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Isolation of hsp70-binding proteins from bovine muscle

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Summary: It has been proposed that the members of the hsp70 major heat shock protein family in non-stressed cells participate in the intracellular transport of newly synthesized proteins and in maintaining such proteins in an unfolded state. Specific binding of other cellular proteins to hsp70 may play a role in hsp70 function. A simple method for isolation of hsp70 binding proteins using hsp70-sepharose column chromatography is described. The column binds hsp70 antibodies and thermodenatured a_2-interferon in an ATP-dependent manner. Low-ionic strength extracts and actomyosin preparations from bovine muscle were passed through the column and eluates in ATP and 2M NaCl were analyzed by electrophoresis. Two proteins that eluted with ATP were identified by immunoblotting as hsp70 and actin. The polypeptides that eluted with 2 M NaCl, among which 51 and 58 kDa proteins were prominent, are suggested to bind tightly by hydrophobic interaction to hsp70. An antiserum raised against total hsp70-binding protein recognized a polypeptide with a mass of 40 kDa as determined both by immunoblotting and immunoprecipitation. Hsp70 binding proteins may thus participate in the function of hsp70 with regards to hsp70s role in protein targeting, unfolding and transport.

The ubiquitous hsp70 family of stress proteins is composed in mammalian cells of several distinct components including glucose regulated protein GRP78 or BIP, mitochondrial P75 protein, constitutively expressed hsc70, and stress-induced hsp70 isoforms (1,2). Numerous studies indicate that the members of the hsp70 family participate in the translocation of newly synthesized proteins through intracellular membranes (3), dissociate certain protein complexes like clathrin vesicles (4) or play a role in protein folding (5,6). All these processes involve ATP binding and hydrolysis (1). To elucidate more precisely the role of hsp70 in these processes, it is important to characterize different hsp70-binding proteins (70BP). It is conceivable that 70BP represents both nascent or denatured proteins that have been incorrectly folded, and

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thus bind to hsp70 in a nonspecific manner, but also proteins that bind through a specific and maybe constitutive manner and which may participate in exerting the many functions of hsp70. During the last years several 70BP were characterized such as mutant form of p53 tumor antigens, calmodulin, cell cycle-dependent proteins, 110K surface glycoprotein and cytoskeletal proteins (reviewed in 4). Usually, coimmunoprecipitation is employed for the detection of 70BP (7). The disadvantages with this method are that antibodies against hsp70 may compete for the binding sites on the hsp70 molecule with the bound proteins, or that using this method it is difficult to isolate 70BP in amounts sufficient for further analysis. We present here a method for 70BP isolation based on chromatography on ATP-agarose to remove excess of endogenous hsp70 followed by chromatography on columns with hsp70 conjugated to sepharose. The hsp70-sepharose column was shown to bind denatured a_2-interferon and an hsp70 antiserum. The method was further applied to the purification of 70BP from bovine muscle.

MATERIALS AND METHODS

Reagents. Chemicals for electrophoresis were obtained from Serva (FRG), CNBr Sepahrose, Protein A Sepharose, Q Sepharose fast flow and AGATP Type 3 ATP agarose were from Pharmacia (Sweden). Nitrocellulose membranes were from Millipore (USA). Recombinant human a2-interferon was from "Ferment" Research Labs (Lithuania). The test kit for interferon detection based on monoclonal antibodies was developed in the Institute of cytology (USSR). Monoclonal antibodies for actin and a-actinin detection were kindly provided by Dr Fridlanskaya, Academy of Sciences USSR, Leningrad.

Purification procedures. Frozen bovine muscle was homogenized in a solution containing 20 mM NaCl, 20 mM tris HCl, pH 7.5, 0.1% Triton X-100, 0.1 mM EDTA, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonylfluoride and the supernatant after centrifugation of the extract at 20.000 g for 20 min, was passed through a 60 ml Q-sepharose Fast Flow column. The fraction eluted from the column with 0.3 M NaCl was loaded over a 10 ml column of ATP-agarose (8,9). The ATP-eluate was shown to contain substantially pure hsp70 (55-60% of inducible hsp70 form, and 40-45% of hsc70). Such eluates were dialyzed against coupling buffer (0.1 M Na bicarbonate and 0.5 M NaCl, pH 8.2) after the addition of EDTA to 4 mM. The protein was coupled to CNBr Sepharose 4B according to the instructions of the manufacturer, Pharmacias "Affinity Chromatography". Column flow-throughs after ion exchange and ATP-agarose chromatography were combined and passed thorugh the hsp70-sepharose column. After washing the column with 6 volumes of tris-buffered saline (TBS= 150 mM NaCl, 20 mM Tris-HCl, pH=7.5) and 2 volumes of TBS+ 0.1 mM EDTA, 70 BP were eluted first in 2 volumes of TBS containing 3 mM ATP and finally 2 M NaCl.

The pellet obtained above after extracting muscle was washed four times in a solution of 60 mM NaCl, 20 mM tris-HCl, pH 7.0, to isolate myofibril-like preparation containing the whole set of contractile proteins (10). The myofibrils were dissolved in a solution of 500 mM NaCl, 20 mM tris-HCl, pH 7.0 containing 0.1 mM EDTA and passed separately through the hsp70-Sepharose column. The latter was washed exhaustively (6 times the column volume) with the above solutions and elution was performed with ATP or 2 M NaCl.

Immunization. Protein eluted with NaCl from hsp70-sepharose column after passing the low ionic strength extract was injected to rabbits 3 times with two-week intervals. IgG fraction was purified from antiserum by chromatography on Protein A Sepharose.
Analytical procedures. Protein concentration was determined according to Bradford method (11). Electrophoresis was performed on 10% acrylamide-0.3% Bis gels (12). Gels were stained by Coomassie or silver according to modified protocol of Oakley and others (13) omitting incubation in glutaraldehyde. Hsp70 antiserum (H7) described elsewhere (14), actin and a-actinin monoclonal antibodies were used in immunoblotting assay (15).

RESULTS

The function of the hsp70-sepharose column was tested in two sets of experiments. First, H7 antiserum (14) raised in rabbit against hsp70 from bovine muscle was passed over the column, and the specificity of eluted IgG was monitored by immunoblotting and immunoprecipitation. As previously noticed, antibodies purified on the hsp70-sepharose column recognized in immunoblotting two components of the hsp70 family and only the inducible form of hsp70 in the immunoprecipitation assay (14). Secondly, 100 μg of intact or thermodenatured (at 70°C) a_2-interferon was loaded on a column carrying 4.5 mg of hsp70. After washing with six column volumes of TBS and the same containing 0.1% Nonidet P-40, elution with 3 mM ATP was performed. According to ELISA data 4-5% and 45-60% of native and denatured protein respectively, was retarded by the column and later detached by ATP. The results indicate that hsp70 coupled to Sepharose retains its ability for interaction with antiserum and denatured protein.

The procedure for 70BP isolation was composed of two steps, removal of excess of hsp70 from the bovine muscle low-ionic strength extract by ATP agarose chromotography, and purification of 70BP from the ATP agarose flow-through by chromatography on a hsp70-sepharose column. Hsp70 was detected by immunoblotting in the ATP-agarose column flow-through independently of the number (up to 4) of passages of the extract over the column (Fig. 1). Major polypeptides eluting from hsp70-sepharose with ATP have molecular masses as follows: 34, 43, 68, 93 and 105 kDa and with NaCl: 34, 40, 43, 51, 58, 93, 105 and 150 kDa. According to immunoblotting data with the hsp70 antiserum the protein is present mostly in ATP-eluate and only trace amounts were found in the high salt eluate (Fig. 1). Actin as determined by immunoblotting was detected in both eluates and the staining of its band was considerably higher in the case of the 2 M NaCl eluate than in the ATP-eluate (Fig. 1).

An actomyosin preparation was isolated from bovine muscle and shown to contain all known contractile proteins and several minor components (Fig. 2 and ref 16). Among the latter we were able to detect by blotting low amounts of hsp70 (Fig 2). Polypeptides found in the ATP-eluate after passing the EDTA-containing actomyosin preparation over the hsp70 sepharose column were as follows: 36, 38, 43, 98 and

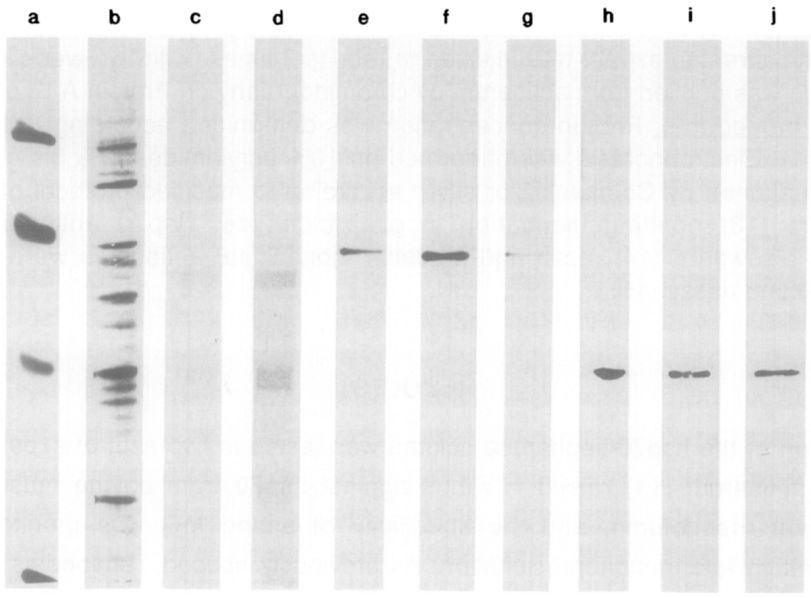

Fig. 1. Patterns of hsp70-binding proteins from muscle extract. The samples were electrophoresed on 10% acrylamide-0.3% Bis gels. In a, molecular weight standards of 94, 67, 43 and 30 kDa (from top) are shown. The gel was stained by Coomassie in a-d. Immunoblotting with the hsp70 antiserum is shown in e-g and with the actin antibodies in h-j. The samples in b, e, h are muscle extract, samples in c, f, i ATP-eluates, and samples in d, g, j NaCl-eluates from the hsp70 column.

200 kDa and in the NaCl eluate: 43 and 105 kDa. The 43 kDa protein present in both eluates was shown to be actin (Fig. 2) and the 105 kDa protein in the NaCl eluate was shown the be a-actinin by immunoblotting (Fig 2).

The 2M NaCl eluate from the hsp70-sepharose column after passing soluble muscle protein was injected into a rabbit in order to generate antiserum reactive with 70BD. Affinity purified IgG from the rabbit was shown to recognize a polypeptide with a molecular mass of 40 kDa and a mobility slightly higher than that of actin by immunoblotting of bovine muscle extract, HeLa cell lysate and rat pancreatic islet cell lysate (Fig. 3) and in mouse heart and muscle extracts (results not shown). The same polypeptide was immunoprecipitated by the IgG purified antiserum from labelled HeLa lysate (Fig. 3). This polypeptide was not detected in the 20000g muscle extract pellet, in the actomyosin preparation, in mouse brain, spleen and liver or in the lysates of primary mouse and human fibroblasts (results not shown).

DISCUSSION

The purpose of this study was to develop a method for isolation of proteins that bind to hsp70 in vitro. The method is based on removal of hsp70 by ATP-agarose chromatography and subsequent passage of hsp70-free extracts over a hsp70-sepharose column. The latter was prepared with protein which according to electrophoresis in non-denaturating conditions seems to consists of hsp70 dimers

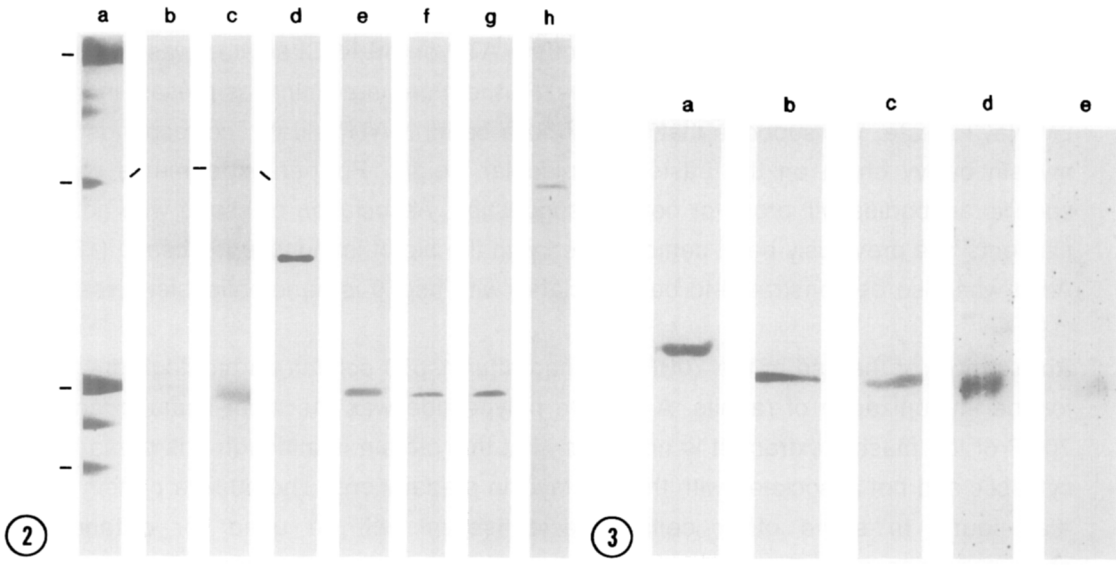

Fig. 2. Patterns of hsp70-binding proteins from myofibril actomyosin preparations. The samples were electrophoresed as in Fig 1. In lanes (a)-(c) the gel was stained by Coomassie blue. The results of immunoblotting with the hsp70 antiserum (lane d), the actin antibody (lanes e-g) and the a-actinin antibody (lane h) are shown. Lanes a, d, e, show the actomyosin preparation, lanes b and f the ATP-eluates and lanes c, g and h the NaCl eluates from the hsp70 column. The positions of myosin heavy chains (200 kDa), a-actinin (105 kDa), actin (43 kDa) and tropomyosin (34 kDa) are indicated from top to bottom.

Fig. 3. Characterization of 70BP antiserum. The samples were electrophoresed as in Fig 1. Lane a shows immunoblotting of the actomyosin preparation with the actin antibody. Lane b shows immunoblotting of the muscle extract using the 70BP antiserum. A [^{35}S]methionine labelled HeLa cell extract was immunoprecipitated with the 70BP antiserum in lane c. Lanes d and e are immunoblotting with the 70BP antiserum of HeLa cell and pancreatic islet extracts, respectively.

and trimers (results not shown). The proper functioning of the column was confirmed in experiments demonstrating binding of antiserum and denatured interferon. The latter was released by ATP elution.

We were not able to remove all the hsp70 from the muscle extract by ATP affinity chromatorgraphy. This could be explained by the existence of a part of hsp70 strongly associated with itself or other molecules thus making it unavailable to ATP binding. Furthermore, under certain conditions ATP hydrolysis may be necessary for the release of hsp70 from the 70BP (1,17). Hsp70 was detected also in the ATP-eluate from hsp70-sepharose column indicating possible leakage of protein from the gel.

The method presented here was applied to the isolation of 70BP from bovine muscle which was found to be a good source of hsp70 (8). Two of the 70BP were identified as actin and hsp70. Among the others two major polypeptides of 51 and 58 kDa were shown to bind strongly to hsp70, probably by a hydrophobic interaction mechanism. Before isolating contractile hsp70-binding proteins from the actomyosin preparation, the presence of hsp70 in myofibrils carefully washed off soluble proteins was

confirmed by blotting. This result corresponds to data indicating association of hsp70 with myofibrils (16). The pattern of 70BP of the ATP or 2M NaCl eluates was distinct except the presence of actin in both eluates. Furthermore, a-actinin was present in the 2M NaCl eluate. We suppose that the 200 kDa band in ATP eluate corresponds to myosin heavy chain on the basis of molecular weight. Further experiments with specific antibodies will prove or not this suggestion. Association of hsp70 with actin filaments hae previously been demonstrated with the aid of immunofluorescence (18). Actin was also demonstrated to be associated with hsp70 is pancreatic islet lysates (19).

An example of the use of the 70BP obtained after hsp70 column chromatography is for the immunization of rabbits. A 40 kDa polypeptide was the most immunogenic 70BP of the muscle extract. It is not clear what this protein is although it is primarily cytosolic and not associated with the actomyosin preparations. The 40 kDa protein is also found in some other cells. The antiserum will be used for detailed characterization of the 40 kDa protein.

In summary, a method is presented for the isolation of 70BP in large amounts for further analysis. It is conceivable that the various 70BP can modify hsp70 function with respect to disrupting protein aggregates (20) and to binding of newly synthesized polypeptides (6).

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