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CHARACTERIZATION OF A NOVEL TRANSFORMATION-SENSITIVE HEAT-SHOCK PROTEIN (HSP47) THAT BINDS TO COLLAGEN

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SUMMARY The synthesis of a major collagen-binding glycoprotein of molecular weight 47,000 was previously shown to be regulated by malignant transformation as well as by heat shock in chick embryo fibroblasts. The 47-kDa protein purified from chick embryos was characterized biochemically, and was found to exist as a monomer in native form. Its composition was enriched in basic amino acids and glycine, with fewer acidic residues and virtually no cysteine. N-terminal amino acid sequencing covering 36 residues revealed a single, novel sequence with an internal tandem repeat of Asp-Lys-Ala-Thr-Thr-Leu-Ala and Asp-Arg-Ser-Thr-Thr-Leu-Ala.

INTRODUCTION The heat shock response is a ubiquitous cellular response to physiological stress characterized by the induction of a specific set of heat shock or stress proteins (1-3). Although a striking evolutionary conservation of the stress proteins suggests some essential role in maintaining cellular homeostasis during cellular trauma, there is a lack of definitive information about the basic functions of these proteins.

We previously reported that a 47-kDa protein of chick embryo fibroblasts characterized earlier as a prominent collagen-binding protein of a variety of cell types (4) was also a novel heat shock protein (5). This heat shock protein (hsp47) is unusual in that its synthesis and phosphorylation are regulated by an oncogene product (6). Hsp47 is also distinctive in terms of its unusually basic isoelectric point (~9.0), since the other hsps are acidic (pI = 5-6; compare references 1-3 and 6). It is located in the endoplasmic reticulum (7,8).

In this paper, we provide a biochemical characterization of purified 47-kDa protein and compare it with a variety of other proteins.

METHODS

Isolation of hsp47--Native hsp47 was isolated from 13-day-old chick embryos for amino acid analysis and sequencing as described previously

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(8). Briefly, crude membranes were extracted with 1% Nonidet P-40 at pH 8.0. The extracts were ultracentrifuged, then mixed with gelatin-Sepharose 4B at 4°C for 16 hours. After a wash with high ionic strength buffer (0.4 M NaCl), the 47-kDa protein was eluted by decreasing the pH to 6.3.

Native Molecular Weight of hsp47--Partially purified hsp47 from chick embryos was analyzed on an Ultrogel AcA34 column (0.24 x 30 cm) to determine the native molecular weight and Stokes radius. The column was pre-equilibrated and eluted with lysis buffer at 200 µl/hour. Each 60 µl fraction eluted was dot immunoblotted by spotting 3 µl aliquots onto nitrocellulose filters (Schleicher and Schuell), then incubating successively with an anti-47-kDa protein rat monoclonal antibody (11D10/B6; 8), with rabbit anti-rat IgG antibody, and then with ^{125}I-labeled protein A essentially as described (9). The radioactivity of each fraction was determined in an LKB 1275 MiniGamma counter (Rockville, MD). Protein standards (Pharmacia, Piscataway, NJ) were chromatographed as described above, and elution positions were determined by densitometry of Coomassie blue-stained SDS slab gels of each column fraction.

Amino Acid Analysis--After further purification by preparative SDS-polyacrylamide gel electrophoresis, the 47-kDa band was briefly stained with Coomassie brilliant blue, excised, eluted electrophoretically, and dialyzed against distilled water containing 0.1% SDS. Aliquots were either subjected to performic acid oxidation (10) or hydrolyzed directly. Three-point hydrolyses were performed using duplicate sets of samples hydrolyzed in 6 N HCl at 110°C for 24, 48, or 72 hours (11). Amino acid compositions were determined by pre-column derivatization with phenylisothiocyanate and reverse-phase HPLC (12). Values were extrapolated to zero time by linear regression analysis. Because only very low quantities of cysteine were found in the 47-kDa protein, hydrolyses and analyses were performed parallel to those of untreated and performic acid-oxidized samples of cystine-containing mixed amino acids and BSA standards to confirm the accuracy of the cysteine determinations.

Amino Terminal Sequence Analysis--Automated Edman degradation and conversion into stable phenylthiodantoin (PTH)-amino acids were performed by Russell Blacher at Applied Biosystems, using a Model 470A gas-phase protein sequencer (13; Applied Biosystems, Foster City, CA). The resultant PTH-amino acids from each cycle were analyzed and quantitated by HPLC with an on-line Model 120A PTH analyzer.

Electrophoresis and Reagents--SDS-polyacrylamide gel electrophoresis used 4% stacking and 10% resolving slab gels (14) and prestained molecular weight markers (Bethesda Research Laboratories, Bethesda, MD). Gelatin-Sepharose 4B was from Pharmacia and Nonidet P-40 from Gallard/Schlesinger (Carle Place, NY). Other reagents were purchased from Sigma (St. Louis, MO), Calbiochem-Behring (La Jolla, CA), or Bio-Rad (Richmond, VA) and were of the highest purity available from each company.

RESULTS

Hsp47 was purified from membrane fractions of homogenates of 13 day-old chick embryos by detergent extraction, gelatin-Sepharose affinity chromatography, and mild acid elution at pH 6.3 (see Methods). The purity of the isolated hsp47 was established by SDS gel electrophoresis and Coomassie blue staining as shown in the inset of Fig. 1.

The native molecular size of purified hsp47 was determined by gel filtration chromatography using Ultrogel AcA34. Based on plots of the

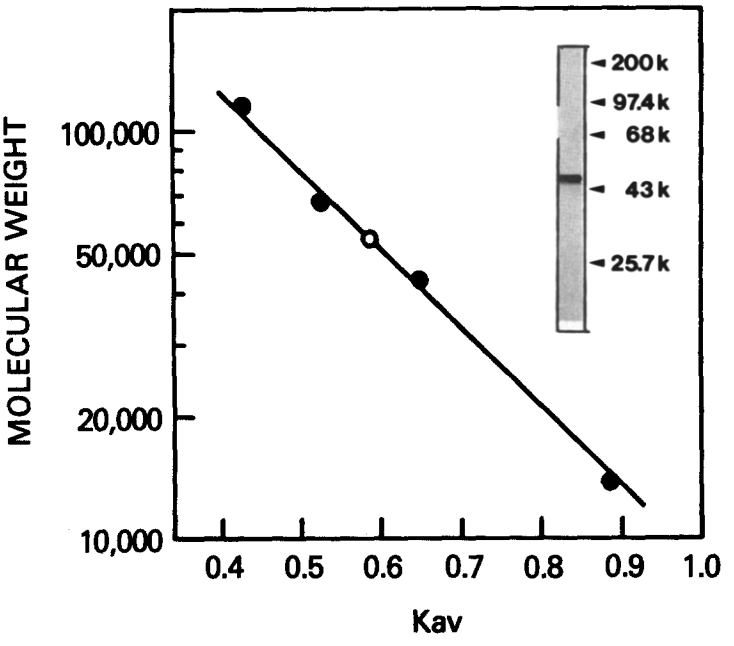

Figure 1. Native molecular weight of the hsp47 protein. Open circle shows the elution position of the 47-kDa protein after gel filtration chromatography on an Ultrogel AcA34 column as described in "Methods." Closed circles indicate the following standard proteins: catalase (dimer M_r = 116,000), BSA (M_r = 67,000, Stokes radius = 35.5 Å), ovalbumin (M_r = 43,000, Stokes radius = 30.5 Å) and ribonuclease A (M_r = 13,700, Stokes radius = 16.4 Å). Inset shows the purity of the hsp47 protein purified from chick embryos. One microgram of purified hsp47 protein was analyzed by SDS-polyacrylamide gel electrophoresis. The slab gel was stained with Coomassie brilliant blue. The molecular weights and mobilities of standard marker proteins are indicated (k = kilodaltons).

molecular weights, distribution coefficients (as K_{av}), and Stokes radii of standard proteins, the native molecular mass of the molecule was estimated to be 53 kDa (Fig. 1) and the Stokes radius to be 33.4 Å, assuming a globular conformation. Since a similar value of 47 kDa was estimated by electrophoresis after denaturation in boiling SDS (Fig. 1), it thus appears to exist in native form as a monomer without detectable intermolecular complex formation.

Hsp47 purified from chick embryos was subjected to amino acid analysis, and its composition was compared with a glycine-rich gelatin-binding protein (GGP) from porcine plasma (15) and chick fibronectin (Table I). Its amino acid composition differed considerably from each of these glycoproteins and from an "average" protein amino acid composition. It was noteworthy that hsp47 was enriched in basic residues, especially lysine, and that potential acidic residues (Asx + Glx) were low compared with the other proteins; this high ratio of basic to acidic residues is consistent with the unusually high isoelectric point of this protein (pI

TABLE I. AMINO ACID COMPOSITION OF THE 47-kDa AVIAN PROTEIN COMPARED WITH PORCINE GGP, AVIAN FIBRONECTIN, AND AN "AVERAGE" PROTEIN COMPOSITION

	47-kDa Protein[a]	residues/1000 GGP[b]	residues/1000 Fibronectin[c]	"Average" Protein[c]
Asx	60	114	94	104
Thr	50	58	100	63
Ser	75	80	74	76
Glx	53	105	118	94
Pro	67	21	77	48
Gly	139	151	106	77
Ala	103	60	48	84
Val	67	58	66	69
Met	16	12	13	17
Ile	36	16	38	48
Leu	92	82	55	75
Cys[d]	3	25	18	28
Tyr	37	41	37	36
Phe	30	42	19	36
Lys	89	59	34	69
His	26	32	16	22
Arg	57	44	53	41

[a]Values from duplicate 24, 48, and 72 hour hydrolysates. Tryptophan was not determined.
[b]From reference 15.
[c]From reference 10.
[d]Determined as cysteic acid after performic acid oxidation (10).

= 9.0, ref. 6). Hsp47 was also moderately enriched in glycine residues, though not as high as in the GGP proteins described previously (15) (Table I). Another unusual feature of the amino acid composition was the low quantity of cysteine, a value equivalent to only 1-2 residues per polypeptide of 47 kDa (Table I).

Gas-phase protein sequencing of the amino terminus of hsp47 yielded a single, unambiguous sequence for the first 36 residues (Table II). A comparison by the FASTP program of Lipman and Pearson (16) with the contents of the National Biomedical Research Foundation protein sequence database (Georgetown University) showed it to be unique, with no apparent homologous sequence matches among 778,224 databank residues. The N-terminal sequence of hsp47 contains the novel, tandemly repeated sequence:

Asp-Lys-Ala-Thr-Thr-Leu-Ala (positions 10-16);

Asp-Arg-Ser-Thr-Thr-Leu-Ala (positions 17-23).

Neither this heptapeptide sequence nor any six-residue match was found in the protein amino acid sequence databank.

TABLE II. AMINO ACID SEQUENCE ANALYSIS OF THE 47-kDa PROTEIN PURIFIED FROM CHICK EMBRYOS

Cycle No.	Amino Acid	Yield[a] pmol	Cycle No.	Amino Acid	Yield[a] pmol
1	Val	296[b]	21	Thr	ND
2	Pro	239	22	Leu	24
3	Ser	ND[c]	23	Ala	29
4	Glu	168	24	Phe	24
5	Asp	167	25	Asn	21
6	Arg	46	26	Leu	19
7	Lys	147	27	Tyr	17
8	Leu	141	28	His	6
9	Ser	ND	29	Ala	17
10	Asp	135	30	Met	16
11	Lys	100	31	Ala	75[b]
12	Ala	102	32	Lys	13
13	Thr	ND	33	Asp	12
14	Thr	ND	34	Lys	48[b]
15	Leu	66	35	Asn	10
16	Ala	58	36	Met	9
17	Asp	70			
18	Arg	20			
19	Ser	ND			
20	Thr	ND			

[a]Net yield in which background was subtracted from the gross yield for each cycle, e.g., cycle number 2 was 256 pmol gross yield, 17 pmol background, and 239 pmol net yield.

[b]Total (gross) yield.

[c]Yield was not quantitated for serine and threonine because of their multiple derivatives.

DISCUSSION

Hsp47 is a collagen-binding glycoprotein (4,6), the synthesis of which is decreased after Rous sarcoma virus transformation of fibroblasts, whereas its phosphorylation is stimulated (6). We also found that its synthesis is transiently induced by temperature-shift to 42°C or 45°C, identifying it as a novel heat shock protein (5). This communication provides the first biochemical characterization of this protein. It exists as a monomer rich in basic amino acids and glycine but with little cysteine. Amino acid sequencing reveals a novel sequence with an internal tandem repeat.

A variety of proteins have been described previously as putative collagen receptors, which differ substantially in molecular size from hsp47. They include: a 65-kDa platelet protein that binds the α_1(I)-chain (17), a 31-kDa chondrocyte glycoprotein termed anchorin CII that binds type II collagen (18), collagen-binding proteins from plasma membranes of nucleated cells of 90-145 kDa (19-22), platelet factor XIII (23), and recently other platelet membrane glycoproteins of 61-kDa and 160-kDa that also bind to collagen (24,25). Gelatin-binding 21-, 70- and 95-kDa glycoproteins are also secreted by some cultured cells (26-28).

A 47-kDa glycine-rich gelatin-binding protein (termed GGP) was isolated by gelatin-Sepharose 4B chromatography from porcine plasma (15). Although identical in apparent subunit size, GGP is distinct from our hsp47 in its estimated native molecular weight without chemical reduction (~450-kDa by SDS-polyacrylamide gel electrophoresis), its ability to bind to heparin, and its amino acid composition, although both proteins do have above-average contents of glycine (Table I and ref. 15). Finally, a 47-kDa phosphoprotein has been purified from human platelets, the phosphorylation of which is enhanced after thrombin activation (29). However, this protein differs substantially from our 47-kDa heat shock protein in isoelectric point. Thus, our hsp47 is distinct from any other collagen-binding protein or receptor.

Hsp47 is also unusual among heat shock proteins: it is membrane-associated and binds to collagen; its synthesis is regulated by two independent phenomena, i.e., malignant transformation and heat shock; and its phosphorylation is concomitantly regulated by transformation (5,6). The results of our analysis thus suggest that this protein is a binding protein with novel biochemical properties unrelated to other stress-related or collagen-binding proteins.

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