hsp70 proteins characteristically have a high-affinity ATP-binding site and an associated ATPase activity (4, 21). Several functions have been attributed to the hsp70 proteins, including uncoating of clathrin-coated vesicles (4), directional translocation of newly synthesized proteins across subcellular membranes (5, 10, 35), and disruption of inappropriate protein-protein interactions that occur after heat shock or related stresses (21). Hsp70 proteins have also been found to associate with a number of cellular proteins. The identities of most of these proteins are unknown; however, hsp70 has been shown to bind to cytoskeletal elements, a cell surface glycoprotein, and calmodulin (7, 16, 31, 33). Oligomeric protein complexes containing hsp70 and p53 tumor antigen have been isolated from transformed cells (15). Hsp70 from both control and heat-shocked cells coprecipitates with a small group of proteins (23). To better understand the nature of these protein-protein interactions, we examined the specific association of hsp70 with calmodulin, the ubiquitous calcium-binding regulatory protein.

Hsp70 from mammalian cells associates with calmodulin. A purified member of the hsp70 family, the constitutively expressed mouse hsc70, bound calmodulin in a calcium-dependent manner (Fig. 1). Hsc70 was prepared from mouse erythroleukemia (MEL) cells. We have previously shown that these cells do not express the heat-inducible hsp70 protein (C. Hunt and S. K. Calderwood, Gene, in press), facilitating the isolation of hsc70 in pure form without contamination by other members of the hsp70 family. The protein was purified from hypotonically lysed MEL cells by successive chromatographic separation on DE-52 anion-exchange resin and ATP-agarose affinity columns, as described previously (32). A single 70-kDa protein band was seen on 10% sodium dodecyl sulfate-polyacrylamide gels (20) (Fig. 1, lane 1). After electrophoretic transfer (29) to Immobilon PVDF membranes (Millipore Corp., Bedford, Mass.), the 70-kDa protein was found to react with a polyclonal antibody raised against the carboxy-terminal 20 amino acids of murine hsp70 (Fig. 1, lane 2). Replicate blots of the 70-kDa protein also bound biotinylated calmodulin (2) (BTI, Stoughton, Mass.), confirming that the purified hsc70 is a calmodulin binding protein (Fig. 1, lane 3). The binding of calmodulin to hsc70 was calcium dependent, with no binding detected in the presence of 2.0 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (Fig. 1, lane 4).

Identification of a potential calmodulin-binding sequence in hsp70. Calmodulin-binding proteins and peptides, including mellitin, mastoparan, and the myosin light-chain kinases (MLCK), contain structurally conserved peptide sequences that are essential to calmodulin binding (3, 9, 14). Common structural features include the juxtaposition of clusters of basic and hydrophobic amino acid residues such that the basic and hydrophobic residues segregate to opposite sides of an amphipathic helix, stabilizing the helical conformation. We used these structural motifs to identify the calmodulin-binding domain of hsp70. The amino acid sequence from the mouse hsp70 gene was analyzed for potential alpha-helix structure by Chou-Fasman probability profile (6). Regions of high alpha-helix probability were in turn scanned for the presence of sequences containing regions of adjacent basic and hydrophobic residues. Two potential calmodulin-binding peptides within hsp70 were identified: a 21-amino-acid residue sequence (257 to 277), designated LTS, and a 14-residue sequence (235 to 248), AFL. The LTS sequence formed an amphipathic helix when viewed in axial helical projection (Fig. 2A). Sequence AFL formed an imperfect helix, with amino acids 244 and 248 (glutamate and lysine) breaking the hydrophobic domain.

Hsp70-derived peptide, LTS, binds to calmodulin. Peptides corresponding to these two sequences were synthesized and tested for their ability to form stoichiometric complexes with

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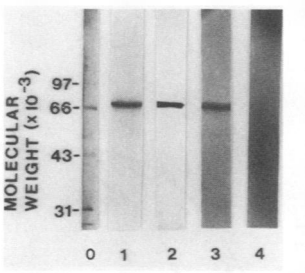

FIG. 1. Binding of calmodulin by purified hsc70 protein. Hsc70 protein fractionated from MEL cells (0.5 μg per lane) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels (lane 1), followed by electrophoretic transfer to Immobilon membranes (lanes 2 to 4). Lane 1 represents the 70-kDa protein band visualized with Coomassie blue stain. The same protein was recognized by rabbit polyclonal antibody raised against mouse hsp70 (lane 2) and bound biotinylated calmodulin in the presence of calcium (1.0 mM) (lane 3). No binding to calmodulin was observed at zero free calcium concentration (2.0 mM EGTA) (lane 4). Lanes 0, M_r markers. Calmodulin and antibody binding was detected by using a tiered biotin-avidin-alkaline phosphatase amplification system (Vector Laboratories Inc., Burlingame, Calif.).

calmodulin in the presence of calcium. Calmodulin purified from bovine testis (BTI) and increasing concentrations of peptides were incubated for 60 min at room temperature in buffer containing 25 mM Tris hydrochloride and 192 mM glycine. Samples were run on 7.5% acrylamide gels under nondenaturing conditions (14), and the protein bands were visualized with Coomassie blue stain. Association of a calmodulin-binding peptide with calmodulin results in a change in electrophoretic mobility of the complex relative to native calmodulin. This shift in mobility is the result of both charge and molecular weight differences resulting from the peptide-calmodulin interaction. In the presence of LTS, the calmodulin band was shifted upward in the gel (Fig. 2B, lanes 2 to 4). The interaction of calmodulin and LTS was a high-affinity association, with a 4:1 ratio of peptide to calmodulin resulting in nearly complete displacement of the native calmodulin band. AFL had no effect on the migration of the calmodulin band when assayed over the same peptide concentrations, implying a lack of association of the peptide with calmodulin (Fig. 2B, lanes 6 to 8). Quantitatively similar results were obtained when gel separations were performed

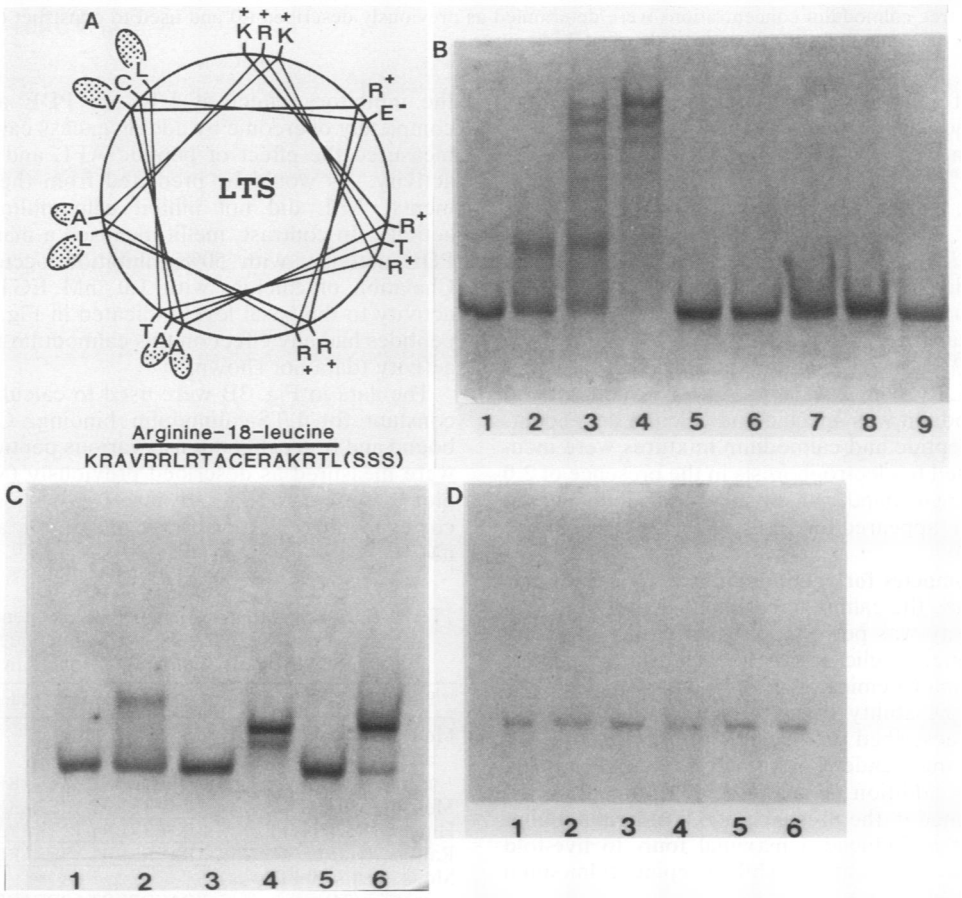

FIG. 2. (A) Axial helical projection of hsp70-derived peptide LTS. Drawing is initiated with the amino terminus of the peptide. Hydrophobic residues are circled and hatched; +, basic residues. (B to D) Analysis of peptide-calmodulin binding by native gel electrophoresis. Increasing concentrations of peptides were incubated with purified calmodulin. The samples were then subjected to nondenaturing gel electrophoresis on 7.5% acrylamide gels. All lanes contained 100 pmol calmodulin. (B) Lanes: 1, calmodulin alone; 2, calmodulin plus LTS (1:1); 3, calmodulin plus LTS (1:2); 4, calmodulin plus LTS (1:4); 5, calmodulin alone; 6, calmodulin plus AFL (1:1); 7, calmodulin plus AFL (1:2); 8, calmodulin plus AFL (1:4); 9, calmodulin alone. (C) Lanes: 1, calmodulin alone; 2, calmodulin plus LTS (1:1); 3, calmodulin alone; 4, calmodulin plus mellitin (1:1); 5, calmodulin alone; 6, calmodulin plus CBP1 (1:1). Experiments were carried out in the presence of 0.1 mM $CaCl_2$. (D) Experiments carried out as for panel C except that $CaCl_2$ was omitted and 2.0 mM EGTA was included.

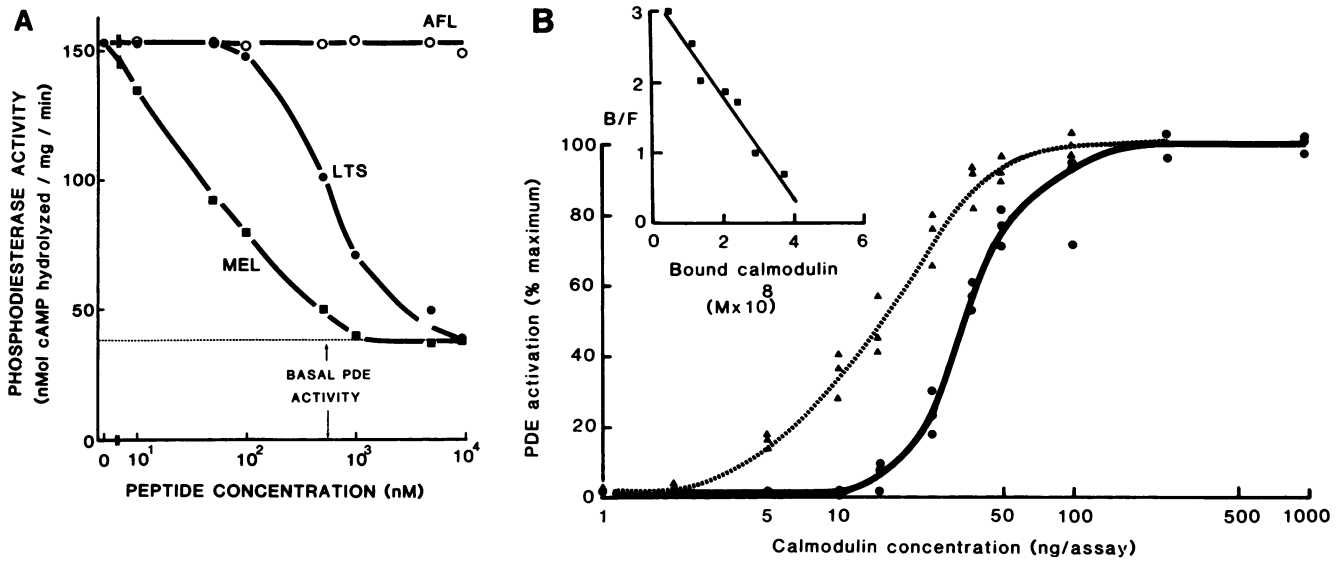

FIG. 3. (A) Effects of synthetic peptides AFL and LTS and mellitin on the activity of calmodulin-dependent PDE. Nanomolar concentrations of the peptides were assayed for competition with PDE for calmodulin. Competition is indicated by inhibition of calmodulin-dependent PDE activity. Datum points are means from duplicate estimations. The data are representative of three individual experiments. (B) Dependence of PDE activity on calmodulin concentration in the presence (●) or absence (▲) of 600 nM LTS peptide. Peptide-bound and free calmodulin concentrations were determined as previously described (8) and used to construct the Scatchard curve (inset). The K_d was determined from the slope of the Scatchard curve.

in the presence of 8 M urea, further indicating a high-affinity interaction between hsp70 and LTS (data not shown).

The interaction between LTS and calmodulin was compared with that of two known high-affinity calmodulin-binding peptides, mellitin (Sigma Chemical Co., St. Louis, Mo.) and CBP1 (Peninsula Laboratories Inc., Belmont, Calif.) (Fig. 2C). The peptides were incubated in equimolar ratios with purified calmodulin and were electrophoresed under nondenaturing conditions as described above. All three peptides cause a similar degree of gel shift, both mellitin and CBP1 binding calmodulin with slightly higher avidity than did LTS. The association of LTS, mellitin, and CBP1 with calmodulin was specific and calcium dependent. When identical peptide and calmodulin mixtures were incubated and subjected to electrophoresis in the presence of 2.0 mM EGTA, a single band, which migrated identically to native calmodulin, appeared for all three calmodulin-binding peptides (Fig. 2D).

LTS peptide competes for calmodulin in a PDE assay. To further characterize the calmodulin-binding activity of LTS, a competition assay was performed by using a calmodulin-dependent enzyme, cyclic nucleotide phosphodiesterase (PDE). PDE (Sigma Chemical Co., St. Louis, Mo.) activity was assayed as the ability to hydrolyze [^{3}H]cyclic AMP, using techniques described previously (30). The enzyme has some calmodulin-independent basal activity but is strongly stimulated by the addition of calmodulin. Competition assays were performed in the presence of 3.0 nM calmodulin, a concentration that induced a maximal four- to five-fold stimulation of the PDE sample while keeping calmodulin limiting (Fig. 3A).

LTS inhibited PDE activity at nanomolar concentrations. The inhibitory activity of the peptide was concentration dependent, with 50% inhibition occurring at a concentration of 700 nM. Since PDE levels were insignificant compared with the peptide concentration, the data indicate that LTS inhibited PDE by competing for calmodulin. This conclusion is further indicated by the data in Fig. 3B, which show that the inhibitory effect of LTS on PDE activity could be completely overcome by adding excess calmodulin. We also measured the effect of peptide AFL and mellitin on PDE activity. As would be predicted from the gel shift experiments, AFL did not inhibit calmodulin-dependent PDE activity. In contrast, mellitin caused a marked inhibition of PDE activity, with 50% inhibition occurring at 50 nM. Chelation of calcium with 1.0 mM EGTA reduced PDE activity to the basal level indicated in Fig. 3A. None of the peptides had any effect on this calmodulin-independent PDE activity (data not shown).

The data in Fig. 3B were used to calculate a dissociation constant for LTS-calmodulin binding. Concentrations of bound and free calmodulin at various peptide concentrations were measured as described previously (8). The Scatchard plot of these data (Fig. 3B, inset) shows a straight line and a corresponding dissociation constant of approximately 10 nM.

TABLE 1. Comparison of amino acid sequences between LTS, the mouse hsp70 calmodulin-binding peptide, and analogous sequences from hsp70 family members in other species

Protein (reference)	Amino acid sequence[a] (positions)
Mouse hsp70 (Hunt and Calderwood, in press)	KRAVRRLRTACERAKRTLSSS (257–277)
Mouse hsc70 (12)	KRAVRRLRTACERAKRTLSSS (257–277)
Human hsp70 (17)	KRAVRRLRTACERAKRTLSSS (257–277)
Rat hsc70 (25)	KRAVRRLRTACERAKRTLSSS (257–277)
Mouse testis 70-kDa protein (34)	KRAVRRLRTACERAKRTLSSS (260–280)
D. melanogaster hsp70 (18)	PRALRRLRTAAERAKRTLSSS (255–275)
S. cerevisiae YG100 (19)	QRALRRLRTACERAKRTLSSS (255–275)
E. coli hsp70 (1)	PLAMQRLKEAAEKAKIELSSA (256–276)
Human grp78 (28)	NRAVQKLRREVEKAK–ALSSQ (281–301)

[a] Conserved amino acids are underlined.

TABLE 2. Comparison of amino acid sequences between LTS, the mouse hsp70 calmodulin-binding peptide, and the calmodulin-binding sequences from skeletal muscle and smooth muscle MLCK

Protein (reference)	Amino acid sequence[a]
Mouse hsp70 (Hunt and Calderwood, in press)	KRAVRRLRTACERAKRTLSSS
Skeletal muscle MLCK (3)	KRRWKKNFIAVSAANRFKKISSS
Smooth muscle MLCK (13)	RRKWQKTGHAVRAIGRLSSM

[a] Sequences showing structural homology are underlined.

Hsp70-derived calmodulin-binding sequence is highly conserved. Comparison of amino acid sequence homology between LTS, the mouse hsp70 calmodulin-binding peptide, and analogous sequences at similar positions in hsc70 and hsp70 from other species showed a high degree of conservation (Table 1). There was 100% homology among sequences derived from mouse hsp70, mouse hsc70, human hsp70, mouse testis 70-kDa protein, and rat hsc70. Sequences from both *Drosophila melanogaster* and *Saccharomyces cerevisiae* hsp70 proteins retained 86% homology as compared with LTS, whereas *Escherichia coli* dnaK and human grp78 showed 57% absolute conservation with LTS at the amino acid level.

This stringent amino acid conservation, which crosses divergent lines of evolution, suggests that the calmodulin-binding sequence may comprise an essential functional, structural, or regulatory domain within the intact hsp70 proteins. Comparison of the mammalian cell-derived hsp70 calmodulin-binding sequence with that of smooth muscle and skeletal muscle MLCK revealed several structural similarities that may provide a framework for understanding the function of the hsp70 calmodulin-binding sequence (Table 2) (3, 13, 22). The carboxy terminus of all three peptides contains a consensus sequence for Ca^{2+}-calmodulin-dependent protein kinase II, consisting of sequential basic and hydrophobic residues followed by two or three serines. In addition, all three peptides contain clusters of basic residues at the amino terminus. The MLCK calmodulin-binding sequence shares these amino-terminal basic residues with an overlapping pseudosubstrate sequence, which appears to inhibit the catalytic site upon binding (26). The specific linear arrangement of the pseudosubstrate and calmodulin-binding domains allows for a tiered regulation of MLCK activity, with the interaction of the pseudosubstrate and the catalytic site being regulated by the binding and dissociation of calmodulin. A similar function could be envisioned for the hsp70 calmodulin-binding site. Heat shock causes a significant increase in intracellular Ca^{2+} concentration (11, 27) and could secondarily promote the binding of calmodulin to hsp70. Dissociation would occur after calcium homeostasis had been reestablished, allowing hsp70 to bind other proteins. Reversible binding of hsp70 to calmodulin would impose spatial, temporal, and structural restrictions on hsp70 as a function of intracellular calcium concentration and could be important in the overall regulation of hsp70 protein-protein interactions.

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