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Ankyrin-B Interacts with the C-terminal Region of Hsp40

Byung-In Min[1], Han Suk Ko[2] and Chong-Rak Kim[3†]

Department of Biology[1], Department of Medicine[2], and Department of Biomedical Laboratory Science[3], Inje University, Kimhae 621-749, Korea

Ankyrins are a ubiquitously expressed family of intracellular adaptor proteins involved in targeting diverse proteins to specialized membrane domains in both the plasma membrane and the endoplasmic reticulum. Canonical ankyrins are 190~220 kDa proteins expressed in most tissues and cell types and comprise a membrane-binding domain (MBD) of 24 ANK repeats, a spectrin-binding domain, a death domain and a C-terminal domain. Rescue studies with ankyrin-B/G chimeras have identified the C-terminal domain of ankyrin-B as the defining domain in specifying ankyrin-B activity, but the function of C-terminal domain of ankyrin-B is, however, not known. We report here that the C-terminal domain of ankyrin-B is capable of interacting with the C-terminal Region of Hsp40. The Hsps are induced not only by heat shock but also by various other environmental stresses. Hsps are also expressed constitutively at normal growth temperatures and have basic and indispensable functions in the life cycle of proteins as molecular chaperones, as well as playing a role in protecting cells from the deleterious stresses. The binding sites required in the interaction between C-terminal domain of ankyrin-B and C-terminal region of Hsp40 were characterized using the yeast two-hybrid system and GST-pull down assay. The interaction between ankyrin-B and Hsp40 represents the first direct evidence of ankyrin's role as chaperones.

Key Words: Ankyrin, Hsp40, Yeast two-hybrid

INTRODUCTION

Ankyrins are a family of proteins involved in the organization of specific membrane domains by mediating the interactions between proteins of the plasma membrane and the subplasma membrane cytoskeleton[1]. Vertebrate ankyrin polypeptides fall into three classes, each containing multiple alternatively spliced variants: ankyrins-R (R for restricted distribution, and the prototypic ankyrin first characterized in erythrocytes; also expressed in a subset of neurons and striated muscle) encoded by Ank1 on human chromosome 8p11; ankyrins-B (B for broadly expressed; first characterized in brain, but now recognized in most cell types) encoded by Ank2 on human chromosome 4q25-27; and ankyrins-G (G for giant size and general expression, first characterized as a 480 kDa polypeptide in the nervous system; expressed in most cell types) encoded by Ank3, on human chromosome 10q21. Canonical ankyrins are 190~220 kDa proteins expressed in most tissues and cell types and comprise a membrane-binding domain (MBD) of 24 ANK repeats, a spectrin-binding domain, a death domain and a C-terminal domain. Whereas death domains in other proteins may function in activation of NF-κB, caspase proteases and cell death, this domain has no known role within ankyrins (Fig. 1A).

Current views of ankyrin function are based on co-localization and biochemical interactions of ankyrin with other proteins. Ankyrin associates with a variety of membrane proteins including ion channels (Na^+/K^+ ATPase, H^+/K^+ ATPase, anion exchangers AE 1-3, voltage-sensitive Na^+ channels, Na^+/Ca^{2+} exchanger), calcium-release channels [ryanodine receptor, inositol (1,4,5)-trisphosphate receptor] cell adhesion molecules [CD44, L1CAMs (L1, NgCAM, neurofascin, LAD-1, NrCAM, neuroglian)], as well as cytoplasmic proteins, including clathrin and tubulin1). Many of these interactions are mediated by ANK repeats within

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†Corresponding author for proof and reprints: Chong-Rak Kim
Tel: +(82) 55-320-3215, Fax: +(82) 55-334-3426
e-mail: bioxgeny@inje.ac.kr
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the MBD, although the Na^+/K^+ ATPase and H^+/K^+ ATPase associate at least in part with the spectrin-binding domain. Ankyrin-$B^{-/-}$ cardiomyocytes display downregulation and mis-sorting of calcium-release channels [ryanodine and inositol (1,4,5)-trisphosphate receptors] within the endoplasmic reticulum in cardiomyocytes that can be rescued by transfection with cDNA encoding ankyrin-B. Both ankyrin-G and ankyrin-R are expressed in cardiomyoctes, but cannot compensate for loss of ankyrin-B. Rescue studies with ankyrin-B/G chimeras have identified the C-terminal domain of ankyrin-B as the defining domain in specifying ankyrin-B activity[13]. A working hypothesis to explain the cellular basis for these phenotypes is that ankyrins play roles as chaperones or guides that direct vesicle transport of a variety of ion channels to sites in the plasma membrane as well as the endoplasmic reticulum.

In response to elevated temperature, almost all organisms synthesize a certain set of proteins[10,16], which are collectively referred to as heat shock proteins (Hsps) due to the method of induction. Studies have revealed that many kinds of environmental stress, such as heavy metals, ethanol, amino acid analogues, anoxia and agents capable of perturbing protein structure, cause a similar response. Hsps are, thus, called stress proteins. Hsp40 is colocalized with Hsp70 in the nucleoli of heat-shocked mammalian cells[9,21]. In spite of drastic alteration of the localization of these Hsps upon heat shock, how and why these Hsps translocate into the nuclei and nucleoli are still unknown at present. These results strongly suggest that both Hsp70 and Hsp40 act together in protecting cellular functions from heat shock.

We report here that the C-terminal domain of ankyrin-B is capable of interacting with the C-terminal Region of Hsp40. The interaction between ankyrin and C-terminal Region of Hsp40 is mediated by C-terminal domain in ankyrin-B, but not in ankyrin-G. The binding sites required in the interaction between C-terminal domain of ankyrin-B and C-terminal Region of Hsp40 were characterized using the yeast two-hybrid system and GST-pull down assay. The interaction between ankyrin-B and Hsp40 represents the first direct evidence of ankyrin's role as chaperones.

MATERIALS AND METHODS

1. Yeast Two-Hybrid System

Yeast two-hybrid screenings were performed utilizing the Matchmaker 2 of Clontech Laboratories (Palo Alto, CA). The C-terminus of human 220 kD ankyrin-B (nt 4327-5517)[13] were amplified by PCR using appropriate primers containing EcoRI/BamHI and cloned into the bait vector pAS2-1. The plasmids were transfected by the lithium acetate method into the yeast reporter strain AH109 together with a cDNA library prepared from human heart (HL4042AH) in the prey vector pACT2. Selection for HIS3 reporter gene activation was performed on agar plates lacking adenine, histidine, tryptophan, and leucine. Colonies appearing after 5~10 days at 30℃ were assayed for β-galactosidase activity utilizing the colony-lift filter assay. The plasmid DNA of positive colonies was isolated with phenol and glass beads as suggested by the supplier of the system. Positive two-hybrid protein interactions were verified by transfection of the plasmids back into the reporter strain AH109 together with the original bait or with selected controls. The plasmids of positive colonies were amplified in Escherichia coli DH5α and sequenced by the dideoxy chain termination method.

2. Deletion Construction of Hsp40

For domain mapping, PCR amplification was used to obtain cDNAs encoding Hsp40. Human Spleen Marathon Ready cDNA (Clontech, Palo Alto, CA) was used as template and custom oligonucleotide primers were generated, based on the sequence of human Hsp40 sequence (Gen Bank™ accession number NM_006145). Additional sets of primers were used for amplification of Hsp40 deletion constructs (Fig. 1B). All sense primers carried an EcoRI recognition sequence, whereas all anti-sense primers contained an XhoI site for insertion into the yeast two-hybrid pACT2 prey vector (Clontech, Palo Alto, CA).

3. GST-pull Down Assay

The cDNA sequences for Hsp40 (residue 1-340) were subcloned into the EcoRI/XhoI restriction site of the expression vector pGEX-6P-1downstream of the gst gene and transfected into competent bacteria (E. coli DH5α). Fusion proteins were expressed after induction with isopropylthio-β-galactoside as suggested by the supplier of the gst gene fusion system (Amersham Pharmacia Biotech). The bacteria were collected by centrifugation and lysed by sonication. Fusion proteins were purified from the lysates by affinity chromatography on reduced glutathione Sepha-

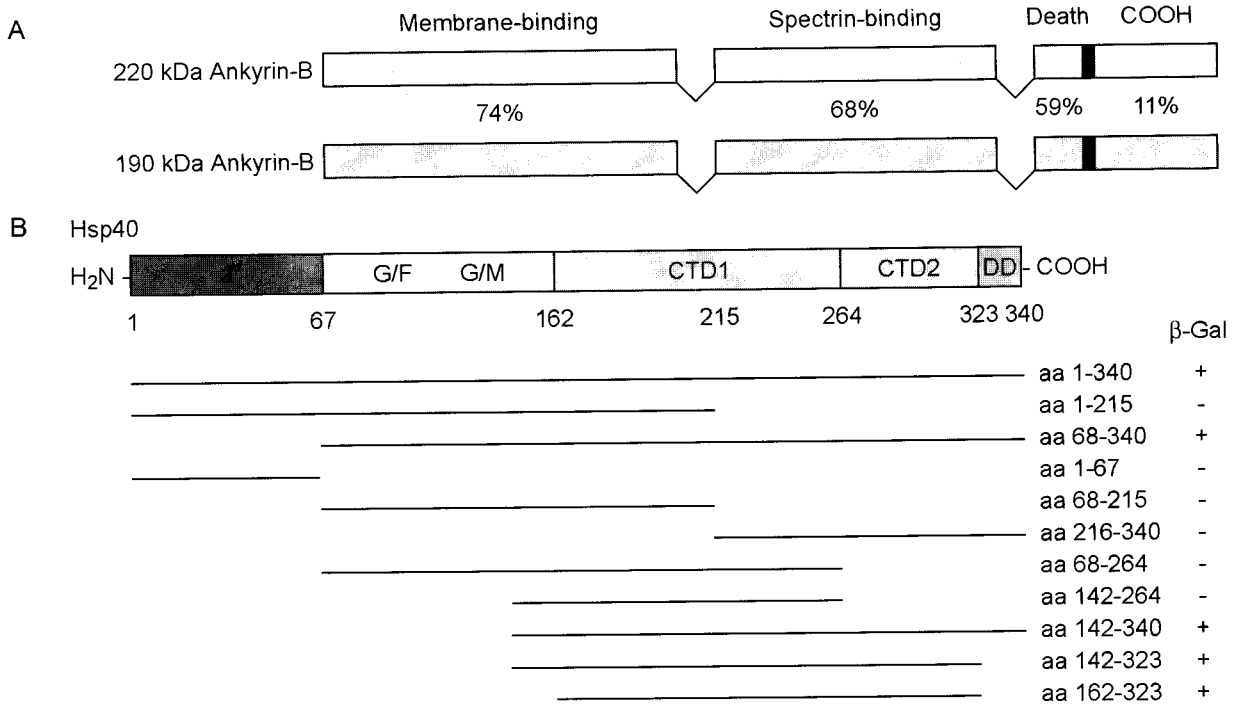

Fig. 1. Association of ankyrin B with the CTD region of Hsp40. **A**, shown schematically are major protein domains of 220-kDa ankyrin-B (top) and 190-kDa ankyrin-G (bottom). Scores represent percent amino acid identity between ankyrin-G and ankyrin-B within the corresponding regions. **B**, schematic representation of the Hsp40 domain structure. The subdomains of Hsp40 are labeled as follows: J, J-domain; G/F, Gly/Phe-rich region; G/M, Gly/Met-rich region; CTD1 and CTD2, carboxyl-terminal domains 1 and 2; DD, dimerization domain. Fragments of the Hsp40 clone were tested for ability to interact with the bait ankyrin-B COOH-terminal region (nt 4327-5517) in the two-hybrid system. The experiment demonstrated that CTD1 and CTD2 of Hsp40 is capable to interact with ankyrin-B COOH-termius. +, detectable activity; −, no detectable activity.

Table 1. Hsp40 clones interacting with ankyrin-B

GenBank number	Start region (aa)	Total clones	Independent clones
NM_006145	J domain (1-67)	13	8
NM_006145	G/F G/M (68-161)	19	7

rose and analyzed on 3.5~17.5% SDS-polyacrylamide gels. The cell lysates (PBS, 20 mM EDTA, 2.5% Triton X-100) were incubated for 20 min at 4℃ with the resin to immobilize the fusion proteins; glutathione Sepharose 4B beads (Amersham Biosciences) for GST. For binding experiments, 50 μg of radioiodinated COOH-terminus fragment of ankyrin B was incubated with 50 μg of fusion protein (50 μl of beads, 50% slurry) in interaction buffer (50 mM NaCl, 1 mM EDTA, 1 mM NaN_3, 0.1% Triton X-100, 0.5% BSA, 20 mM HEPES, pH 7.2) for 1 h at 4℃. Beads were washed in PBS, 1% Triton X-100. Proteins eluted in SDS, β-mercaptoethanol sample buffer were separated by SDS-PAGE.

RESULTS

1. Interaction of ankyrin-B with a COOH-terminal fragment of Hsp40 in two-hybrid experiments

To identify potential interaction partners for the ankyrin-B, a human heart cDNA library was screened using the yeast two-hybrid technique and the C-terminal fragment of the ankyrin-B cDNA as bait (Fig. 1A). Screening of a total of 4×10^6 clones resulted in the identification of 32 positive colonies (Table 1). When their ability to interact with the C-terminal fragment of the ankyrin-G cDNA as bait were tested in a yeast two-hybrid assay, no specific interaction was observed. DNA sequencing revealed that all clones corresponded to overlapping fragments of type II Hsp40 (dnajb1). Members of the Hsp40/DnaJ family (from bacteria to human) have three distinct domains: (1) a highly conserved J domain of approximately 70 amino acids in size, often found near the N-terminus, which has been shown to mediate interaction with Hsp70 and regulate its

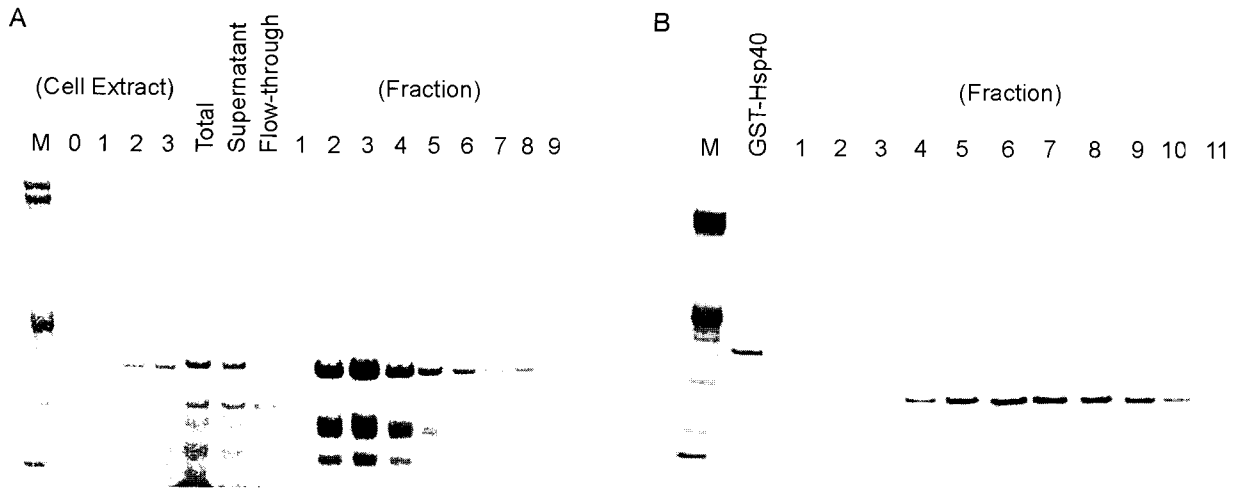

Fig. 2. Purification of GST-Hsp40 fusion protein. **A**, SDS-PAGE analysis of various sample processing steps. M, red blood cell ghost as a molecular weight marker. Cells were induced to overexpress GST-Hsp40 fusion protein with 1 mM IPTG at 24℃ for 0, 1, 2 and 3 h. The expected molecular weight of the fusion protein was about 62 kDa. The cell lysates (Total) were mixed with glutathione Sepharose 4B beads. After incubation at room temperature overnight, unbound proteins (Supernatant) were removed. The beads were transferred to the disposable column and washed with PBS (Flow-through). GST-Hsp40 were eluted from the glutathione Sepharose 4B column with 15 mM reduced glutathione in 50 mM Tris pH 8.0 (Fraction 1~9). **B**, Hsp40 was obtained by cleavage of GST-Hsp40 fusion protein with 8U PreScission protease (Amersham Bioscience). After cleavage of the GST fusion proteins binding to the glutathione Sepharose 4B beads with PreScission protease, the Hsp40 protein was eluted from the glutathione Sepharose 4B column with elution buffer. The eluates were then applied to the glutathione Sepharose 4B column to remove contaminated GST and PreScission protease, which was a GST fusion protein (Fraction 1~11). The expected molecular weight of Hsp40 was about 38 kDa.

Fig. 3. Verification of the two-hybrid protein interaction by GST-pull down assay. After binding to glutathione matrix, recombinant GST-Hsp40, but not GST, adsorbed radioiodinated COOH-terminus fragment of ankyrin-B.

ATPase activity; (2) a glycine and phenylalanine (G/F)-rich region possibly acting as a flexible linker; and (3) a cysteine-rich region (C domain) containing four CXXCXGXG motifs resembling a zinc-finger domain[2]. Recently, Cheetham and Caplan[4] proposed a classification of Hsp40/DnaJ homologues consisting of three groups; Type I homologues have all three domains (J, G/F and C), Type II have the J and G/F but not the C domain, and Type III have the J domain alone. According to this classification, mammalian Hsp40 is a Type II homologue (Fig. 1B). Because the shortest clones can interact with ankyrin-B started at aa 160, we concluded that the interaction site was located in the COOH terminus of Hsp40, downstream of aa 160.

2. Identification of the ankyrin-B binding site in Hsp40

Two-hybrid experiments performed with fragments of the Hsp40 identified a minimal region of 162 aa (residues 162-323, CTD1 and CTD2) that was required for interaction with ankyrin-B (Fig. 1B). To conform the specificity of interaction between ankyrin-B and Hsp40, we performed a GST-pull down assay. We expressed Hsp40 as GST-fusion proteins (Fig. 2). The calculated molecular masses of the GST-fusion protein and PreScission protease-cleaved Hsp40 are ~62 kDa and ~38 kDa, respectively (Fig. 2). Equivalent amounts of these proteins and control GST (25 kDa) were bound to glutathione matrices, and incubated with radioiodinated COOH-terminus fragment of ankyrin. The matrix-bound GST fusion proteins were examined by autoradiograph for their ability to adsorb COOH-terminus fragment of ankyrin. Only GST-Hsp40 specifically retained

COOH-terminus fragment of ankyrin (Fig. 3).

DISCUSSION

Hsp40s represent a structurally-diverse family of co-chaperones that function with Hsp70 to facilitate cellular processes that include protein folding, the suppression of protein aggregation, endocytosis, protein translocation across membranes, signal transduction, DNA replication, protein degradation and prion propagation[5,8,18]. Hsp70 facilitates these processes by utilizing energy derived from ATP hydrolysis to bind and release regions of proteins that exhibit aspects of non-native structure[3,12,14]. Hsp40s function by regulating the Hsp70 ATP hydrolytic cycle[6] and by acting as molecular chaperones which bind and target non-native proteins to the peptide-binding site of Hsp70[20]. To regulate Hsp70 ATPase activity Hsp40 proteins utilize a conserved region, which was identified in E. coli DnaJ, termed the J-domain[7,23]. Hsp40s contain the J-domain, G/F rich region and the CTD and G/M rich region[4]. Biochemical and genetic studies suggest that the G/F region and portions of the conserved carboxyl-terminus enable Hsp40s to function as chaperones[11,18,22]. How Hsp40s function as molecular chaperones to bind and deliver non-native proteins to Hsp70 is not well established[15]. Insight into the nature of the Hsp40 peptide-binding site was provided by the crystal structure of Sis1, an yeast Type II Hsp40 protein, which reveals that CTD of Sis1 forms a crystallographic homodimer that has a wishbone like structure[17]. Sis1 monomers have an elongated shape and contain two barrel-like domains, CTDI and CTDII, and a C-terminal dimerization motif[17].

Here we report that ankyrin-B contains a specific domain capable to selectively interact with the COOH-terminal part of Hsp40. Both CTDI and CTDII of Hsp40 are required for binding activity. Deletion of the dimerization domain (DD) of Hsp40 can still bind to ankyrin-B (Fig. 1B). Thus, Hsp 40 can carry out its essential functions as a monomer, and contrary to a previous suggestion[15], the dimerization domain is not likely to play a direct role in polypeptide binding. These data therefore provide the first experimental evidence of two proteins capable of playing roles as chaperones or guides that direct vesicle transport of a variety of ion channels to sites in the plasma membrane as well as the endoplasmic reticulum. Considering the complexity of the transport of the ion channels, several other proteins are likely to participate in mediating the subcellular localization. A role for ankyrin-B in mediating the assembly of ryanodine and $InsP_3$ receptors at specific domains of the sarcoplasmic reticulum of striated muscle cells has been reported[19]. The region of the ankyrin-B protein necessary for the localization of calcium release channels in the sarcoplasmic reticulum has been restricted to a small region at the COOH terminus of ankyrin-B[13]. On this basis, we propose that the interaction of Hsp40 with ankyrin-B may contributes to the assembly of a large protein complex, which may participate in the mechanisms underlying the localization of Ca^{2+} release channels (i.e., ryanodine and $InsP_3$ receptors). Obviously more work is required for better defining the complexity of these interactions.

REFERENCES

1) Bennett V and Baines AJ (2001): Spectrin and ankyrin-based pathways: metazoan inventions for integrating cells into tissues. *Physiol Rev*, **81:** 1353-1392.
2) Bork P, Sander C, Valencia A and Bukau B (1992): A module of the DnaJ heat shock proteins found in malaria parasites. *Trends Biochem Sci*, **17:** 129.
3) Bukau B and Horwich AL (1998): The Hsp70 and Hsp60 chaperone machines. *Cell*, **92:** 351-366.
4) Cheetham ME and Caplan AJ (1998): Structure, function and evolution of DnaJ: conservation and adaptation of chaperone function. *Cell Stress Chap*, **3:** 28-36.
5) Cyr DM, Langer T and Douglas MG (1994): DnaJ-like proteins: molecular chaperones and specific regulators of Hsp70. *Trends Biochem Sci*, **19:** 176-181.
6) Cyr DM, Lu X and Douglas MG (1992): Regulation of Hsp70 function by a eukaryotic DnaJ homolog. *J Biol Chem*, **267:** 20927-20931.
7) Georgopoulos CP, Lundquist-Heil A, Yochem J and Feiss M (1980): Identification of the E. coli dnaJ gene product. *J Mol Gen Genet*, **178:** 583-588.
8) Hartl FU (1996): Molecular chaperones in cellular protein folding. *Nature*, **381:** 571-579.
9) Hattori H, Kaneda T, Lokeshwar B, Laszlo A and Ohtsuka K (1993): A stress-inducible 40 kDa protein (hsp40): purification by modified two-dimensional gel electrophoresis and co-localization with hsc70 (p73) in heat shocked HeLa cells. *J Cell Sci*, **104:** 629-638.
10) Lindquist S (1986): The heat-shock response. *Annu Rev Bio-*

chem, **55:** 1151-1191.

11) Lu Z and Cyr DM (1998): Protein folding activity of Hsp70 is modified differentially by the Hsp40 co-chaperones Sis1 and Ydj1. *J Biol Chem*, **273:** 27824-27830.

12) Mayer MP, Schroder H, Rudiger S, Paal K, Laufen T and Bukau B (2000): Multistep mechanism of substrate binding determines chaperone activity of Hsp70. *Nat Struct Biol*, **7:** 586-593.

13) Mohler PJ, Gramolini AO and Bennett V (2002): The ankyrin-B C-terminal domain determines activity of ankyrin-B/G chimeras in rescue of abnormal inositol 1,4,5-trisphosphate-and ryanodine receptor distribution in ankyrin-B (-/-) neonatal cardiomyocytes. *J Biol Chem*, **277(12):** 10599-10607.

14) Palleros DR, Reid KL, Shi L, Welch WJ and Fink AL (1993): ATP-induced protein-Hsp70 complex dissociation requires K+ but not ATP hydrolysis. *Nature*, **365:** 664-666.

15) Rudiger S, Schneider-Mergener J and Bukau B (2001): Its substrate specificity characterizes the DnaJ co-chaperone as a scanning factor for the DnaK chaperone. *EMBO J*, **20(5):** 1042-1050.

16) Schlesinger MJ, Ashburner M and Tissieres A (eds) (1982): Heat Shock from Bacteria to Man, (New York: Cold Spring Harbor Laboratory Press), pp. 1-440.

17) Sha B, Lee S and Cyr DM (2000): The crystal structure of the peptide-binding fragment from the yeast Hsp40 protein Sis1. *Structure Fold Des*, **8(8):** 799-807.

18) Sondheimer N, Lopez N, Craig EA and Lindquist S (2001): The role of Sis1 in the maintenance of the [RNQ+] prion. *Embo J*, **20:** 2435-2442.

19) Tuvia S, Buhusi M, Davis L, Reedy M and Bennett V (1999): Ankyrin-B is required for intracellular sorting of structurally diverse Ca^{2+} homeostasis proteins. *J Cell Biol*, **147(5):** 995-1008.

20) Wickner S, Hoskins J and McKenney K (1991): Monomerization of RepA dimers by heat shock proteins activates binding to DNA replication origin. *Proc Natl Acad Sci USA*, **88:** 7903-7907.

21) Yamane M, Hattori H, Sugito K, Hayashi Y, Tohnai I, Ueda M, Nishizawa K and Ohtsuka K (1995): Cotranslocation and colocalization of hsp40 (DnaJ) with hsp70 (DnaK) in mammalian cells. *Cell Struct Funct*, **20:** 157-166.

22) Yan W and Craig EA (1999): The glycine-phenylalanine-rich region determines the specificity of the yeast Hsp40 Sis1. *Mol Cell Biol*, **19:** 7751-7758.

23) Zylicz M, Yamamoto T, McKittrick N, Sell S and Georgopoulos C (1985): Purification and properties of the dnaJ replication protein of Escherichia coli. *J Biol Chem*, **260:** 7591-7598.