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THE TRANSFORMATION-SENSITIVE HEAT SHOCK PROTEIN (HSP47) BINDS SPECIFICALLY TO FETUIN

AKIRA NAKAI[1], KAZUNORI HIRAYOSHI[1], SHINSUKE SAGA[2], KENNETH M. YAMADA[3] AND KAZUHIRO NAGATA[1]

[1]Chest Disease Research Institute, Kyoto University, Kyoto 606, Japan

[2]The Second Department of Pathology, Nagoya University School of Medicine, Nagoya 466, Japan

[3]Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

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SUMMARY The transformation-sensitive heat shock protein of Mr=47,000 (hsp47) has been shown to bind to collagen and gelatin. We examined the binding specificity of hsp47. The binding of hsp47 to gelatin-Sepharose 4B was competitively inhibited by fetuin as effectively as by gelatin or collagen, whereas a variety of other proteins tested had no effect. Fetuin-coupled Sepharose 4B was found to bind hsp47 even at high ionic strength, but the complex was dissociated at pH $\leq$ 5.5.

In response to elevated temperature, almost all organisms induce proteins termed heat shock proteins or stress proteins (1). Functions of some heat shock proteins at normal temperature have been identified using their binding specificities as clues to search for functions (2,3).

We previously reported that i) a collagen-binding membrane glycoprotein of Mr=47,000 (hsp47) was a novel heat shock protein (4), ii) the synthesis of hsp47 decreased after neoplastic transformation with tumor viruses or oncogenes (5), iii) its binding to collagen was inhibited at pH $\leq$ 6.3 (7), iv) its synthesis increased markedly during the differentiation of mouse F9 teratocarcinoma cells (to be published and ref. 6), and v) it was localized in endoplasmic reticulum (7). In this report, we have further examined the binding specificity of hsp47.

METHODS

Cell Culture, Metabolic Labeling, and Competition Assay--- Chick embryo fibroblasts were maintained in Vogt's GM medium (8), and metabolically labeled with [^{35}S]methionine (specific activity 23.62 Tbq/mmol, New England Nuclear) as previously described (5). After extraction with lysis buffer (1% Nonidet p40, 0.15 M NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 2 mM N-ethylmaleimide, 2 mM

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phenylmethylsulfonyl fluoride) on ice for 20 min, the supernatant solution of the centrifugation at 12,000 g for 20 min was mixed with 200 µl of a 50% (v/v) suspension of gelatin-Sepharose 4B (Pharmacia) at 4oC for 1 h as described previously (5). For competition assay, proteins were dissolved at stock concentrations of 5 mg/ml each in lysis buffer and were used as competitors for the binding of the 47-kDa protein to gelatin-Sepharose at the final concentrations indicated. The gelatin-Sepharose beads were collected by centrifugation, washed twice with lysis buffer and once with 10 mM Tris-HCl buffer (pH 6.8). Proteins bound to gelatin-Sepharose were eluted by boiling the beads for 5 min in Laemmli's SDS-gel electrophoresis buffer, and analysed by one- or two-dimensional SDS-polyacrylamide gel electrophoresis (5, 9). Prestained molecular weight markers (Bethesda Research Laboratories) or molecular weight markers (Pharmacia) prestained with remazol brilliant blue (10) were used for electrophoresis.

Isolation of Fetuin Binding Proteins--- Fetuin-Sepharose 4B was prepared by covalent coupling fetuin (Sigma, and Takara, Kyoto, Japan) to cyanogen bromide-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. Fetuin-binding proteins were isolated similarly as for gelatin-binding proteins. To estimate the pH that dissociated the binding of hsp47 to fetuin, radiolabeled cell lysates and excess cold actin (final concentration 2.0 mg/ml) were mixed with fetuin-Sepharose. Thereafter, the beads were packed into a column, and fetuin-binding proteins were eluted with a pH gradient from pH 8.0 (50 mM Tris-HCl) to pH 5.5 (50 mM citric acid). Radioactivity of eluates was scintillation counted, and aliquots were electrophoresed.

RESULTS

The binding specificity of hsp47 was examined based on the competitive inhibition of binding of [^{35}S]methionine-labeled hsp47 to gelatin-Sepharose beads by various proteins. As shown in fig. 1A, the binding of hsp47 in extracts of chick fibroblasts to gelatin-Sepharose was inhibited by gelatin at concentra-

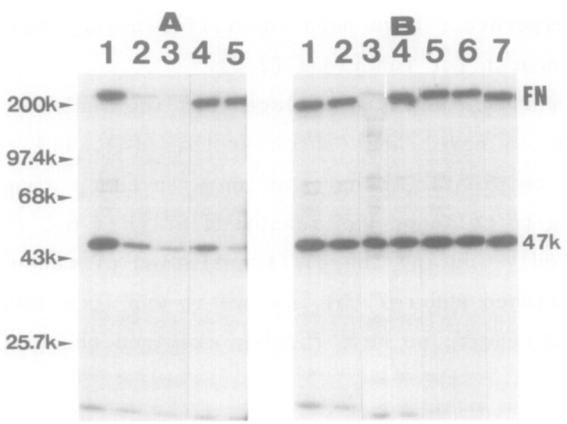


Figure 1. Competition of various soluble proteins for the binding of hsp47 to gelatin. CEF were labeled with [^{35}S]methionine, extracted with lysis buffer, and then aliquots of equal volumes were mixed with gelatin-Sepharose 4B at 4 oC for 1 h with or without competitors at final concentrations of 0.5 mg/ml or 2.0 mg/ml. Gelatin binding fractions were analyzed on SDS-10% polyacrylamide gel electrophoresis. (A) Lane 1, no competitor; lanes 2 and 3, 0.5 and 2.0 mg/ml gelatin respectively; lanes 4 and 5, 0.5 and 2.0 mg/ml fetuin respectively. (B) Lane 1, no competitor; lane 2, transferrin; lane 3, fibronectin; lane 4, fibrinogen; lane 5, ovalbumin; lane 6, hemoglobin; lane 7, myoglobin. The final concentration of each competitor was 2.0 mg/ml. 47K indicates hsp47. FN, fibronectin.

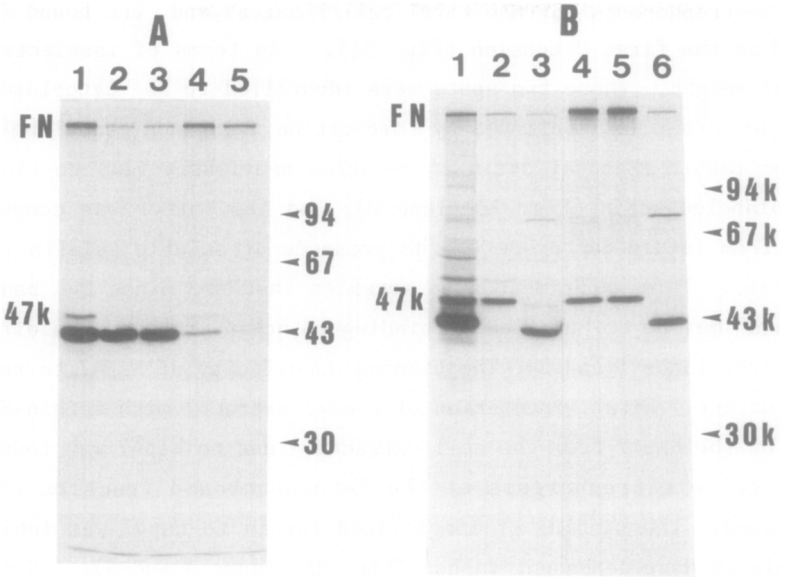


Figure 2. Binding of hsp47 to gelatin- and fetuin-Sepharose 4B beads. Equal volumes of a Nonidet P40 extract from [^{35}S]methionine-labeled chick embryo fibroblasts without or with treatment by 2 μg/ml tunicamycin for 8 h were incubated with fetuin-Sepharose 4B (A and Lanes 1-3 in B) or gelatin-Sepharose 4B (lanes 4-6 in B). Binding was competed by various soluble proteins: (A) Lane 1, no competitor; lane 2, 0.5 mg/ml gelatin; lane 3, 2.0 mg/ml gelatin; lane 4, 0.5 mg/ml fetuin; and lane 5, 2.0 mg/ml fetuin. (B) Lane 1, no competitor; lanes 2 and 3, 1.0 mg/ml actin; lane 4, no competitor; lanes 5 and 6, 1.0 mg/ml actin; cell lysate was prepared from tunicamycin-treated CEF for panel B, lanes 3 and 6 only. 47K, hsp47. FN, fibronectin.

tions of 0.5-2.0 mg/ml. Note that gelatin competed the binding of fibronectin to gelatin-Sepharose beads as well (Fig. 1A, lanes 2 and 3). Native type I collagen (Vitrogen 100) also inhibited the binding similarly (data not shown). Fetuin was also found to inhibit the binding of hsp47 to gelatin-Sepharose beads at concentrations similar to those for gelatin (Fig. 1A, lanes 4 and 5). Except for fetuin, a number of other proteins tested did not affect the interaction between hsp47 and gelatin-Sepharose beads (Fig. 1B). We found no inhibition of the binding of hsp47 to gelatin by transferrin, fibronectin, fibringen, ovalbumin, hemoglobin, and myoglobin at concentrations of 2.0 mg/ml (Fig. 1B). Soluble fibronectin clearly inhibited the binding of [^{35}S]methionine-labeled cellular fibronectin to gelatin-Sepharose (Fig. 1B, lane 3).

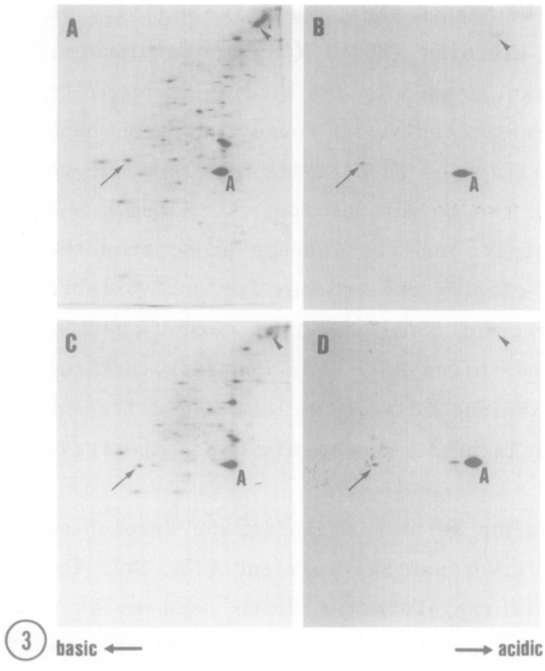
In order to directly confirm that hsp47 can bind to fetuin as well a collagen, we prepared immobilized fetuin affinity matrices. As shown in Fig. 2A, lane 1, a 47-kDa protein was found to bind to fetuin-Sepharose beads. Mock-coupled activated Sepharose did not bind this 47-kDa protein (data not shown, see ref. 5). Fetuin-Sepharose also bound an additional two major proteins from [^{35}S]methionine-labeled chick fibroblast extracts (Fig. 2A, lane 1). To identify the 43- and 47-kDa proteins that bound to fetuin, we performed two-dimen-

sional gel electrophoresis of the total cell lysates and the bound fractions using NEPHGE as the first dimension (Fig. 3A). In terms of isoelectric point and molecular weight, these two spots were identified to be cytoplasmic actin and hsp47. The former was confirmed by competition using non-radiolabeled actin purified from rabbit skeletal actin as reported previously (11) to block binding of radiolabeled actin (Fig. 2B, lane 2), and the latter was competitively dissociated from fetuin-Sepharose by the presence of soluble gelatin (Fig. 2A, lanes 2 and 3). This experiment also revealed that the minor two bands above the hsp47 band may be co-purified by binding to actin, rather than directly to fetuin (Fig. 2B, lanes 1 and 2). The binding specificity of hsp47 to fetuin was confirmed further. First, incubation of a cell extract with fetuin-Sepharose removed all of the hsp47 from the cell extracts, and no hsp47 was found on two dimensional gel electrophoresis of the fetuin-unbound fraction (data not shown). Second, the binding of immobilized fetuin to hsp47 was inhibited by soluble fetuin in dose-dependent fashon (Fig. 2A, lanes 4 and 5). Since hsp47 is a glycoprotein, we examined the effects of glycosylation on its binding specificity. No effect was detected on the binding of hsp47 to fetuin as well as gelatin when the glycosylation of hsp47 was in-hibited by treatment with tunicamycin, and when the binding assay was performed in the presence of purified actin to avoid the binding of cytoplasmic actin to fetuin-Sepharose (Fig. 2B, lanes 3 and 6; Fig. 3C and D).

To examine for possible pH dependence of the binding of hsp47 to fetuin, chick embryo fibroblasts labeled with [^{35}S]methionine were incubated with fetuin-Sepharose beads in the presence of non-radiolabeled actin, packed into a column, washed with high ionic strength buffer, and then eluted by a pH gradient as described in Methods. The radioactivity of eluates reached a peak at pH 5.5, between fractions 14 and 15 (Fig. 4). We performed SDS-polyacrylamide gel electrophoresis of each fraction; only the 47-kDa protein was detected in the peak, without any contamination by actin or fibronectin (data not shown). Thus, the binding of hsp47 to fetuin was strong enough to resist dissociation by high ionic strength (0.4 M NaCl), but was easily dissociated by a change of pH within a physiological range.

DISCUSSION

In a previous study, we reported that hsp47 is a collagen-binding glycoprotein (4), the synthesis of hsp47 decreases after cell transformation (5), and hsp47 localizes in the endoplasmic reticulum (7). In this communication, we examined the binding specificity of hsp47 and found that hsp47 binds to fetuin in addition to collagen, and that this binding is disrupted at a physiological low pH characteristic of some cytoplasmic compartments.



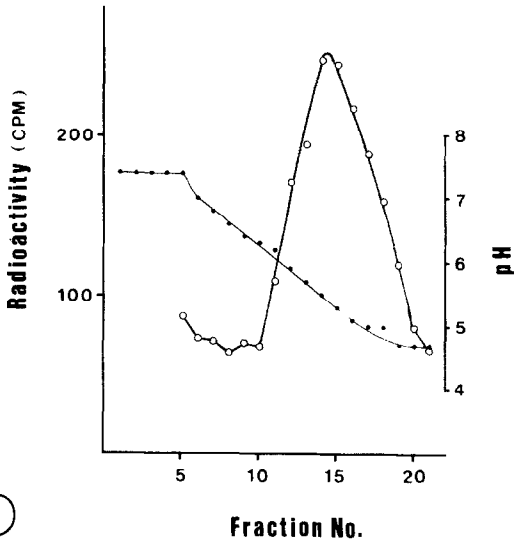


Figure 3. Two-dimensional analysis by NEPHGE/SDS-polyacrylamide gel electrophoresis of total and fetuin-binding fractions. Bound proteins shown in Fig. 2 were analyzed. (A) total cell lysate; (B) fetuin-bound fraction; (C) total cell lysate fraction treated with tunicamycin; (D) fetuin-bound fraction from lysate of tunicamycin treated cells. Arrows indicate hsp47. Arrowheads, fibronectin. A, actin.

Figure 4. Elution of hsp47 from a fetuin affinity column by a gradient of decreasing pH. A Nonidet P40 extract from [^{35}S]methionine-labeled CEF was mixed with 500 µl of fetuin-Sepharose 4 B and purified actin (see Fig. 2B), and then packed to column. After the column was washed with lysis buffer containing 0.4 M NaCl, hsp47 was eluted with a 1.5 ml pH gradient from pH 8.0 (50 mM Tris-HCl) to 4.5 (50 mM citric acid). Each fraction consisted of 10 drops. The radioactivities of aliquots of each fraction were measured, and pH was estimated by pH indicator strips (color pHast, pH 4.0-7.0, EM-Reagents and Spezialindikator, pH 6.5-10.0, Merck).

Soluble gelatin and collagen inhibit the binding of hsp47 to gelatin-Sepharose, indicating that soluble proteins can compete with immobilized collagen or gelatin for binding by hsp47. Soluble fetuin also readily inhibits the binding of hsp47 to gelatin-Sepharose(Fig. 1). Conversely, gelatin inhibits hsp47 binding to fetuin-Sepharose (Fig. 2B). These findings suggest that the binding sites for gelatin and fetuin are either the same or are spatially close enough to produce mutual steric interference with binding.

The inhibition of binding of hsp47 to gelatin-Sepharose by these proteins appears to be specific, since a variety of other proteins, including ovalbumin, hemoglobin, myoglobin, transferrin, fibrinogen, and fibronectin, do not inhibit the binding. In addition, rat IgG, protein A, and heparin were previously shown to be incapable of binding hsp47 (5). Besides these proteins, we examined the

binding specificity of hsp47 to albumin. We tested the competitive activity of many samples of purified bovine serum albumins (BSAs) (Sigma; Calbiochem; Miles; Boehringer-Mannheim; Pentex; and Wako, Japan) in the binding of hsp47 to gelatin-Sepharose (unpublished data). Unexpectedly, we found that some BSAs were active, but others inactive. The differences in activity were not due to differences in purity, in the lots, nor in the commercial source. Albumin and fetuin are known to have structural homology, and these genes arose from the same ancestral gene (12). We cannot yet clarify the reasons for our variable results in terms of the competitive activity of BSA, and thus cannot reach a definite conclusion concerning whether hsp47 binds BSA. In contrast, several preparations of purified fetuin so far examined (different lots and different companies including Sigma, Calbiochem and Takara) showed similar competitive activity without exception.

Fetuin, or α-fetoprotein, is the major serum protein of the developing fetus and is synthesized by the embryonic liver and the yolk sac (13, 14). The concentration of fetuin in the blood falls rapidly after birth and is at a residual low level in adults (13). Hsp47 is not detected in adult chick liver by indirect immunostaining (7). It may be of interest to examine for the existence of hsp47 in fetal liver.

The binding of collagen to hsp47 occurs even at high ionic strength, but is easily disrupted by changing the pH of the buffer from 8.0 to 6.3 (7). The binding of fetuin to hsp47 is also tight enough to be unaffected by 0.4 M NaCl. A similar pH dependency of the dissociation of hsp47 from fetuin is also observed, which may suggest possible roles of hsp47 in transporting collagen, fetuin, and perhaps other as yet unidentified secreted proteins from endoplasmic reticulum to a site of release in the Golgi complex.

REFERENCES

1. Lindquist, S., and Craig, E. A. (1988) Ann. Rev. Genet. 22; 631-677.
2. Pelham, H. R. B. (1986) Cell 46, 959-961.
3. Schlesinger, M. J. (1986) J. Cell Biol. 103, 321-325.
4. Nagata, K., Saga, S., and Yamada, K. M. (1986) J. Cell Biol. 103, 223-229.
5. Nagata, K., and Yamada, K. M. (1986) J. Biol. Chem. 261, 7531-7536.
6. Kurkinen, M., Taylor, A., Garrels, J. I., and Hogan, B. L. M. (1984) J. Biol. Chem. 259, 5915-5922.
7. Saga, S., Nagata, K., Chen, W.-T., and Yamada, K. M. (1987) J. Cell Biol. 105, 517-527.
8. Vogt, P. K. (1969) In Fundamental Techniques in Virology, p. 66. Academic Press, New York.
9. Laemmli, U. K. (1970) Nature 227, 680-685.
10. Saoji, A. M., Jad, C. Y., and Kellar, S. S. (1983) Clin. Chem. 29, 42-44.
11. Nagata, K., Sagara, J., and Ichikawa, Y. (1982) J. Cell Biol. 93, 470-478.
12. Alexander, F., Young, P. R., and Tilghman, S. M. (1984) J. Mol. Biol. 173, 159-176.
13. Abelev, G. I. (1971) Adv. Cancer Res. 14, 295-358.
14. Gitlin, D., and Perricelli, A. (1970) Nature (Lond.) 228, 995-997.