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The induction mechanism of the molecular chaperone HSP70 in the gastric mucosa by Geranylgeranylacetone (HSP-inducer)

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Abstract

To elucidate the induction mechanism of HSP70 by geranylgeranylacetone (GGA), we investigated GGA specific binding proteins using a GGA-affinity column. Alteration of chaperone activity of HSP70 and binding affinity of HSP70 to heat shock factor-1 (HSF-1) was evaluated in the presence or absence of GGA. The binding domain of HSP70 to GGA was also analyzed. A 70-kDa protein eluted by 10 mM GGA from the GGA-affinity column was identical to constitutively expressed HSP70 on immunoblotting. GGA-binding domain of HSP70 was C-terminal of the protein as peptide-binding domain (HSP70C). The chaperone activity of HSP70 and recombinant HSP70C was suppressed by GGA. Furthermore, dissociation of the HSP70 from HSF-1 was observed in the presence of GGA. GGA preferentially binds to the C-terminal of HSP70 which binds to HSF-1. After dissociation of HSP70, free HSF-1 could acquire the ability to bind to HSE (the promoter region of HSP70) gene.

Keywords: Heat shock protein; HSP; Heat shock factor; Geranylgeranylacetone; Stomach; Molecular chaperon

Heat shock proteins (HSPs), also called molecular chaperones, play a crucial role in the folding of newly synthesized proteins and the refolding of denatured proteins [1,2]. Molecular chaperones are highly conserved proteins and are rapidly induced in cells in response to abrupt and advance change in their environment [3,4]. The cytosolic 70-kDa molecular chaperones (HSP70s) are present in cells as two different gene products, but are closely related to each other: a stress-inducible form HSP70 (known as HSP72) and a constitutively expressed form HSP70 (known as HSP72 or 70-kDa heat shock cognate protein, HSC70). HSP70s consist of two domains, NH_2-terminal ATPase domain having a molecular mass of 45-kDa and COOH-terminal peptide-binding domain of 25-kDa [5,6]. The ATP-binding domain of HSP70s binds and releases peptide slowly but more stably [7,8]. Binding of ATP to the ATP-binding domain causes a conformational change, which in turn results in structural alterations in the COOH-terminal, thus leading to substrate release [9,10].

The induction of HSPs in response to various stresses is dependent on the activation of specific transcription factor, the heat shock factor (HSF-1), which binds to the heat shock element (HSE) in the promoters of HSP genes [11]. It has been shown that HSP70 is the preferred candidate for the repressor of HSF-1 [12,13].

GGA is an acyclic isoprenoid compound with a retinoid skeleton that induces HSP70 synthesis in various tissues including the gastric mucosa, intestine, liver, myocardium, retina, and central nervous system [14–18]. Oral administration of GGA rapidly causes up-regulation of HSP70 expression in response to a variety of stresses, although this

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effect is weak under normal conditions [15]. With extremely low toxicity of GGA, this compound has been widely used as an oral anti-ulcer drug. However, the induction mechanism of HSP70 by GGA has not yet been well understood. In the present study, we analyzed the specific binding proteins of GGA by using a GGA-affinity column, and the induction mechanism of HSP70 by GGA is demonstrated and discussed.

Materials and methods

Plasmid or phagemid construction

A full-length human HSP70 cDNA was kindly provided by Dr. Richard Morimoto, Northwestern University (Evanston, Ill, U.S.A.). The polymerase chain reaction-amplified *Bam*HI–*Hin*dIII fragments encoding the cDNA sequence, the N-terminal domain of HSP70 (residues 1–380) or the C-terminal domain of HSP70 (residues 381–640), were inserted into the digested pQE31 plasmid vector (Quiagen, Hilden, Germany). The N- or C-terminal domain of HSP70 was overexpressed in *Escherichia coli* M15 [pREP4] cells as an N-terminal 6× His-tagged protein. Cells were grown in LB at 37 °C to an A600 of 0.6, induced with 0.1 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside), and harvested after 5 h of induction. Cell pellets were resuspended in 10 mM Tris–HCl (pH 7.4) and lysed by a sonicator (TOMY, Tokyo, Japan). The lysate was cleared by centrifugation, and the resulting supernatant was applied to Ni–NTA (nickel–nitrilotriacetic acid) metal-affinity column. After washing the column with 0.3 M NaCl in 10 mM Tris–HCl (pH 7.4), the N- or C-terminal 6× His-tagged proteins were eluted by the linear gradient of 0–0.25 M imidazole in 10 mM Tris–HCl (pH 7.4). Human GST fusion HSF-1 was expressed and purified as previously described [19].

Proteins purification

HSP70 was purified from rat gastric mucosa using an ATP–Sepharose column as described previously [20]. The purified HSP70 was applied to GGA column or mock column and eluted with linear gradient of GGA as described above. The eluants were analyzed on SDS/PAGE [21] and immunoblotted [22] using an antibody against HSP70 as described previously [23].

Affinity column chromatography

GGA. GGA was a gift from Eisai, Inc., (Tokyo) and activated-Thiol Sepharose 4B was purchased from GE Health Care BioScience (Piscataway, NJ, USA). GGA was dissolved in absolute ethanol and diluted with MilliQ water at GGA in 70% ethanol. GGA–Sepharose was prepared according to the manufacturer's instructions. As a control, mock column was prepared the same as GGA–Sepharose column using 70% ethanol in the absence of GGA. Wistar rat stomach (~20 g) was homogenized in 10 mM Tris–HCl (pH 7.4) and centrifuged at 20,000*g* for 10 min at 4 °C. The supernatants were applied to GGA column or mock column equilibrated in the same buffer. The columns was washed with 10 column volumes of 10 mM Tris–HCl (pH 7.4). After washing the column, protein was eluted with 10 mM GGA in 0.4% ethanol, 10 mM Tris–HCl (pH 7.4). Otherwise, Wistar rat gastric mucosal cytosols were applied to GGA column or mock and washed as described above. The binding proteins were eluted with a linear gradient of GGA (0–10 mM).

The HSP70N or HSP70C was applied to GGA-affinity column. After washing the column with 10 mM Tris–HCl (pH 7.4), proteins were eluted by 10 mM GGA.

HSF-1. HSF-1-affinity column was prepared using GST fusion HSF-1 and Glutathione Sepharose 4B (GE Health Care Bio Science) according to the manufacturer's instructions. The purified HSP70 was applied to HSF-1-affinity column. After washing the column with 10 mM Tris–HCl (pH 7.4), proteins were eluted by 10 mM GGA or 5 mM ATP. The HSP70N or HSP70C was applied to HSF-1-affinity column. After washing the column with 10 mM Tris–HCl (pH 7.4), proteins were eluted by 10 mM GGA. The eluted proteins in the eluants from these affinity columns were electrophoresed on SDS/PAGE, followed by staining with Coomassie Brilliant Blue R205 in 25% (v/v) iso-propyl alcohol and 10% (v/v) acetic acid and destaining with 10% (v/v) iso-propyl alcohol and 10% (v/v) acetic acid or by immunoblotting using an anti-HSP70 antibody [23].

Measurement of protein aggregation and chaperone function of HSPs

The thermal aggregation of citrate synthase (CS) (Roche Diagnostics, Mannheim, Germany) was monitored at 45 °C as previously described [24]. Briefly, the concentration of CS used was 0.15 μM in 50 mM Hepes buffer, pH 7.4, in the presence or absence of the wild-type HSP70 (0.5 μM), HSP70N (0.5 μM), or HSP70C (0.5 μM), and in the presence of each HSP70 and 5 μM GGA. Light scattering CS was monitored for 20 min at an optical wavelength of 500 nm by Amersham Biosciences Ultrospec 3000 UV–vis spectrophotometer equipped with a temperature control unit using semi-micro-cuvettes (0.5 ml) with a path-length of 10 mm.

The influence of GGA on the chaperone activity of HSP70C was monitored using rhodanese as previously described [23]. Rhodanese (Sigma) was denatured at a concentration of 100 μM in buffer containing 6 M guanidinium–Cl (GdmCl), 25 mM Hepes–KOH, pH 7.5. Denatured rhodanese was rapidly diluted to 0.5 μM into buffer containing 25 mM Hepes–KOH, pH 7.5, in the presence or absence of HSP70C (500 μM) and GGA (500 μM). Aggregation of rhodanese was monitored over 10 min by measuring the optical density at 320 nm in a GE Health Care Bio Science Ultrospec 3000 UV–vis spectrophotometer, and the data were normalized using the aggregation in buffer alone as the standard.

Results

GGA-affinity column chromatography

We investigated GGA-binding proteins using a GGA-affinity column. In general, the affinity column chromatography is defined as follows: a purified molecule is reversibly adsorbed on the ligand which is fixed to the matrix [25,26]. To avoid nonspecific binding proteins, GGA-affinity columns were thoroughly washed with conditioning buffer. Proteins were eluted with 10 mM GGA from GGA-affinity column or control mock column, and eluants were detected on SDS/PAGE. Although no protein bands were detected in the eluant from the mock column, major protein bands with molecular mass of 70-kDa were detected in the eluant from the GGA-affinity column (Fig. 1).

The protein eluted from the GGA-affinity column was analyzed by immunoblotting using an anti-HSP70 antibody (Fig. 1). The 70-kDa protein was detected in the eluant as a major protein, and the proteins were cross-reacted to an antibody against HSP70. These results suggested that GGA interacted with molecular chaperone HSP70.

To determine whether the association between HSP70 and GGA is direct or indirect (mediates other proteins), we analyzed the interaction applying a purified HSP70 alone under the same conditions on GGA-affinity column. On SDS/PAGE, we could confirm that purified HSP70 could bind to the GGA-affinity column and could be eluted by GGA (Fig. 1). Based on these results, GGA has a high affinity to molecular chaperone HSP70 and the affinity is highly specific.