Cell Stress & Chaperones (1998) 3 (3), 188–199

Constitutive expression of heat shock proteins Hsp90, Hsc70, Hsp70 and Hsp60 in neural and non-neural tissues of the rat during postnatal development

Sandra M. D'Souza and Ian R. Brown

Department of Zoology, University of Toronto, Scarborough Campus, West Hill, Ontario, Canada M1C 1A4

Abstract Heat shock proteins (Hsps) are a group of highly conserved proteins, that are constitutively expressed in most cells under normal physiological conditions. Previous work from our laboratory has shown that neurons in the adult brain exhibit high levels of Hsp90 and Hsc70 mRNA and protein, as well as basal levels of Hsp70 mRNA. We have now investigated the expression of Hsp90, Hsc70, Hsp60 and Hsp70 in neural and non-neural tissues of the rat during postnatal development, a time of extensive cell differentiation. Western blot analysis revealed constitutive expression of these Hsps early in postnatal development. Developmental profiles of these Hsps suggest that they are differentially regulated during postnatal development of the rat. For example, while levels of Hsp90 decrease somewhat in certain developing brain regions, levels of Hsp60 show a developmental increase, and Hsc70 protein is abundant throughout postnatal neural development. Low basal levels of Hsp70 are also observed in the developing and adult brain. A pronounced decrease in Hsp90 and Hsc70 was observed during postnatal development of the kidney while levels of Hsp60 increased. In addition, tissue-specific differences in the relative levels of these Hsps between brain and non-brain regions were found. Immunocytochemical studies demonstrated a neuronal localization of Hsp90, Hsc70 and Hsp60 at all stages of postnatal development examined as well as in the adult, suggesting a role for Hsps in both the developing and fully differentiated neuron. The developmental expression of subunit IV of cytochrome oxidase was similar to that of Hsp60, a protein localized predominantly to mitochondria.

INTRODUCTION

Heat shock proteins (Hsps) are highly conserved proteins which are induced in cells upon exposure to elevated temperatures or other forms of cellular stress (Subjeck and Shyy 1986; Welch 1992; Parsell and Lindquist 1993). In addition, most Hsps are also present in the unstressed cell, where they are thought to play a vital role in normal cellular function (Lindquist and Craig 1988). One of the main roles that Hsps play is that of molecular chaperones. Specifically, Hsps have been shown to function in protein maturation events such as protein folding, unfolding, and translocation across membranes (Ellis and van der Vies 1991; Gething and Sambrook 1992).

Hsps can be classified into major families, based on their molecular weight (Welch 1992); these include Hsp100s, Hsp90s, Hsp70s, Hsp60s and Hsp20s. Hsp90 is an abundant cytosolic protein which is found in association with a variety of other intracellular proteins including calmodulin (Minami et al 1993), actin (Nishida et al 1986), tubulin (Sanchez et al 1988), several kinases (Oppermann et al 1981; Rose et al 1987) and steroid receptors (Kang et al 1994). Among other functions, Hsp90 stabilizes target proteins in an inactive or unassembled state. Members of the Hsp70 multigene

Received 6 October 1997; Revised 7 January 1998; Accepted 8 January 1998

Correspondence to: Ian R. Brown, Tel: +1 416 287 7413; Fax: +1 416 287 7642

family of proteins include the stress-inducible Hsp70 (Wu et al 1985) as well as the constitutively expressed Hsc70 (Chappell et al 1986). Under normal physiological conditions, they function in the stabilization of unfolded protein precursors before assembly and in the translocation of proteins into organelles, such as the endoplasmic reticulum, and the mitochondria. Hsp60 is a mitochondrial matrix protein (Mizzen et al 1989) that exists as a seven-subunit toroidal structure in mammals (Viitanen et al 1992). In conjunction with mitochondrial Hsp70 (grp75), it aids in the folding of translocated mitochondrial proteins (Neupert and Pfanner 1993).

Previous work from our laboratory has shown a glial induction of Hsp70 in the mammalian brain following hyperthermia (Manzerra et al 1993; Foster et al 1995; Foster and Brown 1997). Induction of heat shock protein was not detected in several populations of large neurons which exhibit high levels of constitutive Hsc70 as well as Hsp90 protein in the adult control animal (Manzerra et al 1993; Quraishi and Brown 1995; Manzerra and Brown 1996). In addition, basal levels of Hsp70 mRNA were detected in hippocampal and cortical neurons (Foster and Brown 1996a). Our present study examines the constitutive expression of Hsp90, Hsc70, Hsp70 and Hsp60 protein during postnatal development of the rat using Western blotting and immunocytochemistry.

MATERIALS AND METHODS

Isolation of protein homogenates

Wistar rats (Charles River) were sacrificed by decapitation at postnatal days 1, 5, 10, 15, 20 and 90 days (adult). Tissue was dissected from brain regions, kidney and liver and homogenized in 0.32 M sucrose. Protein concentrations were determined using the BioRad protein assay.

One-dimensional gel electrophoresis and Western blot procedure

Protein samples were solubilized by boiling for 5 min with an equal volume of dissociation buffer (8 M urea, 2% SDS, 2% β-mercaptoethanol, 20% glycerol). Polyacrylamide gel electrophoresis was carried out in the presence of SDS on either 10% (for Hsp90, Hsc70 and Hsp60) or 12% (for Hsp70 and C.OX IV) gels with 5% stacking gels using the discontinuous buffer system of Laemmli (1970). The proteins were transferred onto nitrocellulose membranes for 16–18 h in a solution of 50 mM boric acid, 4 mM β-mercaptoethanol and 2 mM EDTA, at 400 mA.

For Western blotting, the blots were washed 4 × 5 min in TBST buffer (10 mM Tris, 0.25 M NaCl, 0.5% Tween-20, pH 7.5), blocked for 1 h at room temperature in 5% Carnation milk powder in TBST buffer, and then incubated overnight in primary antibody. The following antibodies were used: monoclonal 29A anti-Hsp90 (gift from A. C. Wilkström; Akner et al 1992) detecting both α and β forms of Hsp90, diluted 1:5000, polyclonal 1477 anti-Hsc70 (gift from R. Tanguay) diluted 1:50 000, monoclonal anti-Hsp60 (gift from R. Gupta) diluted 1:16 000, monoclonal C92 anti-Hsp70 (StressGen; Welch and Suhan, 1986) diluted 1:5000, and monoclonal 20E8–C13 anti-cytochrome oxidase subunit IV (Molecular Probes; Taanman et al 1993) diluted 1:10 000. Following incubation with primary antibody, blots were washed 4 × 10 min in 1% BSA (Sigma grade) in TBST, incubated for 2 h at room temperature with secondary antibodies (Sigma) anti-mouse IgG diluted 1:5000 in 1% BSA in TBST (for monoclonal antibodies), or with anti-rabbit IgG diluted 1:10 000 (for polyclonal antibody), and then washed 6 × 5 min in TBST. Immunoreactive bands were visualized by use of enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham, RPN 2106).

Some of the Western blots that were originally incubated with the anti-Hsp90 antibody (Fig. 1A) were stripped with 0.1 M sodium citrate, pH 3.5, and processed again using the anti-Hsp60 antibody (Fig. 6A). Similarly, some of the immunoblots probed with the anti-Hsp70 antibody (Fig. 8A) were stripped and reprobed with the anti-C.OX IV antibody (Fig. 7A). Exposed X-ray film was scanned using a BioRad GS-700 imaging densitometer. Bar graphs represent data from 2 to 4 sets of animals. Values at postnatal day 1 (P1) were standardized to a value of 1. Error bars indicate the SEM. Differences between the means were tested for statistical significance using one-way analysis of variance (ANOVA) followed by Tukey HSD (highly significant differences) multiple comparisons test with P values less than 0.05 indicating significance.

Immunocytochemistry

Rats were perfused with 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Tissue was removed and placed in 4% paraformaldehyde overnight at 4°C and then allowed to equilibriate in 25% sucrose in PBS. Tissue was mounted in OCT embedding compound (Miles Inc.), and kept at −70°C until use. Twenty-micromole cryostat sections were floated on water and collected on gelatin-coated glass microscope slides (1% gelatin, 0.05% chromium potassium sulfate) and air-dried overnight. Sections were rehydrated for 30 min at room temperature in PBT buffer (0.1 M PBS, pH 7.4, 0.2% Triton-X 100, 0.1% BSA), and then blocked for 1 h in PBT buffer with 1% normal horse serum (for monoclonal antibodies) or goat serum (for polyclonal antibody). Sections were incubated overnight

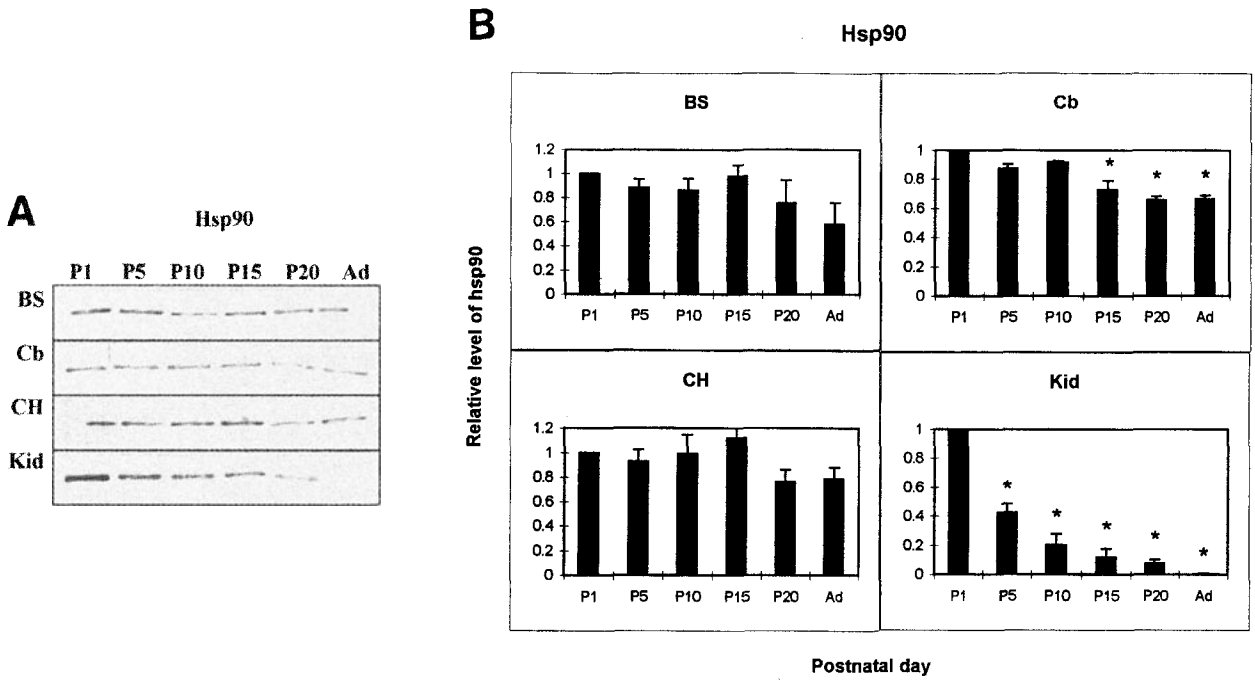

Fig. 1 Western blot analysis of Hsp90 protein levels during postnatal development of the rat. Hsp90 levels in various rat tissues was examined at postnatal days 1, 5, 10, 15, 20 (P1–20), and in the adult (Ad). (A) Fifteen micrograms of protein were loaded per lane for brain stem (BS), cerebellum (Cb), and cerebral hemispheres (CH). Thirty micrograms of protein were loaded per lane for kidney (Kid). For each tissue shown, a parallel gel was stained with Coomassie blue to ensure equal loading. (B) Exposed X-ray film was scanned using an imaging densitometer. Protein levels at postnatal day 1 (P1) were standardized to a value of one. Bar graphs represent the average of independent experiments carried out on 2–4 sets of animals. Error bars indicate the SEM. *Significantly different from postnatal day 1 ($P < 0.05$).

at room temperature in primary antiserum diluted 1:100 for 29A, 1:500 for 1477, 1:200 for Hsp60, and 1:200 for C.OX IV. After washing for 2 × 5 min in PBT buffer, sections were incubated in biotinylated rat-adsorbed anti-mouse IgG (for monoclonal antibodies) diluted 1:200, or biotinylated anti-rabbit IgG (for polyclonal antibody) diluted 1:400 for 1.5 h at room temperature. Following another 2 × 5 min wash in buffer, sections were incubated in 0.3% H_2O_2 in methanol for 30 min to block any endogenous peroxidase activity. After a 20 min wash in buffer, sections were processed with the Vectastain Elite ABC kit (Vector Labs, Burlingame, CA, USA). Diaminobenzidine (DAB) was used as the chromagen. Data shown are representative of independent experiments carried out on 3 sets of animals.

RESULTS

Developmental analysis of Hsp90 protein levels

A Western blot analysis of Hsp90 protein levels during postnatal development of the rat was carried out. As shown in Figure 1A, Hsp90 protein levels decreased slightly during postnatal development in brain regions such as the cerebellum (Cb), and cerebral hemispheres (CH). In the kidney (Kid), a dramatic developmental decrease in Hsp90 was observed. Scanning of exposed X-ray film with an imaging densitometer confirmed the marked developmental decrease in Hsp90 in the kidney, and the less pronounced decrease in brain regions (Fig. 1B). An analysis was performed to determine if these developmental changes were statistically significant. As seen in Figure 1B, the developmental decreases in Hsp90 levels were statistically significant in the cerebellum and kidney.

A comparison of Hsp90 levels between various adult (Ad) rat tissues (Fig. 2A) showed Hsp90 protein levels to be greater in neural regions such as the brain stem, cerebral hemispheres, and cerebellum, compared to non-neural tissues, such as the kidney (K) and liver (L). However, at postnatal day 1 (P1), Hsp90 levels in the kidney were comparable to the neural levels.

Cellular localization of Hsp90 protein in brain and kidney

Immunocytochemical analysis of Hsp90 in the developing rat brain revealed that Hsp90 protein was expressed in the Purkinje neurons of the cerebellum at all stages of postnatal development examined (Fig. 3, Hsp90

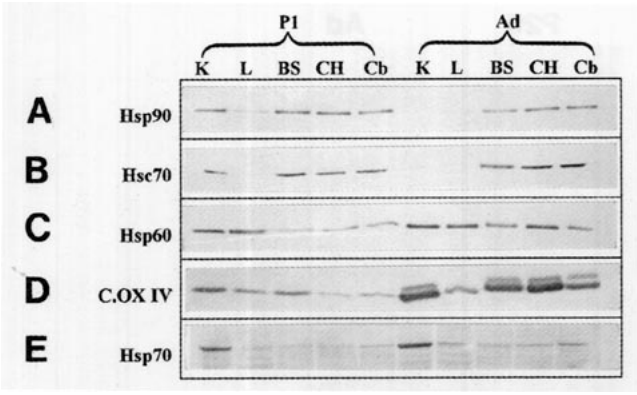

Fig. 2 Tissue comparisons of Hsp90, Hsc70, Hsp60, C.OX IV and Hsp70 protein levels at postnatal day 1 and the adult rat. Levels of Hsp90 (A), Hsc70 (B), Hsp60 (C), C.OX IV (D) and Hsp70 (E) were analyzed in kidney (K), liver (L), brain stem (BS), cerebral hemispheres (CH), and cerebellum (Cb) at postnatal day 1 (P1) and in the adult. For (A) and (C), 15 µg of protein was loaded per lane. For (B), 30 µg of protein was loaded per lane. For (D) and (E), 100 µg of protein was loaded per lane.

panel). Immunoreactivity was localized to the cytoplasm as well as to apical dendrites in these neurons at P1, P15 and in the adult. In addition, neurons in the deep cerebellar nuclei and in the brain stem were immunopositive for Hsp90 (data not shown). Glial-enriched areas of the brain, such as the white matter of the cerebellum, were immunonegative for Hsp90. In the kidney, Hsp90 protein was localized to the convoluted tubules of the renal cortex (Fig. 4, Hsp90 panel). In agreement with the Western data shown in Figure 1, Hsp90 levels appeared to decrease in the kidney through postnatal development.

Developmental analysis of Hsc70 protein levels

As seen by Western blot analysis, levels of Hsc70 protein did not vary greatly during postnatal development of the three brain regions (Fig. 5A). In the kidney, a developmental decrease in Hsc70 protein levels was observed. Densitometric scanning (Fig. 5B) and statistical analysis showed that Hsc70 levels in the adult kidney were significantly decreased compared to P1.

The levels of Hsc70 protein in different rat tissues was investigated at P1 and in the adult (Fig. 2B). In concurrence with the tissue comparison of Hsp90 levels (Fig. 2A), Western blot analysis revealed that Hsc70 protein levels were greater in the three neural regions than in kidney and liver in the adult. In summary, abundant levels of Hsc70 protein appear to be present early in postnatal development of the nervous system and are maintained through to the adult.

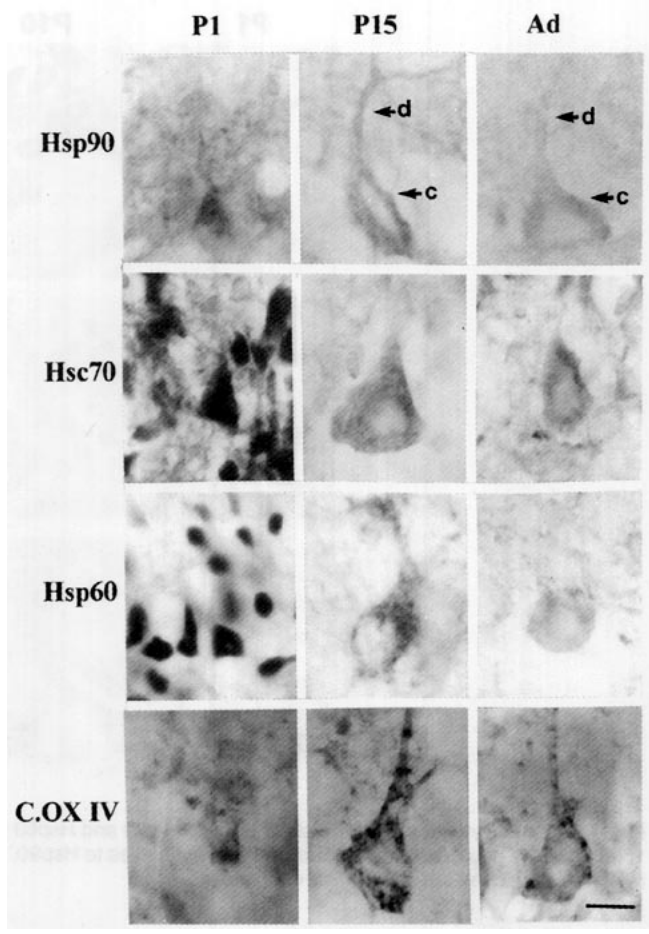

Fig. 3 Immunocytochemical analysis of Hsp90, Hsc70, Hsp60 and C.OX IV in the rat cerebellum during postnatal development. Hsp90, Hsc70, Hsp60 and C.OX IV were all detected in Purkinje neurons of the cerebellum at postnatal days 1, 15 and in the adult. Both the cytoplasm (c) as well as the apical dendrites (d) are immunostained. Bar = 10.9 µm.

Cellular localization of Hsc70 protein in brain and kidney

The cellular localization of Hsc70 protein in the developing rat brain was determined by immunocytochemistry (Fig. 3, Hsc70 panel). Hsc70 protein was expressed in Purkinje neurons of the cerebellum at P1, P15 and in the adult. Immunopositive staining was concentrated in the neuronal cytoplasm as well as to dendritic processes. This pattern of expression of Hsc70 was also observed in neurons in the deep cerebellar nuclei and brain stem (data not shown). In the developing rat kidney, Hsc70 immunoreactivity was detected in the convoluted tubules of the renal cortex (Fig. 4, Hsc70 panel).

Developmental analysis of Hsp60 protein levels

A major increase in Hsp60 protein levels was observed during postnatal development of all three brain regions

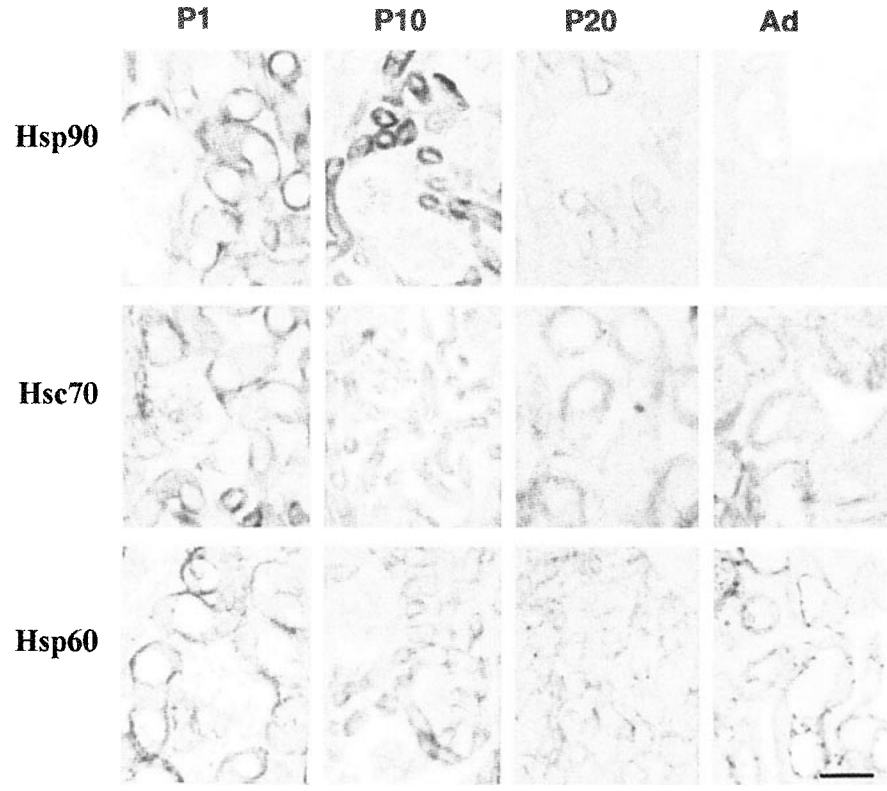

Fig. 4 Immunocytochemical analysis of Hsp90, Hsc70 and Hsp60 in the developing rat kidney. Rat kidney sections from postnatal days 1, 10, 20 and the adult were immunostained with antibodies to Hsp90, Hsc70 and Hsp60. Cross sections of convoluted tubules in the cortex region of the kidney are shown. Bar = 26.4 μm.

examined. A developmental increase of lesser magnitude was observed in the kidney (Fig. 6A). Densitometric scanning of these blots revealed a 25- to 30-fold increase in Hsp60 protein levels during postnatal development in the brain stem, and the cerebral hemispheres, while the cerebellum showed an increase of lesser magnitude (Fig. 6B). Statistical analysis showed that Hsp60 levels were significantly higher at P20 and in the adult compared to P1 in all three brain regions.

A comparative analysis of Hsp60 protein levels in various rat tissues was undertaken by Western blotting (Fig. 2C). In contrast to Hsp90 and Hsc70 protein levels, Hsp60 levels were greater in non-neural tissues, such as the kidney and liver, than in neural regions, such as the brain stem, cerebral hemispheres, and cerebellum, at P1. In the adult, levels of Hsp60 were higher in the kidney, liver, and cerebral hemispheres, and lower in the cerebellum and brain stem.

Cellular localization of Hsp60 protein in brain and kidney

Similar to the immunocytochemical results shown for Hsp90 and Hsc70 protein (Fig. 3, Hsp90 and Hsc70 panels), Hsp60 protein was localized to neurons in the developing rat brain. Hsp60 immunoreactivity was present in the cytoplasm as well as in dendritic processes of the Purkinje neurons of the cerebellum (Fig. 3, Hsp60 panel). The neuronal expression of Hsp60 in other brain regions such as the brain stem, was also comparable to that of Hsp90 and Hsc70 protein (data not shown). In contrast to the Western blot analysis (Fig. 6A), a developmental increase in Hsp60 was not detected by immunocytochemistry, suggesting a developmental change in epitope accessibility. In the developing rat kidney (Fig. 4, Hsp60 panel), a similar pattern of immunostaining of Hsp60 was observed as that seen for Hsc70 (Fig. 4, Hsc70 panel).

Developmental analysis of cytochrome oxidase (subunit IV) protein levels

To investigate whether the observed developmental increase in Hsp60 was due to an increase in mitochondrial content, the developmental pattern of cytochrome oxidase, subunit IV (C.OX IV) was studied. As shown in Figure 7A, a developmental increase in C.OX IV was observed in all brain regions examined as well as in the kidney. However, the magnitude of increase in C.OX IV

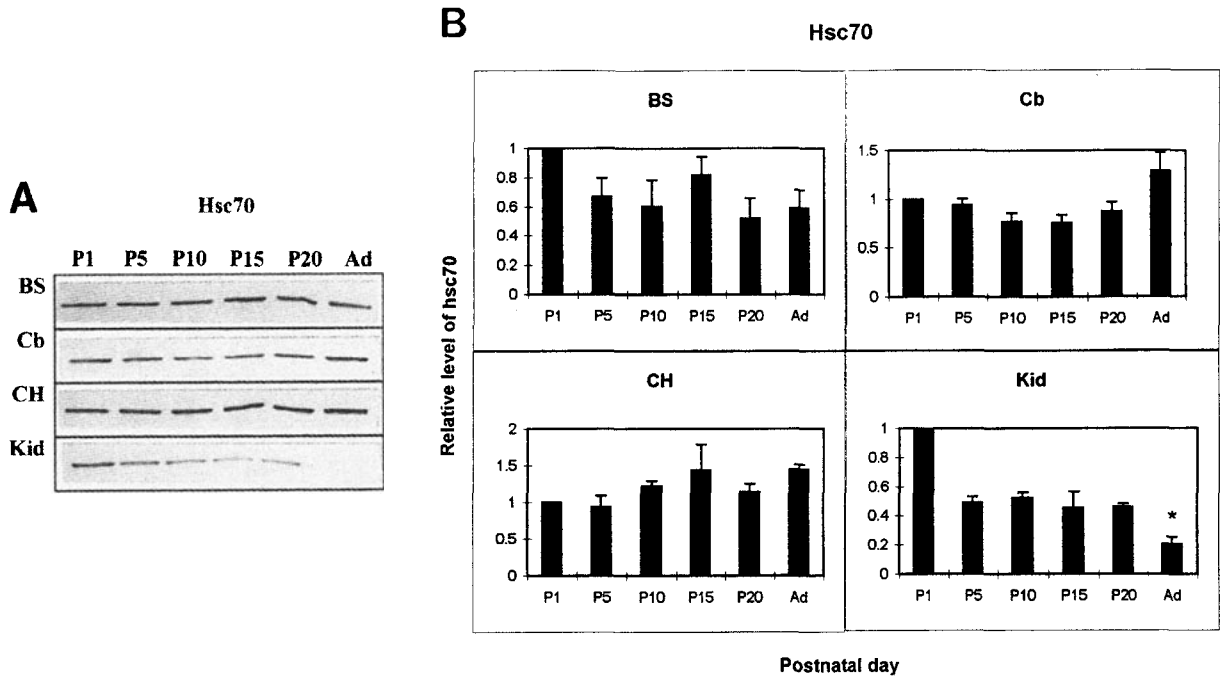

Fig. 5 Western blot analysis of Hsc70 protein levels during postnatal development of the rat. Hsc70 levels in various rat tissues was examined at postnatal days 1, 5, 10, 15, 20 (P1–20) and in the adult (Ad). (A) Thirty micrograms of protein were loaded per lane for brain stem (BS), cerebellum (Cb), cerebral hemispheres (CH) and kidney (Kid). (B) Quantitation of protein levels was carried out as in Figure 1B. Protein levels at postnatal day 1 were standardized to a value of one. Bar graphs represent the average of independent experiments carried out on 3–4 sets of animals. Error bars indicate the SEM. *Significantly different from postnatal day 1 ($P < 0.05$).

for a given region did not always parallel that of Hsp60. For example, Hsp60 levels increased approximately 30-fold in the brain stem (Fig. 6B), whereas C.OX IV levels (Fig. 7B) increased less than 4-fold in the same brain region during postnatal development. In the cerebellum, the magnitude of the developmental increase in C.OX IV levels were greater than that observed for Hsp60. The C.OX IV antibody detected a major and a minor band on SDS-PAGE. Both the major and minor bands showed developmental increases. The identity of the minor band is not resolved. As shown in Figure 7B, developmental changes in levels of C.OX IV were statistically significant only in the cerebellum.

A comparative analysis of C.OX IV protein levels in various neural and non-neural rat tissues was investigated (Fig. 2D). Levels of C.OX IV at P1 appeared higher in non-neural tissues such as the kidney and liver, compared to neural regions such as the cerebral hemispheres and cerebellum. C.OX IV levels in the brain stem were similar to that found in the kidney and liver. In the adult, levels of C.OX IV in neural and non-neural regions were comparable. With the exception of the brain stem at P1, this pattern of expression was similar to that found for Hsp60 (Fig. 2C).

Cellular localization of C.OX IV protein in brain

To determine if the observed cellular localization of Hsp60 correlated with that of C.OX IV, immunocytochemistry was performed on the rat brain with the C.OX IV antibody. As shown in Figure 3 (C.OX IV panel), a neuronal pattern of expression of C.OX IV was observed in the cerebellum at P1, P15 and in the adult. Immunoreactivity was present in the cytoplasm as well as the dendrites of the Purkinje neurons. This neuronal pattern of expression paralleled that of Hsp60 (Fig. 3, Hsp60 panel).

Developmental analysis of Hsp70 protein levels

Previous studies from our laboratory using Western blotting have found basal levels of Hsp70 protein in regions of the unstressed rabbit brain (Manzerra et al 1997); moreover, in situ hybridization techniques have revealed a neuronal localization of basal Hsp70 mRNA (Foster and Brown 1996a). In view of this, the developmental profile of Hsp70 protein was analyzed in the postnatal rat. As shown in Figure 8A, basal levels of Hsp70 increased in the cerebral hemispheres and the kidney until P20, and

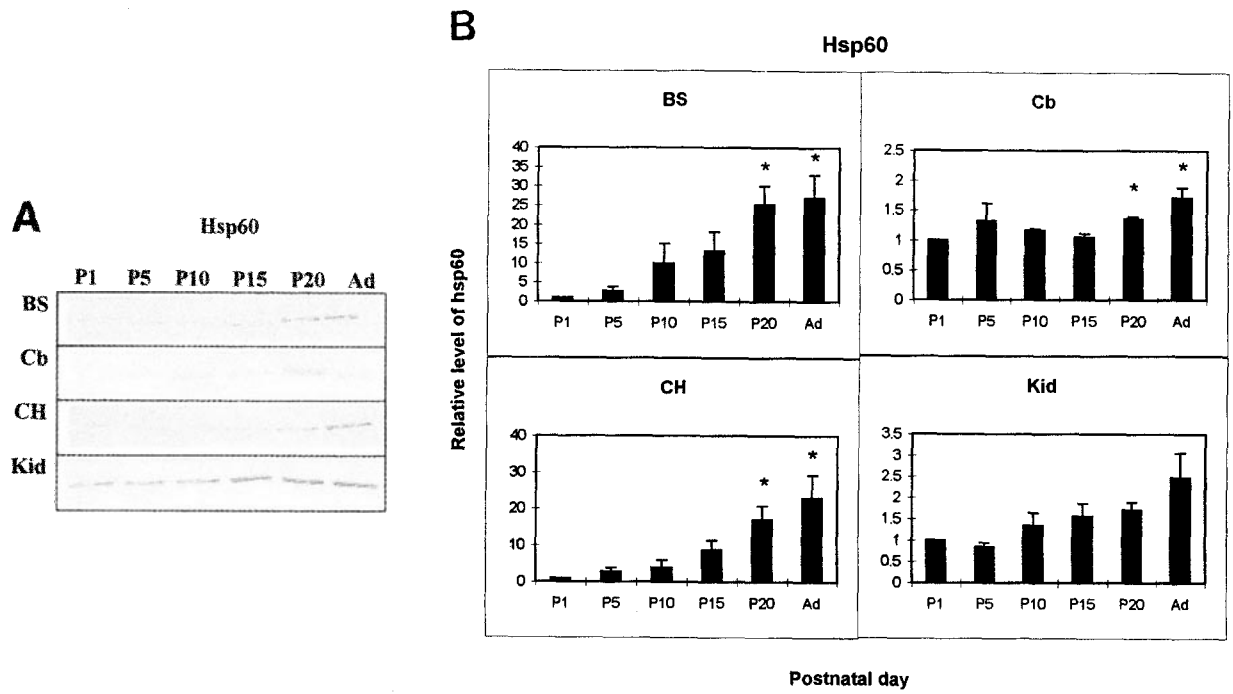

Fig. 6 Western blot analysis of Hsp60 protein levels during postnatal development of the rat. Hsp60 levels in various rat tissues were examined at postnatal days 1, 5, 10, 15, 20 (P1–20) and in the adult (Ad). (A) Fifteen micrograms of protein were loaded per lane for brain stem (BS), cerebellum (Cb) and cerebral hemispheres (CH). Thirty micrograms of protein were loaded per lane for kidney (Kid). (B) Quantitation of protein levels was carried out as in Figure 1B. Protein levels at postnatal day 1 were standardized to a value of one. Bar graphs represent the average of independent experiments carried out on 3–4 sets of animals. Error bars indicate the SEM. *Significantly different from postnatal day 1 ($P < 0.05$).

then decreased in the adult, while protein levels in the cerebellum showed comparatively little change during postnatal development. Statistical analysis showed that Hsp70 levels were significantly higher in the cerebral hemispheres at P20 and in the adult compared to P1. Levels of Hsp70 were highest in kidney compared to brain regions at both P1 and in the adult (Fig. 2E). Cellular localization of Hsp70 in the developing brain was not attempted due to the very low levels of the protein. For the developmental Westerns, the amount of protein loaded per lane had to be increased to 100 µg in order to detect the basal Hsp70 signal.

DISCUSSION

Heat shock proteins are essential proteins that are needed for normal cellular growth and maintenance (Lindquist and Craig 1988). Expression of heat shock proteins has been detected during embryogenesis in various organisms, such as the sea urchin, Drosophila, zebrafish and mouse (Bédard and Brandhorst 1986; Kothary et al 1987; Ding et al 1993; Krone and Sass 1994). While most research on the developmental expression of Hsps focuses on cellular events that occur during embryogenesis, comparatively little work has been carried out on postnatal development, especially in the mammalian brain.

In this study, the constitutive expression of a number of heat shock proteins during postnatal development of the rat was investigated using Western blotting and immunocytochemistry. Our previous work has shown a neuronal localization of Hsp90, Hsc70 and Hsp70 in the adult mammalian brain (Quraishi and Brown 1995; Foster and Brown 1996a; Manzerra and Brown 1996). It was of interest to examine the expression profile of these Hsps in the brain during postnatal development, a time of extensive neuronal differentiation. In addition, the developmental profiles of Hsp60 and another mitochondrial protein, cytochrome oxidase (subunit IV) were investigated. The developmental expression of these Hsps in a non-neural tissue, namely the kidney, was also studied as a comparison to the neural regions.

Western blot analysis revealed high levels of Hsp90 protein early in postnatal development of both brain and non-brain regions. A pronounced developmental decrease in Hsp90 levels was observed in the kidney, whereas comparatively little change in levels of Hsp90 was seen in the three brain regions. A tissue comparison of Hsp90 levels in the adult showed higher amounts of the protein in neural regions compared to non-neural tissues. Immunocytochemical studies demonstrated a neuronal localization of Hsp90 in the rat brain at all stages of postnatal development examined. Previous studies in the

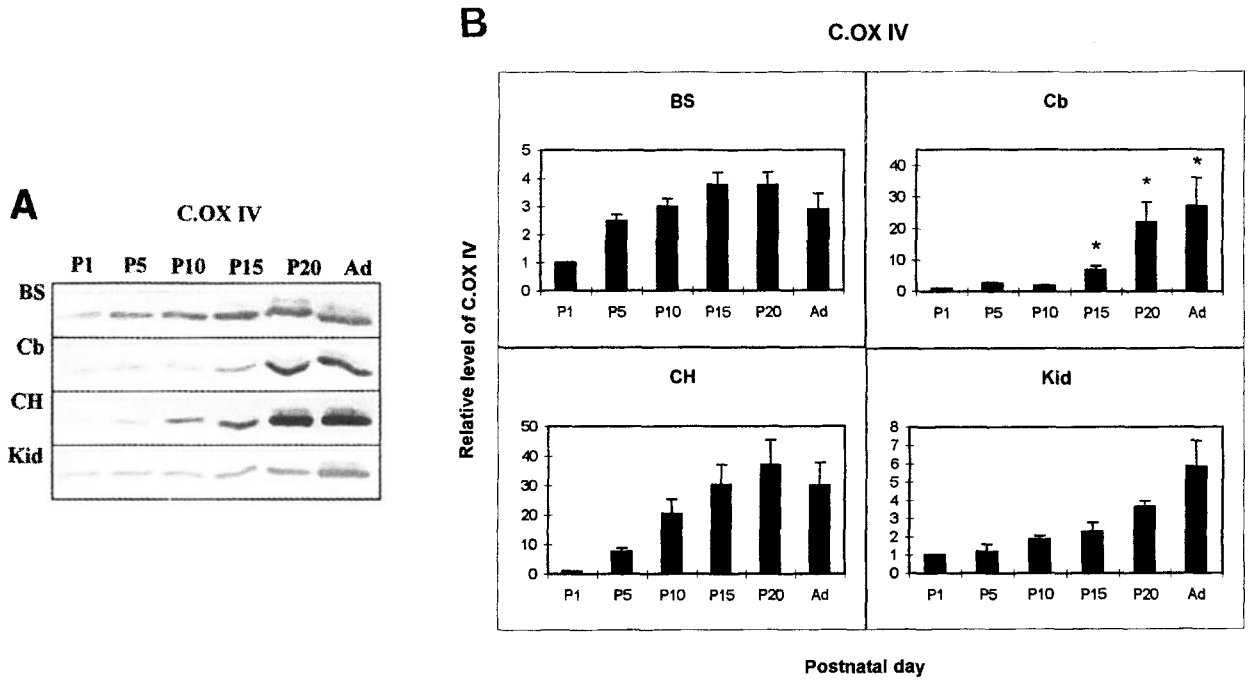

Fig. 7 Western blot analysis of C.OX IV protein levels during postnatal development of the rat. C.OX IV levels in various rat tissues were examined at postnatal days 1, 5, 10, 15, 20 (P1–20) and in the adult (Ad). (A) One hundred micrograms of protein were loaded per lane for brain stem (BS), cerebellum (Cb), cerebral hemispheres (CH) and kidney (Kid). (B) Quantitation of protein levels was carried out as in Figure 1B. Protein levels at postnatal day 1 were standardized to a value of one. Bar graphs represent the average of independent experiments carried out on 3 sets of animals. Error bars indicate the SEM. *Significantly different from postnatal day 1 ($P < 0.05$).

adult mammalian brain have shown Hsp90 protein to be expressed in neurons at abundant levels (Izumoto and Herbert 1993; Quraishi and Brown 1995). In addition, Hsp90 has been found to exist at higher levels in brain regions compared to non-brain regions in the adult rabbit (Quraishi and Brown 1995) as well as in the rat (Itoh et al 1993) by Western blot analysis. In the developing kidney, a dramatic decrease of Hsp90 was observed. Tanguay et al (1993), also found a decrease in Hsp90 protein levels in the adult mouse kidney compared to that of 11-day-old mice, although the magnitude of the decrease was not as large as that observed in this study.

Hsp90 binds to a wide variety of cellular proteins and is thought to play a role in a number of physiological processes, including chromatin condensation (Csermely et al 1994), protein synthesis (Pal et al 1996), protein folding (Wiech et al 1992) and protein trafficking (Pratt 1993). More importantly, it has been shown that Hsp90 functions in the regulation of the cell cycle (Jérôme et al 1993; Galea-Lauri et al 1996), growth and differentiation processes (Catelli et al 1989; Sass et al 1996). In this respect, Hsp90 may be involved in one or more of these phenomena during postnatal development of the rat brain and kidney.

Hsp90 has been shown to interact with several transcription factors, such as myoD (Shaknovich et al 1992), and has thus been implicated in the process of myogenesis (Sass et al 1996). The expression of Hsp90 mRNA was down-regulated in fully differentiated muscle cells, implying a role in muscle differentiation, but not in the maintenance of the mature muscle. In this study, a large developmental decrease in Hsp90 protein levels was observed in the kidney. Hsp90 may play a similar role in the transcriptional regulation of kidney cell growth during postnatal development. Possibly, Hsp90 may function in the developing kidney, but the requirement is decreased in the mature organ.

The developmental profile of Hsc70 protein levels did not vary greatly in the three brain regions which were examined. Abundant levels of Hsc70 were attained early in postnatal development of the nervous system and maintained in the adult. A developmental decrease in protein levels was evident in the kidney by Western blot analysis. Higher amounts of Hsc70 were detected in neural regions than in non-neural tissues at P1 and in the adult. Analysis of Hsc70 distribution in the developing rat brain by immunocytochemistry revealed a neuronal pattern of expression.

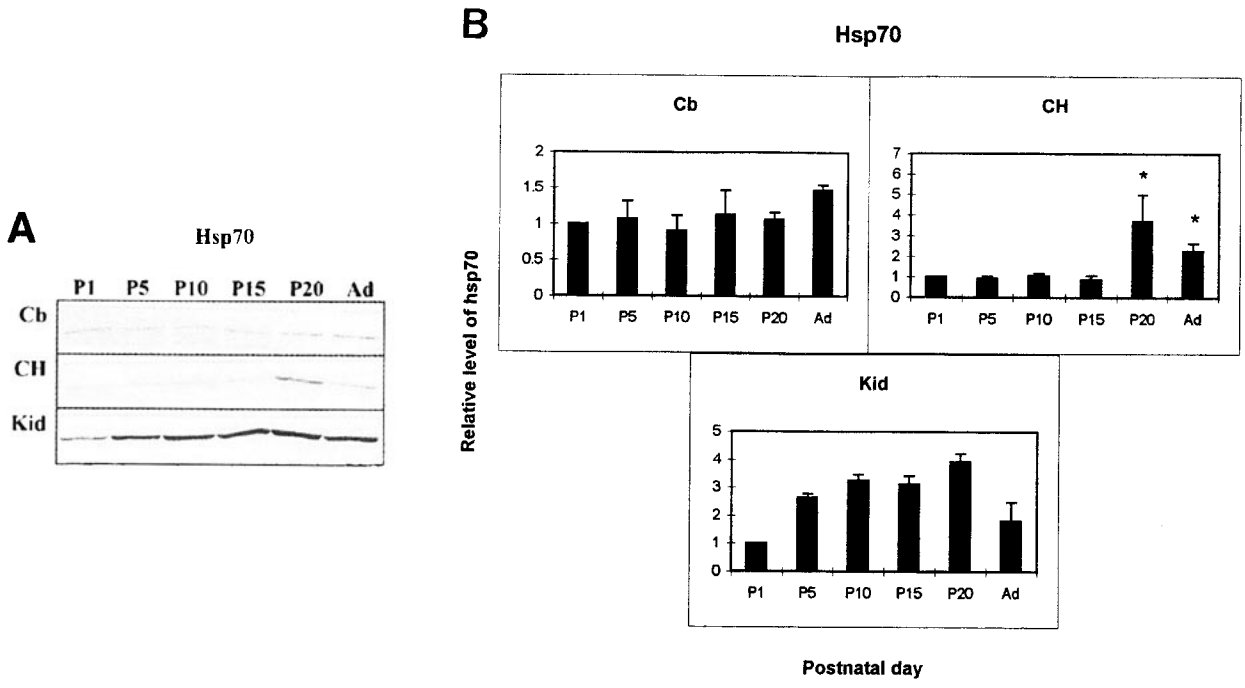

Fig. 8 Western blot analysis of Hsp70 protein levels during postnatal development of the rat. Hsp70 levels in various rat tissues was examined at postnatal days 1, 5, 10, 15, 20 (P1–20) and in the adult (Ad). (A) One hundred micrograms of protein were loaded per lane for cerebellum (Cb), cerebral hemispheres (CH) and kidney (Kid). (B) Quantitation of protein levels was carried out as in Figure 1B. Protein levels at postnatal day 1 were standardized to a value of one. Bar graphs represent the average of independent experiments carried out on 3 sets of animals. Error bars indicate the SEM. *Significantly different from postnatal day 1 ($P < 0.05$).

High amounts of Hsc70 mRNA and protein have been observed in neurons, such as Purkinje cells of the cerebellum, and spinal cord neurons, in the adult mammalian central nervous system (Aquino et al 1993; Manzerra et al 1993; Foster et al 1995). In addition, the adult mammalian brain appears to express higher levels of Hsc70 mRNA and protein compared to non-neural tissues (Giebel et al 1988; Manzerra et al 1997). The present results suggest that Hsc70 plays an important role in the developing as well as adult mammalian brain. Hsc70 has been shown to act as an ATPase in the removal of endocytic clathrin coats (Chappell et al 1986). In neurons, it may function in this manner in the synaptic vesicle recycling pathway. In the brain, Hsc70 has been implicated in axonal transport (deWaegh and Brady 1989) as well as neuronal signalling (Thekkuveettil and Lakhotia 1996).

Western blot analysis of Hsp60 protein levels during postnatal development showed large increases in all brain regions examined. A comparison of Hsp60 levels in neural and non-neural tissues revealed higher amounts of the protein in non-neural tissues at P1, in contrast to results obtained for Hsc70. Immunocytochemical analysis demonstrated a neuronal distribution of Hsp60 protein in the developing rat brain.

Hsp60 is a nuclear-encoded mitochondrial protein, therefore, changes in the expression pattern of Hsp60 during postnatal development may reflect changes in mitochondrial content. Chronic stimulation of skeletal muscle, which induces mitochondrial biogenesis has been shown to lead to an increase in Hsp60 protein levels in the adult rat (Ornatsky et al 1995). The developmental profile of another mitochondrial protein, cytochrome oxidase, subunit IV (C.OX IV), was investigated. In mammals, cytochrome oxidase exists as a 13-subunit complex (Kadenbach et al 1983) which is embedded into the inner mitochondrial membrane. It acts as the terminal component of the electron-transport chain and therefore plays a crucial role in oxidative metabolism. Levels of C.OX IV increased during postnatal development of the brain and kidney. Tissue-specific differences in the relative levels of C.OX IV were also comparable to that of Hsp60. Investigation of C.OX IV localization in the brain revealed a neuronal pattern of expression, similar to that for Hsp60.

These results suggest that the increase in Hsp60 and C.OX IV levels observed during postnatal development of the brain and kidney may reflect an increase in mitochondrial content. Mitochondrial biogenesis has been reported to occur postnatally in the rat brain (Giuffrida et al 1979; Renis et al 1989). A rapidly growing cell would likely require an increase in mitochondria; moreover, the high metabolic demands of neuronal activity might

explain the localization of these two mitochondrial proteins to neurons in the brain. The neuronal expression of C.OX IV protein detected in this study is consistent with that found by other researchers (Hevner and Wong-Riley 1991; Hevner et al 1995).

Analysis of Hsp70 protein levels showed low basal amounts in the unstressed brain and a developmental increase in Hsp70 protein levels in the cerebral hemispheres. In contrast to the brain, the kidney was found to express Hsp70 at high levels throughout postnatal development. Basal levels of Hsp70 mRNA and protein have been previously reported in the unstressed adult brain and kidney (Tanguay et al 1993; Foster and Brown 1996a, 1996b; Manzerra et al 1997). In addition, other tissues, such as the retina (Barbe et al 1988; Manzerra et al 1997) and muscle (Locke et al 1991) have also been shown to express basal levels of Hsp70 protein.

Hsp70 is generally thought of as an indicator of cellular stress. Following heat shock, or other forms of cellular stress, it is induced in both neural as well as non-neural tissues (for review, see Welch 1993; Brown 1994). The presence of Hsp70 under normal physiological conditions may reflect a state of constant stress. For example, tissues such as the kidney, which experience high concentrations of toxic metabolites, may require the protective effects of basal Hsp70.

With the exception of the basal levels of stress-inducible Hsp70, Western blot analysis showed all of the Hsps examined (Hsp90, Hsc70, Hsp60) to be abundant in the adult brain. Hsp90 and Hsc70 were expressed at higher levels in brain regions compared to non-brain tissues in the adult. Immunocytochemical studies demonstrated that these Hsps were localized to neurons in the developing rat brain. Neurons are structurally and functionally very complex cells and therefore may require high constitutive levels of these Hsps. In addition, because neurons are non-mitotic and cannot be replaced, they are vulnerable to damage in the brain and high levels of Hsps may serve a pre-protective purpose.

In summary, the various heat shock proteins investigated appear to be differentially regulated during postnatal development of the rat. While levels of Hsp90 decrease slightly in certain brain regions, such as the cerebellum, Hsp60 levels increase. The developmental profile of neural Hsc70 protein revealed abundant levels through postnatal development and in the adult.

It is known that some of these heat shock proteins exist in the cell as heterocomplexes. For example, Hsp90 has been found in a complex with Hsp70, along with another heat shock protein, Hsp56 (Pratt and Welsh 1994). An interesting question is whether the Hsps in the heterocomplex are regulated together or separately during development. Another question regards the developmental regulation of Hsps. The heat shock transcription factor Hsf2 is activated during hemin-induced differentiation of human K562 erythroleukemia cells (Sistonen et al 1992). This transcription factor is active during mouse embryogenesis and is thought to play a role in cellular differentiation processes, however, the localization of Hsf2 expression during mouse embryogenesis does not parallel the sites of Hsp70 expression in the nervous system (Rallu et al 1997). Other transcription factors and/or promoter elements may play a role in the developmental regulation of heat shock proteins.

ACKNOWLEDGMENTS

We thank A. C. Wikström, R. Tanguay and R. Gupta for the generous donations of the antibodies (29A from A.C.W., 1477 from R.T., anti-Hsp60 from R.G.). This work was supported by grants to I.R.B. from the MRC (Canada). We also thank Sheila Rush for suggestions on the manuscript, Pat Manzerra for advice on Western blotting and immunocytochemistry, and Douglas Bors for assistance with the statistical analysis.

REFERENCES

Akner G, Mossberg K, Sundqvist KG, Gustafsson JA and Wilkström AC (1992) Evidence for reversible, non-microtubule and non-microfilament-dependent nuclear translocation of Hsp90 after heat shock in human fibroblasts. Eur. J. Cell Biol. **58**, 356–364.

Aquino DA, Klipfel AA, Brosnan CF and Norton WT (1993) The 70-kDa heat shock cognate protein (Hsc70) is a major constituent of the central nervous system and is up-regulated only at the mRNA level in acute experimental autoimmune encephalomyelitis. J. Neurochem. **61**, 1340–1348.

Barbe MF, Tytell M, Gower DJ and Welch WJ (1988) Hyperthermia protects against light damage in the rat retina. Science **241**, 1817–1820.

Bédard PA and Brandhorst BP (1986) Translational activation of maternal mRNA encoding the heat-shock protein Hsp90 during sea urchin embryogenesis. Dev. Biol. **117**, 286–293.

Brown IR (1994) Induction of heat shock genes in the mammalian brain by hyperthermia and tissue injury. In: Heat Shock Proteins in the Nervous System, eds RJ Mayer and IR Brown, Academic Press, London, 31–53.

Catelli MG, Ramachandran C, Gauthier Y, Legagneux V, Quelard C, Baulieu EE and Shyamala G (1989) Developmental regulation of murine mammary-gland 90 kDa heat-shock proteins. Biochem. J. **258**, 895–901.

Chappell TG, Welch WJ, Schlossman DM, Palter KB, Schlesinger MJ and Rothman JE (1986) Uncoating ATPase is a member of the 70 kilodalton family of stress proteins. Cell **45**, 3–13.

Csermely P, Kajtar J, Hollosi M, Oikarinen J and Somogyi J (1994) The 90 kDa heat shock protein (Hsp90) induces the condensation of the chromatin structure. Biochem. Biophys. Res. Commun. **202**, 1657–1663.

de Waegh S and Brady ST (1989) Axonal transport of a clathrin uncoating ATPase (Hsc70): a role for Hsc70 in the modulation of coated vesicle assembly in vivo. J. Neuro. Res. **23**, 433–440.

Ding D, Parkhurst SM, Halsell SR and Lipshitz HD (1993) Dynamic *Hsp83* RNA localization during *Drosophila* oogenesis and embryogenesis. Mol. Cell. Biol. **13**, 3773–3781.

Ellis RJ and van der Vies SM (1991) Molecular chaperones. Annu. Rev. Biochem. **60**, 321–347.

Foster JA, Rush SJ and Brown IR (1995) Localization of constitutive and hyperthermia-inducible heat shock mRNAs (Hsc70 and Hsp70) in the rabbit cerebellum and brainstem by non-radioactive in situ hybridization. J. Neurosci. Res. **41**, 603–612.

Foster JA and Brown IR (1996a) Basal expression of stress-inducible Hsp70 mRNA detected in hippocampal and cortical neurons of normal rabbit brain. Brain Res. **724**, 73–83.

Foster JA and Brown IR (1996b) Intracellular localization of heat shock mRNAs (Hsc70 and Hsp70) to neural cell bodies and processes in the control and hyperthermic rabbit brain. J. Neurosci. Res. **46**, 652–665.

Foster JA and Brown IR (1997) Differential induction of heat shock mRNA in oligodendrocytes, microglia, and astrocytes following hyperthermia. Mol. Brain Res. **45**, 207–218.

Galea-Lauri J, Latchman DS and Katz DR (1996) The role of the 90-kDa heat shock protein in cell cycle control and differentiation of the monoblastoid cell line U937. Exp. Cell Res. **226**, 243–254.

Gething MJ and Sambrook J (1992) Protein folding in the cell. Nature **355**, 33–45.

Giebel LB, Dworniczak BP and Bautz EK (1988) Developmental regulation of a constitutively expressed mouse mRNA encoding a 72-kDa heat shock-like protein. Dev. Biol. **125**, 200–207.

Giuffrida AM, Gadaleta MN, Serra I, Renis M, Geremia E, Del Prete G and Saccone C (1979) Mitochondrial DNA, RNA, and protein synthesis in different regions of developing rat brain. Neurochem. Res. **4**, 37–52.

Hevner RF and Wong-Riley MT (1991) Neuronal expression of nuclear and mitochondrial genes for cytochrome oxidase (CO) subunits analyzed by in situ hybridization: comparison with CO activity and protein. J. Neurosci. **11**, 1942–1958.

Hevner RF, Liu S and Wong-Riley MTT (1995) A metabolic map of cytochrome oxidase in the rat brain: histochemical, densitometric and biochemical studies. Neuroscience **65**, 313–342.

Itoh H, Tashima Y, Eishi Y and Okeda R (1993) Localization of Hsp90 in rat brain. Int. J. Biochem. **25**, 93–99.

Izumoto S and Herbert J (1993) Widespread constitutive expression of Hsp90 messenger RNA in rat brain. J. Neurosci. Res. **35**, 20–28.

Jakob U and Buchner J (1994) Assisting spontaneity: the role of Hsp90 and small Hsps as molecular chaperones. Trends Biochem. Sci. **19**, 205–211.

Jérôme V, Vourc'h C, Baulieu EE andCatelli MG (1993) Cell cycle regulation of the chicken Hsp90α expression. Exp. Cell Res. **205**, 44–51.

Kadenbach B, Jarausch J, Hartmann J and Merle P (1983) Separation of mammalian cytochrome c oxidase into 13 polypeptides by a sodium dodecyl sulfate-gel electrophoretic procedure. Anal. Biochem. **129**, 517–521.

Kang KI, Devin J, Cadepond F, Jibard N, Guiochon-Mantel A, Baulieu EE and Catelli MG (1994) In vivo functional protein–protein interaction: nuclear targeted Hsp90 shifts cytoplasmic steroid receptor mutants into the nucleus. Proc. Natl Acad. Sci. USA **91**, 340–344.

Kothary R, Perry MD, Moran LA and Rossant J (1987) Cell-lineage-specific expression of the mouse hsp68 gene during embryogenesis. Dev. Biol. **121**, 342–348.

Krone PH and Sass JB (1994) Hsp90α and Hsp90β genes are present in the zebrafish and are differentially regulated in developing embryos. Biochem. Biophys. Res. Commun. **204**, 746–752.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature **227**, 680–685.

Lindquist S and Craig EA (1988) The heat shock proteins. Annu. Rev. Genet. **22**, 631–677.

Locke M, Noble EG and Atkinson BG (1991) Inducible isoform of Hsp70 is constitutively expressed in a muscle fiber type specific pattern. Am. J. Physiol. **261**, C774–C779.

Manzerra P, Rush SJ and Brown IR (1993) Temporal and spatial distribution of heat shock mRNA and protein (Hsp70) in the rabbit cerebellum in response to hyperthermia. J. Neurosci. Res. **36**, 480–490.

Manzerra P and Brown IR (1996) The neuronal stress response: nuclear translocation of heat shock proteins as an indicator of hyperthermic stress. Exp. Cell Res. **229**, 35–47.

Manzerra P, Rush SJ and Brown IR (1997) Tissue-specific differences in heat shock protein Hsc70 and Hsp70 in the control and hyperthermic rabbit. J. Cell Physiol. **170**, 130–137.

Minami Y, Kawasaki H, Suzuki K and Yahara I (1993) The calmodulin-binding domain of the mouse 90-kDa heat shock protein. J. Biol. Chem. **268**, 9604–9609.

Mizzen LE, Chang C, Garrels JI and Welch WJ (1989) Identification, characterization, and purification of two mammalian stress proteins present in mitochondria, grp75, a member of the Hsp70 family and hsp58, a homolog of the bacterial groEL protein. J. Biol. Chem. **264**, 20664–20675.

Neupert W and Pfanner N (1993) Roles of molecular chaperones in protein targeting to mitochondria. Phil. Trans. R. Soc. Lond. B. **339**, 355–362.

Nishida E, Koyasu S, Sakai H and Yahara I (1986) Calmodulin-regulated binding of the 90-kDa heat shock protein to actin filaments. J. Biol. Chem. **261**, 16033–16036.

Oppermann H, Levinson W and Bishop JM (1981) A cellular protein that associates with the transforming protein of Rous sarcoma virus is also a heat shock protein.. Proc. Natl Acad. Sci. USA **78**, 1067–1071.

Ornatsky OI, Conner MK and Hood DA (1995) Expression of stress proteins and mitochondrial chaperonins in chronically stimulated skeletal muscle. Biochem. J. **311**, 119–123.

Pal JK, Anand S and Joseph J (1996) Association of Hsp90 with the heme-regulated eukaryotic initiation factor 2α kinase – a collaboration for regulating protein synthesis. J. Biosci. **21**, 191–205.

Parsell DA and Lindquist S (1993) The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annu. Rev. Genet. **27**, 437–496.

Pratt WB (1993) The role of heat shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor. J. Biol. Chem. **268**, 21455–21458.

Pratt WB and Welsh MJ (1994) Chaperone functions of the heat shock proteins associated with steroid receptors. Semin. Cell Biol. **5**, 83–93.

Quraishi H and Brown IR (1995) Expression of heat shock protein 90 (Hsp90) in neural and nonneural tissues of the control and hyperthermic rabbit. Exp. Cell Res. **219**, 358–363.

Rallu M, Loones MT, Lallemand Y, Morimoto RI, Morange M and Mezger V (1997) Function and regulation of heat shock factor 2 during mouse embryogenesis. Proc. Natl Acad. Sci. USA **94**, 2392–2397.

Renis M, Cantatore P, Loguercio Polosa P, Fracasso F and Gadaleta MN (1989) Content of mitochondrial DNA and of three mitochondrial RNAs in developing and adult rat cerebellum J. Neurochem. **52**, 750–754.

Rose DW, Wettenhall EH, Kudlicki W, Kramer G and Hardesty B (1987) The 90-kilodalton peptide of the heme-regulated eIF-2α

kinase has sequence similarity with the 90-kilodalton heat shock protein. Biochemistry **26**, 6583–6587.
Sanchez ER, Redmond T, Scherrer LC, Bresnick EH, Welsh MJ and Pratt WB (1988) Evidence that the 90-kilodalton heat shock protein is associated with tubulin-containing complexes in L cell cytosol and in intact PtK cells. Mol. Endocrinol. **2**, 756–760.
Sass JB, Weinberg ES and Krone PH (1996) Specific localization of zebrafish *Hsp90α* mRNA to *myoD*-expressing cells suggests a role for Hsp90α during normal muscle development. Mech. Dev. **54**, 195–204.
Shaknovich R, Shue G and Kohtz DS (1992) Conformational activation of a basic helix-loop-helix protein (MyoD1) by the C-terminal region of murine Hsp90 (hsp84). Mol. Cell. Biol. **12**, 5059–5068.
Sistonen L, Sarge KD, Phillips B, Abravaya K and Morimoto RI (1992) Activation of heat shock factor 2 during hemin-induced differentiation of human erythroleukemia cells. Mol. Cell. Biol. **12**, 4104–4111.
Subjeck JR and Shyy TT (1986) Stress protein systems of mammalian cells. Am. J. Physiol. **250**, C1–C17.
Taanman JW, Hall RE, Tang C, Marusich MF, Kennaway NG and Capaldi RA (1993) Tissue distribution of cytochrome c oxidase isoforms in mammals. Characterization with monoclonal and polyclonal antibodies. Biochim. Biophys. Acta **1225**, 95–100.
Tanguay RM, Wu Y and Khandjian EW (1993) Tissue-specific expression of heat shock proteins of the mouse in the absence of stress. Dev. Genet. **14**, 112–118.
Thekkuveettil A and Lakhotia SC (1996) Regulation of Hsp70 in excitatory neurons: possible implications for neuronal functioning. J. Biosci. **21**, 631–640.
Viitanen PV, Lorimer GH, Seetharam R, Gupta RS, Oppenheim J, Thomas JO and Cowan NJ (1992) Mammalian mitochondrial chaperonin 60 functions as a single toroidal ring. J. Biol. Chem. **267**, 695–698.
Welch WJ (1992) Mammalian stress response: cell physiology, structure/function of stress proteins, and implications for medicine and disease. Physiol. Rev. **72**, 1063–1081.
Welch WJ (1993) How cells respond to stress. Sci. Am. **268**, 57–64.
Welch WJ and Suhan JP (1986) Cellular and biochemical events in mammalian cells during and after recovery from physiological stress. J. Cell. Biol. **103**, 2035–2052.
Wiech H, Buchner J, Zimmermann R and Jakob U (1992) Hsp90 chaperones protein folding in vitro. Nature **358**, 169–170.
Wu B, Hunt C and Morimoto RI (1985) Structure and expression of the human gene encoding major heat shock protein Hsp70. Mol. Cell. Biol. **5**, 330–341.