FEBS Letters 565 (2004) 28–32 FEBS 28309

Hsp25, a member of the Hsp30 family, promotes inclusion formation in response to stress ☆

Yumiko Katoh, Mitsuaki Fujimoto, Kosuke Nakamura, Sachiye Inouye, Kazuma Sugahara, Hanae Izu, Akira Nakai*

Department of Biochemistry and Molecular Biology, Yamaguchi University School of Medicine, Minami-Kogushi 1-1-1, Ube 775-8505, Japan

Received 17 December 2003; accepted 23 December 2003

First published online 10 April 2004

Edited by Jesus Avila

Abstract Protein aggregates are oligomeric complexes of misfolded proteins, and serve as the seeds of inclusion bodies termed aggresomes in the cells. Heat shock proteins (Hsps) prevent misfolding and aggregate formation. Here, we found that only avian Hsp25 dominantly accumulated in the aggresomes induced by proteasome inhibition. Molecular cloning of chicken Hsp25 (cHsp25) revealed that it belongs to the Hsp30 family, which is a subfamily of the α-crystallin/small Hsp gene family. Unexpectedly, overexpression of cHsp25 into HeLa cells promoted inclusion formation whereas overexpression of mouse Hsp27 and its chicken homologue did not. These results suggest that cHsp25 acts differently from other small Hsps on protein aggregates.

Keywords: Aggresome; Molecular chaperone; Crystallin; Heat shock protein; Ubiquitin

1. Introduction

Aggresomes are microtubule-dependent cytoplasmic inclusion bodies that arise from aggregates of misfolded proteins [1]. Folding of newly synthesized proteins is facilitated by molecular chaperones, but substantial amounts of proteins are misfolded and degraded shortly after their synthesis. Therefore, the blockade of protein degradation pathways such as the ubiquitin-proteasome pathway facilitates the formation of aggresomes [2]. Protein aggregation is also facilitated by various stresses such as heat shock and alterations in the primary structures caused by mutation. Accumulation of the protein aggregates is in most cases associated with many diseases such as neurodegenerative diseases.

Heat shock proteins (Hsps) are associated with misfolded proteins and prevent aggregate formation [2–4]. Here, we examined which Hsps are associated with aggresomes caused by proteasome-inhibition in avian cells. We found that chicken Hsp25 (cHsp25) dominantly accumulates in the aggresomes. cHsp25 is distinguished from cHsp24, a chicken homologue of human Hsp27 [5], and forms perinuclear large aggregates under conditions of heat shock [6,7]. Molecular cloning of cHsp25 revealed that cHsp25 belongs to the Hsp30 subfamily among the α-crystallin/small Hsp superfamily. Mammalian cells lack any homologue of cHsp25 [5]. Unexpectedly, overexpression of cHsp25 in HeLa cells accelerated inclusion formation.

2. Materials and methods

2.1. Cell culture

Quail fibroblast QT6 cells were maintained at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum, 4% chicken serum. Chicken embryo fibroblasts (CEF), chicken B lymphocyte DT40 cells, and human HeLa cells were maintained in DMEM containing 10% fetal bovine serum at 37 °C in 5% CO_2.

2.2. Immunostaining

QT6 cells were fixed with 4% paraformaldehyde for 15 min at room temperature, and washed with phosphate-buffered saline (PBS). After soaking in 0.2% Nonidet P-40/PBS for 15 min, the cells were blocked with 5% dried milk/PBS for 1 h, and then treated with rabbit antiserum against cHsp90 (1:100 dilution of αcHsp90a) [8], cHsp70 (1:100 dilution of αcHsp70a) [8], cHsp24 (1:100 dilution of αcHsp24a) [5], cHsp25 (1:100 dilution of αcHsp25U) [5], rat Hsp27 (1:100 dilution) [9], or mouse monoclonal antibodies against ubiquitin (1:100 dilution of sc-8017, Santa Cruz) and γ-tubulin (1:200 dilution of T6557, Sigma) at room temperature for 1 h. After washing with PBS, the cells were treated with FITC-conjugated goat anti-rabbit IgG (1:1000 dilution, Cappel) or Alexa Fluor 568-conjugated anti-mouse IgG (1:1000 dilution, Nacalai Tesque). After washing, the cells were mounted in VECTASHIELD mounting medium with DAPI (Vector Lab., CA), and visualized using fluorescence microscopy (Axioplan 2, Zeiss).

2.3. Generation and screening of chicken cDNA library

Total RNA was isolated from DT40 cells, which were heat-shocked at 45 °C for 1 h and allowed to recover for 5 h, using TRIZOL reagent (Invitrogen). An oligo (dT)-cellulose column (Molecular Research Center, Inc, OH) was used to isolate the poly A^+RNA fraction from the total RNA. The ZAP-cDNA library was generated using the ZAP-cDNA synthesis kit (Stratagene) according to the instruction manual. Approximately 1×10^5 plaques were screened using antiserum against cHsp25, αcHsp25U (1:500 dilution in 2% milk/PBS) [5] as a first antibody and peroxidase-conjugated anti-rabbit antibody (1:1000 dilution) (Cappel) as a second antibody, and signals were detected using the ECL system (Amersham Pharmacia). Two clones were isolated and converted into pBluescript vector by in vivo excision (plasmids pcHsp25-1 and pcHsp25-5, respectively). Sequencing reactions were performed using an AutoRead sequencing kit (Amersham Pharmacia) with synthetic oligonucleotides and Cy5-dATP labeling mix. Sequences were analyzed using an ALF express DNA sequencer (Amersham Pharmacia) and assembled with GENETYX-MAC.

☆ Nucleotide sequence data reported are available in the DDBJ database under the accession number AB154518.

* Corresponding author. Fax: +81-836-22-2315.
E-mail address: anakai@yamaguchi-u.ac.jp (A. Nakai).

0014-5793/$22.00
doi:10.1016/j.febslet.2003.12.085

2.4. Generation of cells expressing cHsp25, cHsp24, and mHsp27

cHsp25 expression vector, pcDNA3.1neo-cHsp25, was constructed by inserting a 0.9 kb *Eco*RI/*Xho*I fragment of pcHsp25 into the pcDNA3.1(+) vector (Invitrogen). The DNA was transfected into HeLa cells by a calcium-phosphate method. Four hours after transfection, the cells were treated for 2 min with 15% glycerol in HBS buffer (140 mM NaCl, 25 mM HEPES, and 1.4 mM Na_2HPO_4). Eighteen hours after transfection, the cells were incubated in medium containing 1.5 mg/ml of G418 disulfate (Nacalai Tesque, Kyoto, Japan). cHsp24 expression vector (pcDNA3.1neo-cHsp24) was generated by insertion of a 0.9 kb *Bam*HI/*Xho*I fragment of pGC27-5 [10] into pcDNA3.1(+) vector, and mHsp27 expression vector (pcDNA3.1neo-mHsp27) was generated by insertion of a 0.8 kb *Bam*HI/*Nhe*I fragment of pQE-mHsp27 into pcDNA3.1(+) vector.

2.5. Western blot analysis

HeLa cells expressing each Hsps were harvested, and cell extracts were prepared with NP-40 lysis buffer {150 mM NaCl, 1.0% Nonidet P-40,50 mM Tris (pH 8.0), 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin}. Western blot analysis was performed as described previously [8] using each specific antiserum.

3. Results

3.1. Avian Hsp25 dominantly accumulates in perinuclear inclusions

Perinuclear inclusions called aggresomes are generated in cells treated with proteasome inhibitors [2]. To examine which Hsps actually accumulate in inclusions, we performed immunostaining in quail fibroblast QT6 cells treated with a proteasome inhibitor lactacystin. After the treatment, any distinct structure of Hsp90, Hsp70, and Hsp24 was not observed (Fig. 1B–D and F–H). In marked contrast, Hsp25 localization was quite different. Hsp25 was hardly detected in control cells (Fig. 1A). Eight to twelve hours after the treatment of lactacystin, Hsp25 was induced and became to accumulate in the perinuclear regions (data not shown). Twenty-four hours after the treatment Hsp25 accumulated in a perinuclear inclusion in a cell, which was associated with a decrease in the diffuse cytoplasmic staining of Hsp25 (Fig. 1E). The inclusions were co-localized with γ-tubulin (Fig. 1I–K), and the inclusion formation was not observed in the presence of ndcodazole that disrupts microtubules (data not shown). These data indicate that Hsp25 is unique among major Hsps and dominantly accumulates in the aggresomes. To rule out the possibility of cell-type specificity, we examined the localization of Hsp25 and ubiquitin in CEF treated with lactacystin. We found that Hsp25 localized at the perinuclear inclusions in 20–30% of cells (Fig. 1M). Parts of the inclusions stained with Hsp25 were also stained with antibody for ubiquitin, the accumulation of which is also a hallmark of the aggresomes (Fig. 1M–O).

It is also known that heat shock causes protein aggregation at the centriole [11]. To examine the effect of heat shock on the localization of Hsps, QT6 cells were treated at 43 °C for 24 h. Remarkably, Hsp25 was stained as perinuclear inclusions in more than half of the cells after the heat shock (Fig. 1L). In contrast, Hsp90, Hsp70, and Hsp24 were induced, but did not accumulate in any specific inclusion within the cells (data not shown).

3.2. Hsp25 belongs to the Hsp30 subfamily

Previously, we distinguished cHsp24, a chicken homologue of human Hsp27 [12], from a chicken major heat-inducible protein cHsp25 [5]. To identify the avian Hsp25 at the molecular level, we isolated cDNA for cHsp25 by expression screening of a heat-shocked chicken B lymphocyte DT40 cDNA library using cHsp25 antiserum. From the two independently isolated cDNA clones, one clone (referred to as pcHsp25-1) contained an insert of 918 nucleotides with a single open reading frame encoding 172 amino acids. The predicted amino acids contained an α-crystallin domain, which is a hallmark of the α-crystallin/small Hsp superfamily (Fig. 2, underlined). The sequence comparison of cHsp25 revealed that it is highly related to Xenopus Hsp30C and Hsp30D [13], minnow Hsp30B [14], and salmon Hsp30 (unpublished, SP accession No. P42931), and is less related to cHsp24. The cHsp25 mRNA was heat-inducible in DT40 cells only in the presence of heat shock transcription factors, HSF3 and HSF1 [15]. These results indicate that cHsp25 belongs to the Hsp30 subfamily among the α-crystallin/small Hsp superfamily (Fig. 3).

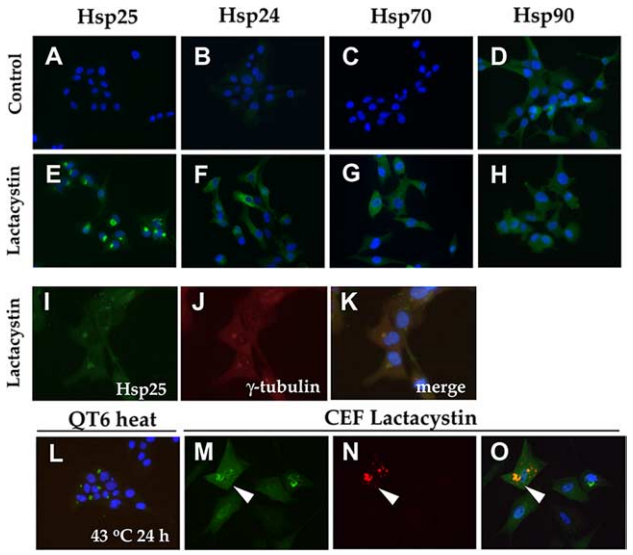

Fig. 1. cHsp25 accumulates in perinuclear inclusion bodies. QT6 cells treated with (E–H) or without (A–D) 10 μM lactacystin for 24 h were stained using each specific antiserum for cHsp25 (A, E), cHsp24 (B, F), cHsp70 (C, G), or cHsp90 (D, H). Signals of each Hsp (green) and DNA stained with DAPI (blue) were visualized using fluorescence microscopy (Axioplan 2, Zeiss). Lactacystin-treated QT6 cells were also stained with antibodies for cHsp25 (I) and γ-tubulin (J), and the signals were merged (K). Immunostaining of cHsp25 was further performed in heat-shocked (43 °C for 24 h) QT6 cells (L). CEF cells treated with 10 μM lactacystin for 36 h were stained with antibodies for cHsp25 (M) and ubiquitin (N), and the signals were merged (O). Arrowheads indicate the location of intense cHsp25 staining that is co-localized with the ubiquitin localization.

3.3. Ectopic expression of cHsp25 into human cells accelerates inclusion formation

Mammalian cells contain a member of the Hsp27 family, but lack any homologue of cHsp25 [5,14]. To further understand differential roles of cHsp25, we generated human HeLa cells stably expressing cHsp25, cHsp24, or mouse Hsp27 (mHsp27). When cells were treated with lactacystin (data not shown) and another proteasome inhibitor MG132 and were immunostained with antisera for cHsp24, or mHsp27, any cytoplasmic inclusion was not detectable (Fig. 4C–H). In marked contrast, perinuclear inclusions containing cHsp25 were clearly observed in cHsp25-expressing HeLa cells treated with MG132

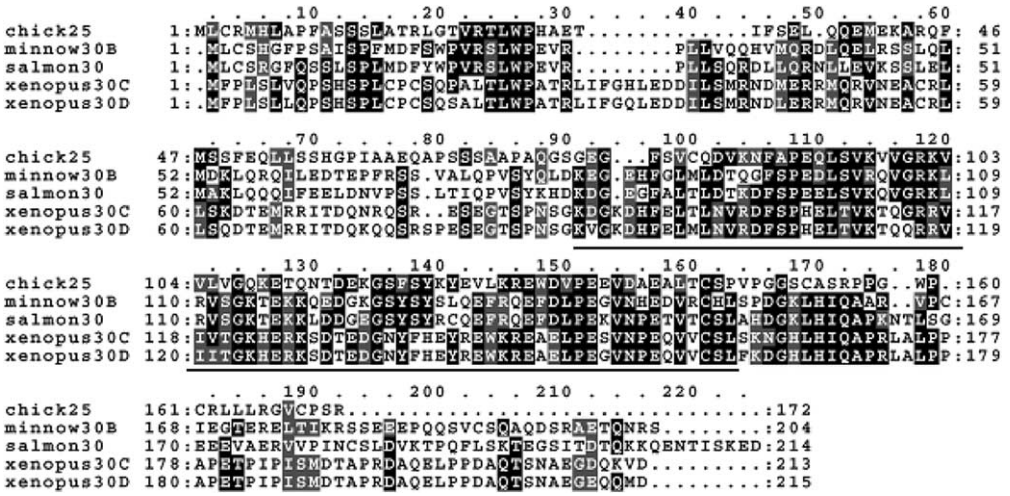

Fig. 2. Molecular cloning of complementary DNA for cHsp25. Comparison of predicted amino acid sequences. The sequence of cHsp25 is aligned with sequences of minnow Hsp30B [14], salmon Hsp30 (SP accession No. P42931, unpublished), and Xenopus Hsp30C and 30D [13]. CLUSTAL W [32] was used to generate an alignment of the Hsp30 family. Underlines indicate position of the crystallin domain that is highly conserved among the α-crystallin/small Hsp superfamily [16]. Identical residues are shadowed and similar residues are half shadowed.

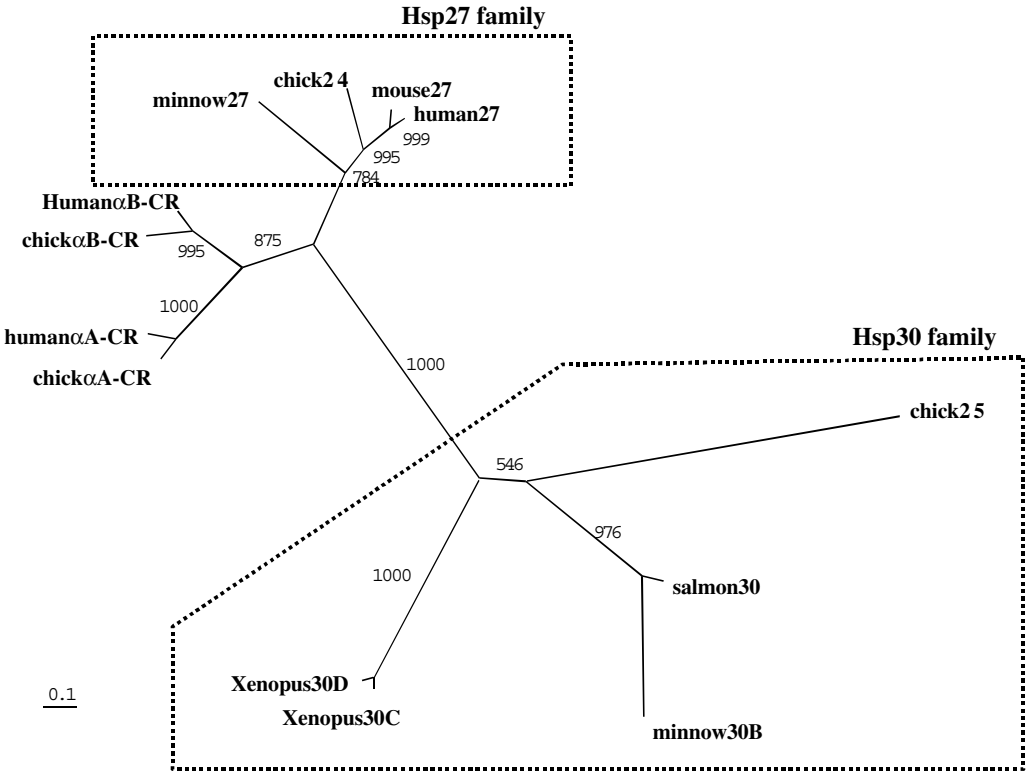

Fig. 3. The phylogenetic tree generated in CLUSTAL W for members of the α-crystallin/small Hsp superfamily. The molecular tree was constructed by the neighbor-joining method. Gaps were excluded from all phylogenetic analysis. The numerals showed bootstrap values (1000 bootstrap replicates were performed). The unrooted tree was drawn with the TREEVIEW program [33]. Bar represents 0.1 substitutions per site. The amino acid sequences used in the tree construction are Hsp27 from human (SP accession No. P04792), mouse (SP accession No. P14602) and minnow (DAD accession No. U85501), Hsp24 from chicken (SP accession No. Q00649), Hsp30 from salmon (SP accession No. P42931), Hsp30B from minnow (DDBJ accession No. U85502), Hsp30C and Hsp30D from Xenopus (SP accession No. P30218 and SP accession No. P30291, respectively), αA-crystallin from chicken (SP accession No. P02504) and human (SP accession No. P02489), and αB-crystallin from chicken (SP accession No. Q05713) and human (SP accession No. P02511).

(Fig. 4B) or lactacystin (data not shown). The inclusion containing of cHsp25 was not observed in cells just overexpressing cHsp25 (Fig. 4A), The levels of endogenous Hsp27 were similar even in the presence of MG132 in each cell line (Fig. 4I). These results indicate that cHsp25, but not Hsp27, accelerates inclusion formation in response to stress.

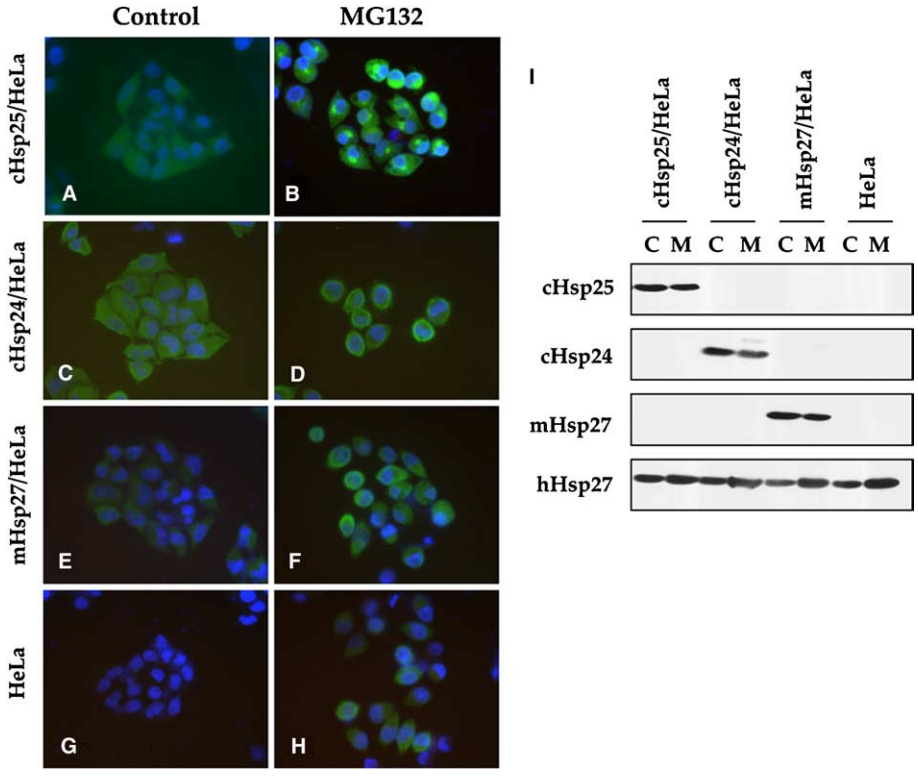

Fig. 4. cHsp25 accelerates inclusion formation. HeLa and cells stably expressing cHsp25, cHsp24, or mHsp27 were treated with 5 μM MG132 for 24 h. Immunostaining was performed using antiserum for cHsp25 (A, B), cHsp24 (C, D), or rat Hsp27 that recognized mouse and human Hsp27 (E–H). The signals were merged with DNA stained with DAPI. Cell extracts were prepared from HeLa cells expressing each Hsps with (M) or without (C) treating of MG132 for 24 h. Western blot analysis of each small Hsps was performed using antiserum specific for cHsp25, cHsp24, mHsp27, or hHsp27.

4. Discussion

The α-crystallin/small Hsp superfamily [16] consists of nine members in human: αA-crystallin, αB-crystallin, Hsp27 [17], p20 [18], HspB3 [19], MKBP/HSPB2 [20,21], cvHsp [22], HspB8 [23,24], and HspB9 [23]. The expression of human small Hsps are restricted to some specific tissues such as the heart/muscle or lens even in the presence of stresses, while cHsp25 is not expressed in the absence of stress, but is highly expressed after hyperthermia in all the tissues of developing embryos [5]. The expression profile of cHsp25 is similar to those of Xenopus Hsp30 [25] and of minnow Hsp30 [14]. Taken together with the relatedness of the amino acid sequences (Figs. 2 and 3), cHsp25 was revealed to be a member of the Hsp30 subfamily, which is lacking in mammalian cells [5,14]. Since members of the Hsp30 family were isolated in fish [14] and frog [13] as well as in avian, these may be oviparous animal-specific genes. These animals develop in eggs, where the temperature cannot be kept constant even in the avian, which is warm-blooded later after hatching. Therefore, developing embryos may be frequently exposed to stresses such as thermal stress. This may be why the Hsp30 family is restricted to oviparous animals.

Previous studies showed that aggresomes are enriched in Hsps including Hsp70, Hsp40, and Tric/TCP [2–4]. Because Hsps bind to misfolded proteins that form aggregates, it is not surprising that only a small fraction of Hsps co-localized with aggresomes. The major question is whether Hsps actively move to aggresomes or not. We showed that Hsp90, Hsp70, and Hsp24 showed diffuse cytoplasmic staining in avian cells treated with a proteasome inhibitor (Fig. 1). Therefore, these Hsps do not dominantly accumulate in the aggresomes. Among the members of small Hsps, αB-crystallin is known to be a component of abnormal inclusions within astrocytes in Alexander's disease [26]. However, small Hsps do not co-localize to aggresomes or polyglutamine aggregates [27,28]. This study firstly showed the co-localization of a small Hsp with the aggresomes. Why does cHsp25 specifically accumulate in aggresomes? cHsp25 may preferentially bind to non-native proteins and maintain them in a folding-competent state ready for refolding by the Hsp70 system like Hsp27 [29,30]. Small Hsps form large complexes of 200–800 kDa and the size dramatically increases at high temperatures (~1.3 MDa) [31]. Especially, cHsp25 tends to form larger structures [6,7], which may prevent the non-reversible interaction of misfolded proteins. As heat shock-induced aggresomes could be reversibly disappeared only 24 h after the cells were allowed to recover (data not shown), cHsp25 may facilitate the dissociation of the aggresomes.

Acknowledgements: We thank Dr. K. Kato and Y. Inaguma for providing antiserum and cDNA, and T. Nakamoto, R. Katsumoto, and T. Katoh for assistance. This work was supported in part by Grants-in-Aid for Scientific Research and on Priority Areas "Life of Protein" and "Cancer", from the Ministry of Education, Culture, Sports, Science and Technology, Japan, Naitoh Foundation, and Yamaguchi University Foundation.

References

[1] Kopito, R.R. (2000) Trends Cell. Biol. 10, 524–530.
[2] Johnston, J.A., Ward, C.L. and Kopito, R.R. (1998) J. Cell Biol. 143, 1883–1898.
[3] Wigley, W.C., Fabunmi, R.P., Lee, M.G., Marino, C.R., Muallem, S., DeMartino, G.N. and Thomas, P.J. (1999) J. Cell Biol. 145, 481–490.
[4] Gartia-Mata, R., Bebok, Z., Sorscher, E.J. and Sztul, E.S. (1999) J. Cell Biol. 146, 1239–1254.
[5] Kawazoe, Y., Tanabe, M. and Nakai, A. (1999) FEBS Lett. 455, 271–275.
[6] Collier, N.C. and Schlesinger, M.J. (1986) J. Cell Biol. 103, 1495–1507.
[7] Collier, N.C., Heuser, J., Levy, M.A. and Schlesinger, M.J. (1988) J. Cell Biol. 106, 1131–1139.
[8] Kawazoe, Y., Tanabe, M., Sasai, N., Nagata, K. and Nakai, A. (1999) Eur. J. Biochem. 265, 688–697.
[9] Kato, K., Ito, H., Kamei, K., Inaguma, Y., Iwamoto, I. and Saga, S. (1998) J. Biol. Chem. 273, 28346–28354.
[10] Tanabe, M., Kawazoe, Y., Takeda, S., Morimoto, R.I., Nagata, K. and Nakai, A. (1998) EMBO J. 17, 1750–1758.
[11] Vidair, C.A., Huang, R.N. and Doxsey, S.J. (1996) Int. J. Hyperthermia 12, 681–695.
[12] Miron, T., Vancompernolle, K., Vandekerckhove, J., Wilchek, M. and Geiger, B. (1991) J. Cell Biol. 114, 255–261.
[13] Krone, P.H., Snow, A., Ali, A., Pasternak, J.J. and Heikkila, J.J. (1992) Gene 110, 159–166.
[14] Norris, C.E., Brown, M.A., Hickey, E., Weber, L.A. and Hightower, L.E. (1997) Mol. Biol. Evol. 14, 1050–1061.
[15] Inouye, S., Katsuki, K., Izu, H., Fujimoto, M., Sugahara, K., Yamada, S., Shinkai, Y., Oka, Y., Katoh, Y. and Nakai, A. (2003) Mol. Cell. Biol. 23, 5882–5895.
[16] Ingolia, T.D. and Craig, E.A. (1982) Proc. Natl. Acad. Sci. USA 79, 2360–2364.
[17] Hickey, E., Brandon, S.E., Sadis, S., Smale, G. and Weber, L.A. (1986) Gene 43, 147–154.
[18] Inaguma, Y., Hasegawa, K., Kato, K. and Nishida, Y. (1996) Gene 178, 145–150.
[19] Boelens, W.C., Van Boekel, M.A. and De Jong, W.W. (1998) Biochim. Biophys. Acta 1388, 513–516.
[20] Iwaki, A., Nagano, T., Nakagawa, M., Iwaki, T. and Fukumaki, Y. (1997) Genomics 45, 386–394.
[21] Suzuki, A., Sugiyama, Y., Hayashi, Y., Nyu-I, N., Yoshida, M., Nonaka, I., Ishiura, S., Arahata, K. and Ohno, S. (1998) J. Cell Biol. 140, 1113–1124.
[22] Krief, S., Faivre, J.F., Robert, P., Le Douarin, B., Brument-Larignon, N., Lefrere, I., Bouzyk, M.M., Anderson, K.M., Greller, L.D., Tobin, F.L., Souchet, M. and Bril, A. (1999) J. Biol. Chem. 274, 36592–36600.
[23] Kappe, G., Verschuure, P., Philipsen, R.L., Staalduinen, A.A., Van de Boogaart, P., Boelens, W.C. and De Jong, W.W. (2001) Biochim. Biophys. Acta 1520, 1–6.
[24] Benndorf, R., Sun, X., Gilmont, R.R., Biederman, K.J., Molloy, M.P., Goodmurphy, C.W., Cheng, H., Andrews, P.C. and Welsh, M.J. (2001) J. Biol. Chem. 276, 26753–26761.
[25] Lang, L., Miskovic, D., Fernando, P. and Heikkila, J.J. (1999) Dev. Genet. 25, 365–374.
[26] Iwaki, T., Kume-Iwaki, A., Liem, R.K. and Goldman, J.E. (1989) Cell 57, 71–78.
[27] Chai, Y., Koppenhafer, S.L., Bonini, N.M. and Paulson, H.L. (1999) J. Neurosci. 19, 10338–10347.
[28] Jana, N.R., Tanaka, M., Wang, G. and Nukina, N. (2000) Mol. Genet. 9, 2009–2018.
[29] Lee, G.J., Roseman, A.M., Saibil, H.R. and Vierling, E. (1997) EMBO J. 16, 659–671.
[30] Ehrnsperger, M., Graber, S., Gaestel, M. and Buchner, J. (1997) EMBO J. 16, 221–229.
[31] Ehrnsperger, M., Lilie, H., Gaestel, M. and Buchner, J. (1999) J. Biol. Chem. 274, 14867–14874.
[32] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) Nucl. Acids Res. 22, 4673–4680.
[33] Page, R.D. (1996) Comput. Appl. Biosci. 12, 357–358.