Presence of both Constitutive and Inducible Forms of Heat Shock Protein 70 in the Cerebral Cortex and Hippocampal Synapses

Heat shock proteins serve as molecular chaperones in a protein 'holding and folding' system. Protein sequencing, extraction and immunoblot analyses indicate that Hsc70, a constitutive form, is a major component of the rat postsynaptic density (PSD) fraction, while Hsp70, an inducible form, is present at the basal level. Immunohistochemical studies show that expression of Hsc70 is high, but that of Hsp70 is low, in the cerebral cortex and hippocampal formation. In dissociated hippocampal neurons, both Hsp70 and Hsc70 immunoreactivities are distributed throughout the soma and dendrites. In dendrites, there are many stained puncta which are mostly co-localized with PSD-95, a postsynaptic marker. Interestingly, variation in staining intensity of the puncta is significantly larger for Hsp70 than for Hsc70 in 2-week-old cultures, but becomes less significant in 5-week-old cultures. At the electron microscopic level, both Hsp70 and Hsc70 are mainly associated with asymmetrical PSDs. However, Hsc70 is also associated with amorphous subsynaptic structures and spine apparatus-like cisternae. Our data indicate that both Hsp70 and Hsc70 are present in PSDs but are differentially distributed at subsynaptic sites, and provide a potential candidate system for a 'synaptic tag'.

Materials and Methods

Subcellular Fractionation

The One-Triton PSD fraction (Cho et al., 1992) was prepared from adult rat (Sprague-Dawley) forebrains by washing synaptosome-enriched fraction with 0.5% Triton X-100 as described previously (Carlin et al., 1980; Cho et al., 1992). The One-Triton PSD fraction was resuspended and incubated for 15 min in ice-cold detergents at the indicated concentrations. The pellet and supernatant were separated by centrifugation at 201800 g for 1 h, and the pellets were resuspended in 40 mM Tris–HCl (pH 8.0).

Purification and Sequencing of Internal Peptides of n-Octyl Glucoside-soluble and -insoluble PSD-72

The One-Triton PSD fractions were treated with 1% n-octyl glucoside (OG) (4°C, 15 min), and soluble and insoluble proteins were separated by centrifugation at 200 000 g for 30 min at 4°C. About 7 mg (∼10 nmol) of the soluble fraction was concentrated in a Speed-Vac concentrator and fractionated on seven preparative 6% SDS–polyacrylamide gels. The 72 kDa proteins were electroeluted as described previously (Moon et al., 1994) and ∼500 µg of the protein was electrophoresed in a 6% SDS gel (50 µg/1.2 mm thick and 10 mm wide well). The protein band was

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visualized by Coomassie R-250 staining, cut into small pieces (~3 × 4 mm) and fragmented by cyanogen bromide (CNBr, 200 mg/mL in 70% formic acid) as described previously (Sokolov et al., 1989). The CNBr-cleaved peptides were separated on a 12.5–17.5% gradient Tricine–SDS gel (Schagger and von Jagow, 1987) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The peptide bands were visualized by staining with Coomassie R-250, cut and sequenced. The 72 kDa protein, which remains insoluble in 1% OG, was isolated similarly as above by SDS–polyacrylamide gel electrophoresis (PAGE) and electroelution. About 140 μg (~2 nmol) of the protein was electrophoresed in a 6% SDS gel, transferred to nitrocellulose membrane (NC) and digested with trypsin as described previously (Cho et al., 1992). Tryptic peptide sequences were determined by Edman degradation in the Caltech Protein and Peptide Microanalysis Laboratory.

**Immunoblot**

After SDS–PAGE, proteins were transferred to NC, which was blocked in 5% non-fat dry milk in 0.2% Tween-20, 10 mM Tris–HCl, pH 7.5 and 0.2 M NaCl. Blots were incubated with primary antibodies [rat anti-Hsc70 monoclonal antibody (MAb), 1:2000 (StressGen Biotechnologies Corp., BC, Canada); mouse anti-Hsp70 MAb, 1:2000 (StressGen); mouse anti-Hsp70/Hsc70 MAb, 1:2000 (Boehringer Mannheim); rabbit anti-NR2B polyclonal antibody (PAb), 1:5000 (Moon et al., 1999)] for 2 h at room temperature. Blots were rinsed in TTBS four times (20 min each) and incubated with alkaline phosphatase-conjugated secondary antibodies (Boehringer Mannheim) for 2 h. The anti-Hsc70 blot was incubated with biotinylated anti-rabbit immunoglobulin G (IgG; StressGen) at 2 h, rinsed with TTBS, then incubated with alkaline phosphatase-conjugated streptavidin. Blots were developed according to the supplier’s instructions. For quantification, blots were scanned and the signal strengths were measured with the NIH Scion Image Beta 5.0 software. The solubility of each protein was expressed as mean ± SD.

**Immunocytochemistry of Dissociated Hippocampal Cultures**

Cultures of embryonic day 18 rat hippocampal neurons were grown as described previously (Brewer et al., 1993). After 2–5 weeks, cultures were fixed (Kornau et al., 1995) and double-labeled with anti-Hsc70 (rat monoclonal SPA-815, StressGen) at 1:500 or anti-Hsp70 (mouse monoclonal SPA-810, StressGen) at 1:50 (Milarski et al., 1989) and 10 μg/ml affinity-purified anti-PDI-95 (rabbit PAb) (Apperson et al., 1996; Kornau et al., 1995). After three washes in the preblock solution (Apperson et al., 1996), coverslips for the Hsc70/PSD-95 double-label were incubated with biotinylated anti-rat IgG (Vector, 1:100) and Cy3-conjugated streptavidin. Blots were developed according to the supplier’s instructions. For quantification, blots were scanned and the signal strengths were measured with the NIH Scion Image Beta 5.0 software. The solubility of each protein was expressed as mean ± SD.

**Identification of HSP70 (Hsp70 and Hsc70) in the PSD Fraction by Protein Sequencing and Immunoblot Analyses**

The One-Triton PSD fraction was isolated from rat forebrains and fractionated into 1% OG-soluble and -insoluble fractions (Moon et al., 1994). Densitometric analyses indicated that the PSD-72 protein band (Fig. 1, PSD-72) represented ~1% of the total One-Triton PSD fraction (data not shown). The 72 kDa proteins in each fraction were electroeluted separately. Tryptic peptides of OG-insoluble PSD-72 were purified through HPLC. One large HPLC peak was successfully sequenced, producing mixed sequences. However, the major (peptide 1) and a minor (peptide 2) sequences were unambiguously identified by the size of the signals. The OG-soluble PSD-72 was fragmented by CNBr and two of the fragments were successfully sequenced (peptide sequences 3 and 4 in Fig. 1A). All four amino acid sequences were found in those of Hsc70 (Sorger and Pelham, 1987) and Hsp70 (Longo et al., 1993) (Table 1). However, the amino acid sequences were aligned better with Hsc70 (Table 1). Identification of both of the OG-soluble and insoluble PSD-72 as members of the Hsp70 family by protein sequencing revealed previous reports that Hsp70, an inducible form, is also expressed in the brain at the basal level prompted us to investigate whether both constitutive and inducible forms of HSP70 are present in the PSD fraction. An antibody specific for Hsc70 recognized two protein bands (Fig. 1B, arrowheads): one relatively strong band at ~72 kDa, and a weak one at ~75 kDa. The molecular size (72 kDa) of the lower band corresponded to the sequenced protein (Fig. 1B, α-Hsc70, lower arrowhead). Densitometric analyses showed that the immunoblot signal strength of the upper band (75 kDa) is 11.2 ± 1.7% (n = 5) that of the lower one (72 kDa). The molecular size (75 kDa) of the upper band in the immunoblots corresponded well with that of the band seen just above the PSD-72 in Coomassie stain (Fig. 1, arrowhead). However, the amount of protein in the 75 kDa band, deduced from densitometric analyses of the Coomassie stain, was 58.8 ± 2.0% (n = 3) that of the PSD-72, indicating that the protein recognized by the Hsc70 antibody in the 75 kDa band represents only a small part of the band. When an antibody specific for Hsp70 was used to probe a similar blot of PSD proteins, a single band appeared (Fig. 1B, upper arrowhead)
Figure 1. Identification of HSP70 in the PSD fraction. (A) Protein composition of the One-Triton PSD fraction and peptide sequences of PSD-72. The One-Triton PSD fraction was prepared by washing synaptosomes of rat forebrains, as described in Materials and Methods, and separated on a 6% SDS gel. Proteins were stained with Coomassie blue R-250. Peptide sequences derived from PSD-72 are shown on the right. Peptide sequences 1 and 2 were derived from tryptic digestion of, and 3 and 4 were from CNBr cleavage of, the PSD-72. The ∼75 kDa protein band which may correspond to the upper band of the immunoblot signal for Hsc70 in panel B is marked with an arrowhead. Some of the known proteins (bars) in the PSD fraction are identified. NR2B and α-CaMKII refer to the NMDA receptor subunit 2B and α isoform of the types II calcium/calmodulin-dependent protein kinase, respectively. (B) Immunoblots. Aliquots (40 µg) of One-Triton PSD fractions were fractionated on a 6% SDS gel, transferred to NC and immunoblotted with either anti-Hsc70 (α-Hsc70) or anti-Hsp70 (α-Hsp70) MAb. The signals for Hsc70 at the 72 and 75 kDa positions are marked with arrowheads in the Hsc70 immunoblot (α-Hsc70), and the signal for Hsp70 in the Hsp70 immunoblot is marked with an arrow (α-Hsp70). The positions of molecular weight markers are indicated on the left of each panel in kilodaltons.

α-Hsp70, arrow) at the 72 kDa position. The signal, however, was weaker than that of Hsc70 (usually the Hsc70 signals were visualized in 5–10 min, while that of Hsp70 appeared in 1–2 h), indicating that the amount of Hsp70 present in the PSD fraction is much less than that of Hsc70.

The Nature of the Association of HSP70 with the PSD Fraction

To understand the characteristics of the association of HSP70 with the PSD fraction, we extracted the One-Triton PSD fraction with various detergents or with salt. The soluble and insoluble fractions were probed for the presence of each protein with specific antibodies (Fig. 2) and the solubility for each protein fractions were probed for the presence of each protein with specific antibodies (Fig. 2) and the solubility for each protein was shown in Table 2. About one-third of the lower band (72 kDa) of Hsc70 was solubilized in 0.5–1.0% Triton X-100. However, 1.0% OG solubilized 31.9 ± 1.4% (n = 3) of the Hsp70, which is significantly different from the solubility (15.0 ± 4.2%, n = 4) of the Hsp70, which is significantly different from the solubility of the Hsp70 (57–71) (Hsp70 (238–248)).

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Internal peptides were generated through trypsin digestion of OG-insoluble PSD-72 (peptides 1 and 2) or CNBr-cleavage of the OG-soluble PSD-72 (peptides 3 and 4). Peptides were purified on peptide gels (for peptide 3 and 4) or on HPLC (for peptide 1 and 2). Each peptide was sequenced on an automatic sequencer in the Caltech Protein and Peptide Microanalysis Laboratory with approximate initial yields of 15, 5, 23 and 10 pmol for peptides 1, 2, 3, and 4, respectively. The sequences were aligned with those of the rat Hsc70 (Sorger and Pelham, 1987) and Hsp70 (Longo et al., 1993), and the positions of the peptides were shown in parentheses. A space, indicated with a bar (–), was introduced in peptide 4 for a better alignment. The higher sequence similarity of PSD72 to Hsc70 than to Hsp70 strongly indicate that the sequenced PSD-72 is a member of the Hsc70 family.

When the PSD fraction was extracted with 1 M NaCl, a condition that disrupts hydrophilic protein–protein interactions (Apperson et al., 1996), 61.3 and 54.9% of the Hsc70 and Hsp70, respectively, were solubilized (Fig. 2B). Interestingly, only 36.3 ±

1.4% (n = 3) of the upper band (75 kDa) of Hsc70 was solubilized in 1 M NaCl (Fig. 2B, Hsc70, upper arrowhead).

To study subcellular distributions, the amounts of HSP70...
Figure 2. Association properties of HSP70 with the PSD. (A,B) Extraction of the PSD with detergents and salt. Aliquots (40 µg) of the One-Triton PSD fractions (PSD) were extracted with various detergents at indicated concentrations (A) or 1 M NaCl (B), and the soluble (S) and insoluble (P) fractions were electrophoresed in 6% SDS gels. Immunoblots were obtained with antibodies indicated at the left of each blot. Note that most of the Hsp70 and the lower band of Hsc70 (lower arrowhead) remain insoluble by detergent extraction, while most of the upper band of Hsc70 is solubilized (A). However, when One-Triton PSD fractions were extracted with 1 M NaCl, most of the Hsp70 and the lower band of Hsc70 (lower arrowhead) are solubilized, while most of the upper Hsc70 band (upper arrowhead) remains insoluble (B). (C) Subcellular distribution. The Hsp70 and lower band of Hsc70 are not specifically enriched in the synaptosome (S) or PSD fraction compared with brain homogenate (H). Note that the amount of the upper Hsc70 band (upper arrowhead) is dramatically reduced in the PSD fraction (upper arrowhead). (D) Expression of Hsc70 in various tissues. Tissue homogenates were immunoblotted with anti-Hsc70 MAb. Expression of Hsc70 in the spinal cord and thymus is very similar to that of the forebrain in respect to the relative amount of the two Hsc70 bands (arrowheads). Additional bands in the liver and kidney are marked with bars.

The immunoreactivity of Hsc70 was much stronger than that of Hsp70 in forebrain (Fig. 4, Cx, right panel). In the hippocampal formation, perikarya of pyramidal cells in cornus ammonis areas 1–3 (CA1–3) (Fig. 3, CA1, upper right panel), polymorphic neurons in CA4 (Fig. 3, CA4, inset) and granule cells in the dentate gyrus (DG) (Fig. 3, DG, inset) were stained. Staining of dendrites was also evident in CA1 pyramidal cells (Fig. 3, CA1, lower right panel) but was not in cells in CA4 and DG (Fig. 3, insets of CA4 and DG). In control images, for which the primary antibody was omitted, no signals were detected even at higher contrasts (Fig. 3, Control-Cx and Control-HC, insets). The immunoreactivity of Hsc70 was stronger than that of Hsp70 in the cerebral cortex, pyramidal cells were strongly positive for Hsp70 (Fig. 4, Cx). The strongest immunoreactivity was associated with perikarya and dendrites (Fig. 4, Cx, right panel). Staining of nucleoplasm was not evident, and that of nucleoli was very weak or non-existent (Fig. 4, inset of right panel). In the hippocampal formation, pyramidal neurons in CA1–3 (Fig. 4, CA1), polymorphic neurons in CA4 (Fig. 4, CA4) and granule neurons in DG (Fig. 4, DG) were strongly stained. The subcellular distribution of immunoreactivity was more or less similar to that of the pyramidal cells in the cerebral cortex. Subcellular distributions of both Hsp70 and Hsc70 were not homogeneous but patchy.

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*Only the major signal of the Hsc70 (Fig. 1B, a-Hsc70, lower arrowhead) was analyzed.

*Difference between two concentrations significant at P < 0.05. n, repeat number.

Table 2

Solubility of HSP70 proteins

Expression of HSP70 in Rat Cerebral Cortex and Hippocampal Formation at the Light Microscopic Level

The presence of HSP70 in the PSD fraction prompted us to compare the expression of Hsc70 and Hsp70 in vivo with specific antibodies. In general, immunoreactivities of both proteins were distributed throughout the brain (Figs 3 and 4), although staining for Hsp70 was stronger. As expected from the immunoblot analyses, the immunoreactivity of Hsp70 was weak in all regions of the forebrain, but the signals were definitely above the background (Fig. 3). In the cerebral cortex, pyramidal neurons were positively stained (Fig. 3, Cx). Perikarya and dendrites were also stained. Staining of nucleoplasm was not evident, but nucleoli were stained almost as strongly as perikarya (Fig. 3, Cx, upper right panel). In the hippocampal formation, perikarya of pyramidal cells in cornus ammonis areas 1–3 (CA1–3) (Fig. 3, CA1, upper right panel), polymorphic neurons in CA4 (Fig. 3, CA4, inset) and granule cells in the dentate gyrus (DG) (Fig. 3, DG, inset) were stained. Staining of dendrites was also evident in CA1 pyramidal cells (Fig. 3, CA1, lower right panel) but was not in cells in CA4 and DG (Fig. 3, insets of CA4 and DG). In control images, for which the primary antibody was omitted, no signals were detected even at higher contrasts (Fig. 3, Control-Cx and Control-HC, insets). The immunoreactivity of Hsc70 was much stronger than that of Hsp70 in forebrain (Fig. 4). In the cerebral cortex, pyramidal cells were strongly positive for Hsp70 (Fig. 4, Cx). The strongest immunoreactivity was associated with perikarya and dendrites (Fig. 4, Cx, right panel). Staining of nucleoplasm was not evident, and that of nucleoli was very weak or non-existent (Fig. 4, Cx, inset of right panel). In the hippocampal formation, pyramidal neurons in CA1–3 (Fig. 4, CA1), polymorphic neurons in CA4 (Fig. 4, CA4) and granule neurons in DG (Fig. 4, DG) were strongly stained. The subcellular distribution of immunoreactivity was more or less similar to that of the pyramidal cells in the cerebral cortex. Subcellular distributions of both Hsp70 and Hsc70 were not homogeneous but patchy.

Immunocytochemical Localization of HSP70 in Dissociated Hippocampal Neurons

Biochemical and histological evidence for the presence of HSP70

Present in brain homogenate, synaptosome and PSD fractions were compared. As shown in Figure 2C, Hsp70 and the lower band of Hsc70 are not specifically enriched in the PSD fraction. Interestingly, the amount of the upper band (75 kDa) of Hsc70 is dramatically reduced (14.1 ± 2.2%, n = 2, that of synaptosome) in the One-Triton PSD fraction (Fig. 2C, Hsc70, upper arrowhead), indicating that the majority of this protein is removed by 0.5% Triton X-100 and that it may not be an intrinsic member of the PSD. The tissue distribution of the two Hsc70 proteins was also investigated through immunoblot analyses of several tissue homogenates. As shown in Figure 2D, the spinal cord and thymus were similar to the brain in the relative amounts of the two Hsc70 proteins. In all other tissues tested, i.e. liver, kidney, muscle, testes, pancreas, heart and lung, the expression levels of the upper band (75 kDa) of Hsc70 were higher than those of the lower one (72 kDa). Interestingly, the expression levels of the upper band of Hsc70 were very high in the liver, kidney, pancreas, and heart. Moreover, there was an additional band in the liver and kidney (Fig. 2D, bars), indicating that there is another isoform of Hsc70 in these tissues.
in brain neurons encouraged us to perform immunocytochemical localization of the two proteins in dissociated hippocampal neurons. Hippocampal neurons, dissociated at embryonic day 18, were double-stained with antibodies specific for one of the HSP70 and PSD-95 proteins. The latter is known to be highly localized at the PSD in the forebrain (Kornau et al., 1995; Hunt et al., 1996). Confocal imaging revealed that the Hsp70 immunoreactivity was distributed throughout the neuron (Fig. 5). Immunoreactivity was stronger in perikarya and proximal dendrites than in distal processes, and was not uniform but punctate in both perikarya and processes. Staining intensity varied significantly among dendritic puncta and protrusions in 2-week-old neurons (Fig. 5A, upper right panel), while the intensities of punctate staining for PSD-95 were relatively

Figure 3. Expression of Hsp70 in the cerebral cortex and hippocampal formation. Sections of adult rat forebrains were stained with anti-Hsp70 MAb as described in Materials and Methods. Cx, expression in the cerebral cortex. Hsp70 is expressed at a low level throughout the cerebral cortex (left panel). However, note that expression is evident in perikarya and nucleoli of pyramidal cells in enlarged images (right panels). CA1, expression in the CA1 region of hippocampal formation. The Hsp70 is expressed weakly throughout the hippocampal formation (left panel), but expression in perikarya and nucleoli is evident in enlarged images of neurons (right panels). The pyramidal cell layer is marked as a bracket. CA4 and DG, expression in CA4 and the dentate gyrus, respectively. Enlarged images are shown in insets. Control-Cx and Control-HC, control images of neocortex and hippocampal formation. Sections were processed in the absence of the primary antibody. No immunoreactivities are associated with neurons even at high contrasts (insets). Scale bar, 50 µm.
uniform (Fig. 5A, lower right panel). When the two images were superimposed, most of the punctate staining overlapped (Fig. 5A, left panel, yellow color), indicating that the two proteins are mostly co-localized. There were, however, many puncta which were apparently labeled only with Hsp70 (green color, arrowheads in the insets of Fig. 5A, left panel), but pure red puncta was rare and most of the apparently red puncta actually had yellow hues (Fig. 5A, inset a, arrows) in 2-week-old cultures. Interestingly, when 5½-week-old cultures were double-stained, most of the dendritic puncta and protrusions were superimposed (Fig. 5B). Occasionally, however, dendritic protrusions labeled with only the Hsp70 antibody were found (Fig. 5B, left panel, large arrow and arrowhead in the inset). Moreover, variations in the staining intensities were small (see the yellow color of uniform intensities in most of the double-labeled puncta in Fig. 5B, left panel), suggesting that the amount of Hsp70 in the synapse stabilizes with synaptic maturation. A control image, which was single-labeled with the Hsp70 antibody (Fig. 5C, left panel), was very similar to the double-labeled ones, indicating that images were not altered by double-labeling. In another control, in which primary antibodies were omitted, no signals were detected in processes and only very weak signals were associated with perikarya (Fig. 5C, right panel, arrows). There was absolutely no bleed-over of signals between the two light channels during the recording of images by confocal microscopy (not shown).

The subcellular distribution of Hsc70 immunoreactivity was similar to that of Hsp70 in that most of the puncta were

Figure 4. Expression of Hsc70 in the cerebral cortex and hippocampal formation. Sections of adult rat forebrains were stained with rat anti-Hsc70 MAb. Cx, expression in the cerebral cortex. Hsc70 is expressed at high level throughout the cerebral cortex (left panel). High expression of Hsc70 in perikarya and major dendrites of pyramidal cells is shown in the lower right panel and the patchy staining in the top right panel. Scale bar, 500 µm. CA1, expression in the CA1 region of the hippocampal formation. Hsc70 is expressed at high level throughout the hippocampal formation (left panel). The pyramidal cell layer is marked as a bracket. Scale bar, 50 µm. CA4 and DG, expression in the CA4 and dentate gyrus regions, respectively. Enlarged (2× zoom) images are shown in insets. Scale bar, 50 µm.

Figure 5. Expression of Hsp70 in dissociated hippocampal neurons. Hippocampal neurons dissociated at E18 were grown on coverslips for 2 weeks (A,C) or 5½ weeks (B). For double-labeling, coverslips were immunostained with a mouse anti-Hsp70 MAb and a rabbit anti-PSD-95 PAb, followed by incubation with goat anti-mouse IgG (Alexa 568) and goat anti-rabbit IgG (Alexa 488), as described in detail under Materials and Methods. Images were taken with a fluorescence laser-scanning confocal microscope. Images of double-labeled cells were colorized and combined using Adobe Photoshop 5.0 software. Red and green pseudocolors represent Alexa 488 and Alexa 568 stainings, respectively, and regions of overlap are yellow. (A) Expression of Hsp70 and PSD-95 in hippocampal neurons cultured for 2 weeks in vitro (double-label). Strong immunoreactivities for Hsp70 are associated with the perikaryon and proximal dendrites. Note that puncta or spine-like protrusions are stained with Hsp70 antibodies in various intensities along processes (upper right panel). In contrast, PSD-95 is associated primarily with puncta at more or less uniform intensities (right lower panel). A combined image is shown at the left panel and areas a and b are magnified twofold (insets). Hsp70 is colocalized with PSD-95 at most puncta (yellow). However, note that the hue of the yellow color varies significantly among overlapped puncta. Green puncta, which are most evident with dendritic spine-like protrusions, are frequently seen (insets a, arrowheads). Red puncta are seen infrequently and most of the apparently red puncta have yellow hues (inset a, arrow). (B) Expression of Hsp70 in hippocampal neurons cultured for 5½ weeks in vitro (double-label). Hsp70 is colocalized with PSD-95 in most puncta (left panel, yellow puncta). In this neuron, intensities of the Hsp70-associated puncta are more or less uniform (Hsp70), which is evident in the combined image (left panel). An enlarged image of an area (rectangle) is shown in the inset (note similar hues of yellow color associated with puncta). Dendritic protrusions stained with Alexa 568 (green) are indicated with a large open arrow and an arrowhead in the inset. Note that red puncta are rare. (C) Control images. Two-week-old cultures were single-labeled with the Hsp70 antibody (left panel) or without primary antibodies (right panel). Scale bar, 10 µm.
double-labeled with both Hsc70 and PSD-95 antibodies (Fig. 6A). However, the signals were generally stronger and intensity variations among the puncta were small even in 2-week-old cultures (Fig. 6A, upper right panel; see also the more or less uniform intensities of yellow color in Fig. 6A, left panel). As in Hsp70 images, dendritic protrusions labeled only with the Hsp70 antibody were frequently seen (arrowheads in Fig. 6A, inset a and in Fig. 6B, panels a and b). However, dendritic protrusions labeled only with PSD-95 antibodies were very rare, and the apparently red puncta actually had yellow hues in the double-labeled images (arrow in Fig. 6A, inset and 6B, panel a).

Immunoelectron Microscopic Localization of HSP70 Proteins in the PSD

Synaptic localization of HSP70 proteins in the adult rat hippocamal neurons was further studied at the immunoelectron microscopic level. The most immunoreactive structures for Hsp70 in synapses were the asymmetrical PSDs (Fig. 7C, asterisk), some of which were axospinous synapses (Fig. 7C, asterisk). Presynaptic thickenings and synaptic vesicles were also stained, but less strongly than the PSD. Patch-like staining of cytoplasmic matrix was also detected. Symmetric synapses were stained very weakly (Fig. 7A,C, large open arrows). The DAB reaction products were also associated with some mitochondria (Fig. 7A, small open arrow), but other mitochondria were stained very weakly (Fig. 7A, small solid arrow). The DAB reaction product associated with mitochondria may be an artifact, as mitochondria frequently contain a high level of free radicals.

Immunoreactive structures for Hsp70 were similar to those staining for Hsp70. Asymmetrical synapses (Fig. 8, asterisks), including axospinous ones (Fig. 8B, asterisk), were strongly stained, but symmetrical ones were stained weakly (Fig. 8A,

Figure 6. Expression of Hsc70 in dissociated hippocampal neurons. (A) Hsc70/PSD-95 double-label. E18 hippocampal neurons grown for 2 weeks in vitro were double-stained with a rat anti-Hsc70 MAb and rabbit affinity-pure anti-PSD-95 PAb. The Hsc70/PSD-95 double-labeled coverslips were incubated with biotinylated anti-rat IgG and Cy3-conjugated anti-rabbit IgG, and incubated further with fluorescein-conjugated streptavidin. Confocal images were obtained and processed as in Figure 5. Green and red represent Hsc70 and PSD-95 immunostainings, respectively. Regions of overlap are yellow. Hsc70 is distributed throughout the somata and processes, and punctate staining is seen along processes (upper right panel). PSD-95 is mainly associated with synapse-like puncta (lower right panel). In the combined image (left panel), most of the puncta are overlapped (yellow color). Single-labeled puncta with green color are occasionally seen (inset a, arrowheads), but single-labeled puncta with red color are rare and most of the apparently red puncta have yellow hues (inset a, arrow). Note the more or less uniform hues of yellow color. A process with many dendritic spine-like protrusions is marked with a rectangle (asterisk) and shown in panel B. Scale bar, 10 µm. (B) Dendritic spine-like protrusions associated with Hsc70. The rectangular region in panel A (asterisk) is shown in magnification (3× zoom). Punctate staining along the dendritic shaft is evident. Positions of the two knobby spine-like protrusions labeled only with Hsc70 antibodies are indicated with arrowheads. A punctum apparently labeled only with PSD-95 is marked with an arrow. But note the yellow hue in this punctum (c) and weak signal in Hsc70 staining (b). a, combined image. b, Hsc70 image. c, PSD-95 image. Scale bar, 2 µm.
Although the immunoblot signals for Hsp70 in the forebrain PSD (~90% identity) and molecular size (O’Malley et al., 1985) are very similar in amino acid sequence and molecular size (O’Malley et al., 1985), the Hsp70 and Hsc70 are very similar in amino acid sequence (O’Malley et al., 1985).

Presence of both Hsp70 and Hsc70 in the PSD

The Hsp70 and Hsc70 are very similar in amino acid sequence (~90% identity) and molecular size (O’Malley et al., 1985). Although the immunoblot signals for Hsp70 in the forebrain PSD fraction were weak compared with those for Hsc70, we argue, for several reasons, that the presence of Hsp70 in normal brain synapses is real and that the Hsp70 signals seen in this study are not from cross-reaction with Hsc70. First, the binding strength of Hsp70 with the PSD is larger than that of Hsc70 (Fig. 2A). Second, the amount of Hsp70 associated with the One-Triton fraction is not reduced compared with that in brain homogenate or in synaptosomal fractions (Fig. 2C), indicating strong association of the Hsp70 with the PSD. Third, although the signal from immunohistochemical staining for Hsp70 is much weaker than that for Hsc70, it is above the background level in forebrain neurons (Figs 3 and 4). Fourth, confocal images of immunostained hippocampal neurons show that Hsp70 is associated with spine-like structures or dendritic protrusions, and that the pattern of punctate staining is different from that of Hsc70 in regard to variation in signal intensities and co-localization with PSD-95 (Figs 5 and 6). Last, both the Hsp70 and Hsc70 immunoperoxidase reaction products were associated primarily with the PSD but only Hsc70 was associated with the amorphous subsynaptic structures and spine apparatus-like structures (Figs 7 and 8). Together with the finding that three out of four amino acid sequences from the 72 kDa band were aligned better to Hsc70 than to Hsp70 (Table 1), we conclude that both Hsp70 and Hsc70 are present in the PSD and that the major HSP70 associated with the forebrain PSD is Hsc70. Our data are inconsistent with a recent report by Suzuki et al. (Suzuki et al., 1999), who reported that only Hsc70, but not Hsp70, is present in the PSD fraction.

Discussion

Together with various cohorts, the HSPs function as molecular chaperones in the protein life cycle (Kelley, 1988; Frydman et al., 1994; Morimoto et al., 1994). In this report we have demonstrated, by biochemical, immunocytochemical and histological studies, that both Hsp70 and Hsc70 are present in CNS synapses, suggesting that mechanisms for receptor-mediated endocytosis and a protein ‘holding and folding’ system which would serve as a part of ‘synaptic tag’ (Frey and Morris, 1997, 1998) are present in postsynaptic sites.

Biochemical Characteristics of HSP70 (Hsc70 and Hsp70) Associated with the PSD

The PSD is thought to be composed of highly insoluble ‘core’ proteins and other proteins which are associated with the ‘core’ (Kennedy, 1993, 1997). Proteins tightly bound to the PSD ‘core’ proteins and other proteins which are associated with the ‘core’ (Kennedy, 1993, 1997). Proteins tightly bound to the PSD ‘core’
are insoluble in mild detergents such as OG or Triton X-100 (Apperson et al., 1996). The fact that PSD-associated HSP70 proteins were efficiently dissociated from the PSD ‘core’ by salt (1.0 M NaCl) (Fig. 2A), but not by OG (0.5–1.0%) or Triton X-100 (0.5–1.0%) (Fig. 2A), indicates that both Hsp70 and Hsc70 are closely associated with the PSD ‘core’ mainly through hydrophilic protein interactions. In contrast to Hsc70, however, the solubility of Hsp70 is increased about twofold (from 15.0 to 31.9%) in 1.0% OG compared with 0.5% OG, suggesting a slight difference in binding strength or characteristics of the two proteins with the PSD ‘core’. The HSP70 proteins are not enriched in the PSD (Fig. 2C). This may be due to the fact that the HSP70 proteins are also distributed in somata, dendrites and spines (Figs 3–6), and that not all PSDs contain HSP70 proteins (Figs 7 and 8). We did not address the cognate proteins that associate with the HSP70 proteins. However, the HSP70 contain a highly conserved calmodulin-binding domain (Stevenson and Calderwood, 1990). Since calmodulin is the major PSD protein that binds to a number of proteins there (Grab et al., 1979; Carlin et al., 1981), HSP70 proteins may be associated with calmodulin in the PSD.

Differential Distribution of HSP70 at Synaptic Sites

Our immunoelectron and confocal microscopic studies have shown differential distribution of the HSP70 proteins at synaptic sites. Only Hsc70 was associated with spine apparatus-like cisternae and unidentified amorphous subsynaptic structures (Figs 7 and 8). The latter may be the ‘subsynaptic webs’ which have been previously described as associated with Hsc70 (Suzuki et al., 1999). At the confocal microscopic level, the Hsp70 was frequently not co-localized with PSD-95 at puncta or spine-like protrusions of dissociated hippocampal neurons, and variations in amount among these structures were large in young cultures (Fig. 5A). Interestingly, most of the Hsp70 became co-localized with PSD-95 in older cultures (Fig. 5A), suggesting that Hsp70 may be involved in an early stage of synaptic development, such as formation of new dendritic sproutings, in addition to synaptic maturation and/or activity-dependent modulation of synaptic strength.

Possible Roles of PSD-associated HSP70

The most straightforward interpretation of the function of the HSP70 proteins in the cytoplasm of synaptic sites and dendritic shafts would be that they are involved in facilitation of folding of nascent proteins and in the repair of partially denatured proteins. Rapid input-specific growth of small filopodia-like protrusions (Maletic-Savatic et al., 1999) and formation of new spines (Hosokawa et al., 1995; Engert and Bonhoeffer, 1999) are induced by synaptic stimulation. Recently, tetanic stimulation has been shown to cause local protein synthesis in dendrites of hippocampal CA1 pyramidal neurons (Ouyang et al., 1999) and dentate granule cells (Steward and Halpain, 1999). Therefore, HSP70 proteins may function in the process of local synthesis of new proteins required for synaptic plasticity, remodeling, neurite outgrowth and/or the stabilization of existing or nascent synapses.

Hsc70 may function in clathrin-dependent endocytosis. The clathrin-mediated synaptic vesicle endocytosis (Gad et al., 1998; Palfrey and Artalejo, 1998) and postsynaptic receptor internalization (Craven and Breit, 2000; Man et al., 2000; Wang and Linden, 2000) have been reported. With a clathrin-uncoating ATPase activity (Rothman and Schmid, 1986; DeLuca-Flaherty et al., 1990), Hsc70 may participate in vesicle trafficking in both pre- and postsynaptic compartments.

The HSP70 associated with the PSD may function in a protein ‘holding and folding’ system as part of a ‘synaptic tag’. A potential ‘synaptic tag’ would be capable of both sequestering the plasticity proteins in stable form and releasing and/or activating them upon synaptic stimulation (Frey and Morris, 1997, 1998). Since the HSP70 proteins can act as both a ‘holding’ and a ‘folding’ system depending on the chaperone cofactors associated with them (Zeiner et al., 1997; Bimston et al., 1998; Takayama et al., 1999), they would be good candidates for part of a ‘synaptic tag’.

Notes

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