Cloning, characterization, and functional studies of a human 40-kDa catecholamine-regulated protein: implications in central nervous system disorders

Joseph Gabriele · Giuseppe F. Pontoriero · Nancy Thomas · Christy A. Thomson · Kevin Skoblenick · Zdenek B. Pristupa · Ram K. Mishra

Received: 20 November 2008 / Revised: 9 February 2009 / Accepted: 19 February 2009 / Published online: 12 March 2009
© Cell Stress Society International 2009

Abstract Catecholamine-regulated proteins (CRPs) have been shown to bind dopamine and other structurally related catecholamines; in particular, the 40-kDa CRP (CRP40) protein has been previously cloned and functionally characterized. To determine putative human homologs, BLAST analysis using the bovine CRP40 sequence identified a human established sequence tag (EST) with significant homology (accession #BQ224193). Using this EST, we cloned a recombinant human brain CRP40-like protein, which possessed chaperone activity. Radiolabeled dopamine binding studies with recombinant human CRP40 protein demonstrated the ability of this protein to bind dopamine with low affinity and high capacity. The full-length human CRP40 nucleotide sequence was elucidated (accession #DQ480334) with RNA ligase-mediated rapid amplification of complementary DNA ends polymerase

chain reaction, while Northern blot hybridization suggested that human CRP40 is an alternative splice variant of the 70-kDa mitochondrial heat shock protein, mortalin. Human SH-SY5Y neuroblastoma cells treated with the antipsychotic drug, haloperidol, exhibited a significant increase in CRP40 messenger RNA expression compared to untreated control cells, while other dopamine agonists/antagonists also altered CRP40 expression and immunolocalization. In conclusion, these results show that we have cloned a splice variant of mortalin with a novel catecholamine binding function and that this chaperone-like protein may be neuroprotective in dopamine-related central nervous system disorders.

Keywords Catecholamine-regulated protein · CNS disorders · Dopamine · Molecular chaperone · Mortalin

Summary Previous studies have found a 40-kDa catecholamine-regulated protein (CRP40) with chaperone-like activities in the bovine brain. The experiments detail the discovery of a human isoform of said protein and that this isoform is an alternative splice variant of mortalin. The catecholamine-regulated properties of the protein also provide implications for the role of CRP40 in central nervous system disorders.

Joseph Gabriele and Giuseppe F. Pontoriero made equal contribution to this work.

J. Gabriele (✉) · G. F. Pontoriero · N. Thomas · K. Skoblenick · Z. B. Pristupa · R. K. Mishra
Department of Psychiatry and Behavioural Neurosciences,
McMaster University,
Hamilton, ON, Canada
e-mail: gabriejp@mcmaster.ca

C. A. Thomson
Biomedical Research Center, University of British Columbia,
Vancouver, BC, Canada

Abbreviation List

CRP	catecholamine-regulated protein
DA	dopamine
EST	established sequence tag
GST	glutathione <i>S</i> -transferase
HAL	haloperidol
HSP	heat shock proteins
PBS	phosphate-buffered saline
PKA	protein kinase A
STR	striatum

Introduction

One of the primary function of molecular chaperones is to aid in the proper folding and transport of proteins (Lanneau et al. 2008; Liberek et al. 2008; Tomala and Korona 2008). Moreover, molecular chaperones, also known as heat shock

proteins (HSPs), have additional functions such as: (1) acting as a transcription factors (Bagchi et al. 2001); (2) providing transient scaffolds in order to maintain elements of cellular networks (Tomala and Korona 2008; van Noort 2008); and (3) regulating apoptosis (van Noort 2008). Following apoptosis, HSPs also promote antigen presentation in adjacent cells (Buck et al. 2007; Calderwood et al. 2007). Protein denaturation caused by environmental and oxidative stresses (Buck et al. 2007; Liberek et al. 2008) can stimulate the expression of molecular chaperones, which subsequently bind to the damaged protein's hydrophobic amino acids (Dobson 2004). By stabilizing and refolding partially denatured proteins, chaperones are able to prevent aggregation and ultimately prevent apoptosis (Buck et al. 2007; Dobson 2004; Lanneau et al. 2008; van Noort 2008).

When normal cellular processes are disrupted by oxidative stress or other environmental factors, cells become more vulnerable to mutations and posttranslational aberrations, which in turn, contribute to improper folding and subsequent protein accumulation (Giffard et al. 2004; Muchowski and Wacker 2005; van Noort 2008). It is becoming clear that the mechanisms contributing to abnormal protein accumulation may be related to deficiencies in antistress responses, either through decreased molecular chaperone synthesis or inefficiencies in proteasomal and lysosomal degradation pathways (Min et al. 2008). This process becomes especially important for the senescence of postmitotic cells, including neurons, where such deficiencies can lead to greater aggregation of proteins (Soti and Csermely 2002a). Recent reports have now implicated inefficient molecular chaperone recruitment to the progression of many neurodegenerative and neurodevelopmental diseases such as Parkinson's disease, Alzheimer's disease, and schizophrenia (Irvine et al. 2008; Ohtsuka and Suzuki 2000; Sherman and Goldberg 2001; Soti and Csermely 2002a).

We have previously reported the presence of a unique class of brain-specific chaperone-like proteins, termed catecholamine-regulated proteins (CRP) and isolated three distinct species with molecular weights of 26, 40, and 47 kDa, based on their ability to bind to dopamine (DA) and structurally homologous catecholamines (Ross et al. 1993, 1995). Based on pharmacological and biochemical studies, no similarities to already classified receptors have emerged, indicating the novel nature of this protein (Modi et al. 1996; Ross et al. 1993, 1995). Molecular cloning of bovine brain CRP40 (GenBank #AF047009) revealed that this protein is related to the 70-kDa heat shock protein, HSP70, family (Nair and Mishra 2001). Bovine CRP40 shares significant sequence homology with human HSP70 (GenBank #BC024034), HSC70 (GenBank #NM006389), GRP78/BIP (GenBank #NM5347), and with the human established sequence tag (EST; GenBank #BQ224193).

Unlike HSP70, CRP40 is primarily modulated by DA receptor agonists and antagonists, as well as psychostimulants such as amphetamine and cocaine (Gabriele et al. 2002, 2005, 2007; Modi et al. 1996; Nair and Mishra 2001; Sharan et al. 2001, 2003). Interestingly, our laboratory has observed reduced CRP40 protein expression within human schizophrenic postmortem brain specimens, relative to control patients (Gabriele et al. 2005). Additionally, the study also showed that increased CRP40 protein expression is correlated with increased antipsychotic treatment in the schizophrenic profile group (Gabriele et al. 2005).

In this study, we describe the cloning, characterization, and cellular distribution of human CRP40. Alignment of the bovine CRP40 complementary DNA (cDNA) sequence relative to the human EST, BQ224193, allowed for the generation of specific primers. The entire human CRP40 messenger RNA (mRNA) sequence was elucidated using RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE). Our findings demonstrate that the entire human CRP40 nucleotide and subsequent amino acid sequence are identical to the carboxy-terminal portion of the human mitochondrial HSP70 family member, mortalin (Wadhwa et al. 1993a; Xie et al. 2000). Moreover, we show that recombinant human CRP40 (rhCRP40) has similar chaperone and catecholamine-regulated functions as bovine CRP40, in that it binds DA with low affinity and high capacity. Finally, we provide evidence that human CRP40 is localized within the mesocorticolimbic and nigrostriatal DA systems and is differentially expressed in response to various pharmacological treatments.

Methods and materials

Northern blot hybridization

Total RNA was isolated from human neuroblastoma SH-SY5Y cells using TRIzol reagent according to the manufacturer's protocol (Invitrogen Life Technologies, Burlington, ON, Canada). PolyA mRNA was isolated from 200 µg of total RNA using Oligo dT cellulose with a capacity of 10-mg RNA per gram of resin (Nair and Mishra 2001).

Twenty micrograms of polyA RNA from SH-SY5Y cells was separated on 1.0% formaldehyde agarose gel and transferred to Hybond nylon filters (Amersham, Piscataway, NJ, USA) which were baked at 80°C for 2 h. Prehybridization was carried out using ExpressHyb Hybridization Solution (Clontech Laboratories Inc., Mountain View, CA, USA) and 70 µl of heat-denatured salmon sperm DNA (Invitrogen), by rotating for 2 h at 68°C. A 740-bp cDNA probe corresponding to the EST, BQ224193, was next generated using the sense primer, 5'-ATG GAT TCT TCT GGA CCC AAG CAT -3' and 5'- TCG TTC CTT CTT TGG CCG GTT TTT T -3' and

labeled with α - 32 P-dCTP. The polymerase chain reaction (PCR) was carried out for 40 cycles, under the following conditions: 95°C for 15 s, 60°C for 30 s, and 72°C for 55 s. The Northern blot was then hybridized to α - 32 P-dCTP-labeled BQ224193 cDNA probes for 22 h at 45°C and washed with 2× saline–sodium citrate buffer. The same filters were hybridized with a β -actin probe as an internal control of both the RNA integrity and amount.

RNA ligase-mediated rapid amplification of cDNA ends

RLM-RACE PCR was performed using the GeneRacer Core Kit (Invitrogen Life Technologies, Burlington, ON, Canada) according to the manufacturer's detailed protocol. Briefly, total RNA from a human brain library (Ambion Inc., Austin, TX, USA) was used to amplify the BQ224193 fragment using the 5' BQ224193 primer, 5'-ATG GAT TCT TCT GGA CCC AAG CAT -3', and the 3' GeneRacer Oligo dT primer. The amplified PCR products were cloned into a pCR4-TOPO vector (Invitrogen) and transformed into One Shot TOP10 chemically competent BL-21 *Escherichia coli* cells (Invitrogen). The DNA was extracted from an overnight culture of the transformed colonies and sequenced.

Cloning of human CRP40

Human brain RNA (Ambion Inc., Austin, TX, USA) was first reverse-transcribed using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA). A 1,047-bp DNA fragment was amplified using the sense primer, 5'-ATG GAT TCT TCT GGA CCC AAG CAT-3', and the antisense primer, 5'-TTA CTG TTT TTC CTC CTT TTG ATC TTC TCG TTC-3', derived from the EST, BQ224193. The PCR was carried out for 40 cycles under the following conditions: 95°C for 45 s, 60°C for 60 s, and 72°C for 90 s. The 1,047-bp band was extracted and purified from a 1.5% agarose gel using the Qiagen gel extraction kit (Qiagen Inc., Mississauga, ON, Canada) and sequenced to confirm its identity.

*Bam*HI and *Sma*I restriction enzyme sites were introduced at the 5' end of the sense and antisense primers, respectively, to facilitate cloning. Subsequent PCR using the same conditions as above revealed a fragment, which was subsequently purified from an agarose gel as described above. The pGEX-2T vector (GE Healthcare Life Sciences, Piscataway, NJ, USA) and the 1,047-bp fragment were digested using the restriction enzymes, *Bam*HI (Invitrogen Life Technologies, Burlington, ON, Canada) and *Sma*I (Fermentas, Burlington, ON, USA), according to the manufacturers' recommended protocols. The digested vector and human CRP40 fragments were run on a 0.7% agarose gel and purified. The 1,047-bp fragment was ligated to the pGEX-2T vector using T4 DNA ligase (Fermentas) over-

night at 25°C. The ligation reaction was then transformed into One Shot TOP10 chemically competent BL-21 *E. coli* cells (Invitrogen) according to the manufacturer's recommended protocol, then plated on Luria–Bertani (LB) media with ampicillin (100 μ g /ml) overnight at 37°C. Colonies were then picked, regrown at 37°C, and sequenced to determine successful glutathione *S*-transferase (GST)-CRP40 clones.

Expression of recombinant human CRP40

Colonies positive for GST-CRP40 were grown at 37°C overnight with shaking in LB media with ampicillin. Bacterial cultures were grown until the optical density at 600 nm was at least 0.6. Cultures were subsequently induced with isopropyl β -D-1-thiogalactopyranoside (0.1 mM) at 16°C for 22 h in order to initiate transcription of the GST-CRP40 fusion protein. Cultures were then spun down and the supernatants were discarded. The bacterial pellets were resuspended in phosphate-buffered saline (PBS) with Complete Mini Protease Inhibitor tablets (Roche) and lysed with a French press. Following lysis, lysates were centrifuged and the clear supernatants were saved. A 50% glutathione sepharose 4B slurry (Amersham, Piscataway, NJ, USA) was then added. The bound GST-CRP40 fusion protein–glutathione sepharose matrix was added to an empty disposable column and incubated at 4°C overnight with shaking. Following the washes (1× PBS containing 0.1% Tween 20 and 1 M NaCl), the bound fusion protein was liberated from the glutathione sepharose beads by adding thrombin protease (100 units) overnight at 22°C to cleave the GST protein tag. Fractions were eluted from the column (1 ml PBS×4) and protein concentration was determined using the Bradford (1976) method. SDS-PAGE electrophoresis was carried out as previously described by our laboratory (Gabriele et al. 2003, 2005), followed by Coomassie gel staining. Protein sequencing analyses carried out by the University of Alberta, confirmed the presence of rhCRP40. Phenylmethylsulfonyl fluoride (PMSF; 0.15 mM) was added to each fraction and incubated at 37°C for 15 min. Fractions were stored at –20°C for future experiments.

Luciferase chaperone assay

To assess rhCRP40 chaperone activity, firefly luciferase (100 nM, Sigma-Aldrich, Oakville, ON, Canada) alone or mixed with rhCRP40 (100 nM) was equilibrated to room temperature in buffer (40 mM HEPES, pH 7.9) and incubated at 42°C in a 24-well optical plate. Light scattering by protein aggregation was determined in quintuplicate by measuring the increase in optical density at 320 nm with a Safire² plate reader (TECAN Group Ltd., Durham, NC, USA).

Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay was used to assess cell viability. SH-SY5Y cells were first transfected with either the pEGFP-C1 mammalian expression vector encoding human CRP40 or the empty vector alone, using Lipofectamine 2000 (Invitrogen), according to the manufacturer's recommended protocol. Cells were then treated with a 10 μ M H₂O₂/10 μ M FeSO₄ solution for 16 h at 37°C. Following H₂O₂/FeSO₄ treatment, cells were incubated with MTT (5 mg/mL) for 4 h at 37°C and subsequently treated with dimethyl sulfoxide, to liberate formazan crystals. The absorbance of individual wells was read at 560 nm, with background correction at 670 nm, using a Safire² plate reader (TECAN Group Ltd., Durham, NC, USA). Experiments were performed in triplicate and data are presented as the percentage of viable cells as compared with untransfected untreated SH-SY5Y cells.

Radiolabeled dopamine binding

The ability of rhCRP40 to bind DA was assessed by competitively binding [³H]-DA in the presence of different concentrations of unlabeled DA (in 0.1% ascorbic acid). Briefly, the binding of [³H]-DA to the rhCRP40 was carried out in triplicate in 1.0 mL of assay buffer (pH 7.4, 50 mM Tris-HCl, 1 mM EDTA, 5 mM MgCl₂, 0.1 mM dithiothreitol, 0.1 mM PMSF, 100 mg/mL bacitracin, and 5 mg/mL soybean trypsin inhibitor) containing 5 nM of radioligand, the indicated concentrations of unlabeled DA (0.1, 1, 5, 10, 50, 100, 500 nM, 1, 5, 10, 50, 100, 500 μ M, 1, and 2.5 mM), and 20 μ g of rhCRP40. Incubation of the rhCRP40 with ligands was carried out at 37°C for 2 h in darkness. At the end of the incubation period, the bound and free ligands were separated by vacuum filtration through Whatman GF/B filters. The filters were washed with 3 $\times$ 5 mL of Tris-EDTA buffer (pH 7.4, 50 mM Tris-HCl, 1 mM EDTA) and the radioactive counts were determined on a Beckman scintillation counter (model 1780). Nonspecific binding was assessed through the addition of excess DA (10 mM).

Real-time PCR

DA D_{2L} receptor-transfected SH-SY5Y cells were first treated with 1 μ M haloperidol for 24 h in serum-free medium and compared to untreated control cell samples. Total RNA was extracted from the cells using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's recommended protocol. One microgram of total RNA was next reverse-transcribed using SuperScript II as per the protocol provided by the supplier (Invitrogen). One hundred nanograms of the cDNA were used for each

real-time PCR experiment and were executed in triplicate. PCR reactions lacking cDNA template and reverse transcriptase were also included as controls. Human CRP40 mRNA transcript levels were measured by two-step real-time PCR using a StratGene MX300P sequence detection system (StrataGene, La Jolla, CA, USA). The primers utilized for the reaction were: forward, 5'-TTG GCC GGC GAT GTC ACG GAT GTG-3', and reverse, 5'-ACA CAC TTT AAT TTC CAC TTG CGT-3'. Real-time PCR reaction conditions were optimized to ensure that amplification was occurring in the exponential phase and that the efficiencies were consistent throughout the course of the reaction. The real-time PCR consisted of: 10 μ l of 2 $\times$ SYBR green (Qiagen), 300 nM of forward primer, 300 nM of reverse primer, 100 ng of template cDNA, and nuclease-free H₂O mixed to a final volume of 20 μ l. Real-time PCR conditions were: 50°C for 30 s (1 cycle), 95°C for 15 min (1 cycle), 95°C for 15 s, 60°C for 30 s, 72°C for 40 s (40 cycles). A final dissociation curve step was included to ensure that a single PCR product was obtained. To calculate the absolute CRP40 mRNA copy number, a standard curve was constructed using a double-amplified and purified cDNA sample (Bio-Rad, Hercules, CA, USA) with six concentrations ranging from 1 pg to 10 ag. Statistical analysis of the data was performed in GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA). Student's *t* test was used to compare the mean values and to determine any significant differences. The expression of the mRNA encoding human cyclophilin was assessed in control experiments using the forward primer, 5'-GCA AGA CCA GCA AGA AGA-3', and reverse primer, 5'-CAG CGA GAG CAC AAA GAT-3'.

Polyclonal antibody production

rhCRP40 protein was loaded onto a 12% SDS-PAGE gel and electrophoresis separation was performed. The gel was then transferred to a nitrocellulose membrane and a distinct single band at approximately 40 kDa was identified using Ponceau-S staining. The band was carefully cut out, minced into very small pieces, and ground to a smooth slurry in 1 ml of PBS using a 1.5 ml dounce hand homogenizer. Two New Zealand rabbits (Charles River Laboratories, Wilmington, MA, USA) were subcutaneously injected (30 μ g of protein per injection) on the nape of the neck with the slurry containing the rhCRP40 protein, at four injection sites on days 0, 14, 42, and 134, while bleeds were performed on days 13, 32, 50, 89, and 146 (Diano et al. 1987). Antibodies were purified using the Protein A antibody purification kit (Sigma-Aldrich).

Primary culture of murine midbrain neurons

Midbrain sections were removed from 15-day old mouse embryos and mechanically dissociated using a Pasteur

pipette tip against a 0.05-mm² wire mesh. Cells passing through the wire sieve were collected into Ca²⁺- and Mg²⁺-free PBS solution (Sigma-Aldrich). The number of cells in the suspension was determined and cells were subsequently plated onto 10-mm round coverslips at a density of 10⁴ cells per coverslip. Five hundred microliters of Neurobasal media supplemented with B27 and Glutamax 2 mM (Sigma-Aldrich) were added to each well and cells were cultured at 37°C and 5% CO₂.

Cell cultures were used 7–9 days postplating and received treatment overnight with one of the following diluted in PBS: haloperidol (1 µM), SCH23390 (1 µM), SKF28393 (10 µM), and a protein kinase A inhibitor (10 µM). Control slides received an equivalent volume of PBS.

Immunofluorescence

Following antipsychotic drug treatment, primary cell cultures were fixed using 4% formaldehyde diluted in PBS. Following a 30-min fixation period, coverslips were washed twice for 5 min with PBS and incubated in 5% donkey serum (Sigma-Aldrich) for 1 h. After blocking, the serum solution was replaced with rabbit anti-rhCRP40 primary antibody at a concentration of 1:150 diluted in PBS containing 0.6% Triton X-100 (Bio-Rad). Cells were incubated overnight in the primary antibody solution then washed 3×5 min in PBS. Fluorescein-isothiocyanate-conjugated antirabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) was applied to the cells at a concentration of 1:100, diluted in PBS containing 0.6% Triton X-100 for 4 h at room temperature. The coverslips were then washed 6×5 min in PBS and mounted to slides using VectaShield mounting media (Vector Labs, Burlingame, CA, USA). In control experiments, incubation reactions with rabbit anti-rhCRP40 primary antibody preabsorbed with rhCRP40 failed to yield any appreciable signal. Slides were visualized on a Carl Zeiss LSM 510 confocal microscope (Carl Zeiss, North York, ON, Canada) connected to a computer running the LSM 510 Image Examiner software. Scale bars were measured using the LSM 510 Image Browser software and Adobe Photoshop 7.0. ImageJ (National Institutes of Health, Bethesda, MD, USA) software was used to quantify illuminated pixel count per image field with identical threshold values maintained between all images. Statistical analysis of the data was performed in GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA). A one-way analysis of variance was used to compare the mean values and to determine where significant differences existed among these groups a Tukey's multiple-comparison post hoc test was used.

In order to determine the *in vivo* distribution of human CRP40, three specimens of the ventral striatum region of

the brain, generously donated by the Stanley Foundation Neuropathology Consortium, were utilized. The slides consisted of a normal control, a schizophrenia patient free from antipsychotic treatment, and a schizophrenic patient treated with haloperidol (lifetime dose was measured as 1.5×10⁷ mg fluphenazine equivalents). Human CRP40 immunolocalization was carried out as described above.

Statistical analysis

All data are presented as the mean±standard error of the mean (SEM), while Student's *t* test was used to compare differences between experimental and control groups. Statistical analyses were performed using GraphPad Prism software (Intuitive Software for Science, San Diego, CA, USA).

Results

Determination of the human CRP40 mRNA sequence

Previous studies from our laboratory have described the cloning and functional characterization of bovine CRP40 (Nair and Mishra 2001). BLAST analysis revealed that bovine CRP40 shares significant sequence homology with the EST, BQ224193. Thus, specific primers, identical to distinct BQ224193 sequences, were generated to clone and characterize the human CRP40 isoform. These primers were first utilized in order to generate an mRNA probe that was subsequently hybridized to a population of polyA-selected mRNA derived from the human SH-SY5Y neuroblastoma cell line. Northern blot analysis identified the presence of two distinct bands, of approximately 2,850 and 1,750 bp in length, following hybridization with the BQ224193 mRNA probe (Fig. 1a). This finding was also observed following hybridization to polyA-selected mRNA derived from rat, bovine, and human striata (data not shown).

In order to clone and determine the human CRP40 mRNA nucleotide sequence, the specific BQ224193 primers were used for RLM-RACE. Amplification from the 3' end of the CRP40 mRNA transcript using 3' RLM-RACE yielded a number of different fragment sizes (Fig. 1b). The most intriguing RLM-RACE product obtained from the PCR reaction was 1,743 bp in length, corresponding to the smaller 1,750-bp fragment observed following Northern blot hybridization.

DNA sequencing of the 3' RLM-RACE product revealed the human CRP40 mRNA sequence (Fig. 2). The human CRP40 mRNA transcript consists of 1,743 bp that encodes a 346-amino-acid polypeptide. Based on this amino acid sequence, the predicted molecular weight is approximately 38 kDa. Nucleotide and protein BLAST analyses of the human CRP40 mRNA transcript against all other human

Fig. 1 Characterization of human CRP40. **a** Northern blot hybridization probing 50 μ g of polyA RNA, derived from human SH-SY5Y neuroblastoma cells, with a [32 P]-cDNA probe. The probe corresponds to a fragment of the EST, BQ224193, which is homologous to bovine CRP40. Hybridization revealed the presence of two homologous RNA sequences that were 2,850 and 1,750 bp in length. The blot was stripped and reprobed with a β -actin probe to control for accurate RNA loading and to ensure adequate RNA integrity. **b** 3' RLM-RACE PCR using primers derived from BQ224193 and human brain RNA revealed the presence of a 1,743-bp band. BLAST analysis confirmed its identity as a splice variant of mortalin

mRNA and protein sequences revealed that human CRP40 is 100% identical to a portion of the mRNA transcribed from the human HSPA9 gene, encoding the 70-kDa mitochondrial heat-shock-like protein, mortalin.

Recombinant human CRP40 possesses distinct protective effects and dopamine binding activity

In order to functionally characterize the CRP40 protein, forward and reverse primers flanking the predicted open reading frame of the mRNA transcript were generated. These primers were then employed to amplify a 1,047-bp DNA fragment (encoding the predicted human CRP40 peptide sequence) from a human brain cDNA library (Fig. 3a). The 1,047-bp amplicon was subsequently cloned within the PGEX-2T expression vector system to produce GST-rhCRP40 fusion protein (66 kDa; Fig. 3b). Following column purification and thrombin protease digestion to remove the 26-kDa GST tag, a single pure 40-kDa fragment was liberated. In gel digestion followed by trypsin digestion and mass spectrometry, the 40-kDa protein band was correctly identified to be rhCRP40, a variant of human mortalin.

Fig. 2 Human CRP40 sequence. Sequencing of the 3' RLM-RACE PCR product revealed the identity of the 1,743-bp human CRP40 mRNA transcript. mRNA sequence is represented in *lower case letters*. The corresponding 344 amino acid sequence is positioned above each codon in *upper case letters*. Underlined sequences represent the 3'-untranslated region, while *asterisk* indicates the presence of a stop codon

The mitochondrial heat-shock-like protein, mortalin, is a chaperone protein that functions in a number of intracellular processes such as mitochondrial biogenesis, cell proliferation, and protein trafficking (Kaul et al. 2007). Thus, the ability of human CRP40 to also act as a multifunctional chaperone protein was also determined. Firstly, firefly luciferase was incubated at 42°C, in the presence and absence of rhCRP40 protein; the degree of luciferase light scattering was then assessed by observing the absorbance at 320 nm. Incubation in the presence of rhCRP40 significantly reduced the degree of denaturation and aggregation following both 20 and 30 min of heat shock at 42°C (Fig. 4a). Furthermore, in order to determine the protective effects of human CRP40 overexpression, SH-SY5Y neuroblastoma cells were transfected with either human CRP40 or the empty expression vector. Following transfection, cells were cultured in the presence of H₂O₂/FeSO₄ and assayed for cell viability. As compared with untransfected and untreated control cells, empty vector-transfected and H₂O₂/FeSO₄-treated SH-SY5Y cells displayed an approximately 85% reduction in cell viability (Fig. 4b). In contrast, SH-SY5Y cells transfected with human CRP40 and treated with H₂O₂/FeSO₄ only exhibited an approximately 68% reduction in cell viability when compared with untreated control cells (Fig. 4b). Thus, CRP40 overexpression significantly increased the viability of cells (~116% increase over empty vector-transfected cells) following H₂O₂/FeSO₄ treatment, demonstrating its ability to protect cells from oxidative stress.

Previous studies from our laboratory have demonstrated an intimate association between alterations in dopaminergic activity, through DA receptor modulation and CRP40 expression (Gabriele et al. 2002, 2003, 2005, 2007; Modi et al. 1996; Nair and Mishra 2001; Sharan et al. 2001, 2003). As such, the catecholamine binding characteristics of rhCRP40 were determined through radioligand binding assays. rhCRP40 protein was incubated with [3 H]-labeled DA in the absence and presence of different concentrations of unlabeled DA. Competitive inhibition of [3 H]-DA by unlabeled DA indicated that rhCRP40 possesses distinct DA binding capabilities. Furthermore, the one-site competition curve indicated that rhCRP40 binds to DA in a low-affinity and high-capacity manner with an approximate IC₅₀ of 25 μ M (Fig. 4c). Similar binding studies were carried out in the presence of the radiolabeled dopamine D₁/D₂ receptor agonist, [3 H]-N-propyl-norapomorphine, which yielded similar findings (data not shown).

```

      M   D   S   S   G   P   K   H   L   N   M   K   L   T   R
1  atg gat tct tct gga ccc aag cat ttg aat atg aag ttg acc cgt
      A   Q   F   E   G   I   V   T   D   L   I   R   R   T   I
46 gct caa ttt gaa ggg att gtc act gat cta atc aga agg act atc
      A   P   C   Q   K   A   M   Q   D   A   E   V   S   K   S
91 gct cca tgc caa aaa gct atg caa gat gca gaa gtc agc aag agt
      D   I   G   E   V   I   L   V   G   G   M   T   R   M   P
136 gac ata gga gaa gtg att ctt gtg ggt ggc atg act agg atg ccc
      K   V   Q   Q   T   V   Q   D   L   F   G   R   A   P   S
181 aag gtt cag cag act gta cag gat ctt ttt ggc aga ccc cca agt
      K   A   V   N   P   D   E   A   V   A   I   G   A   A   I
226 aaa gct gtc aat cct gat gag gct gtg gcc att gga gct gcc att
      Q   G   G   V   L   A   G   D   V   T   D   V   L   L   L
271 cag gga ggt gtg ttg gcc ggc gat gtc acg gat gtg ctg ctc ctt
      D   V   T   P   L   S   L   G   I   E   T   L   G   G   V
316 gat gtc act ccc ctg tct ctg ggt att gaa act cta gga ggt gtc
      F   T   K   L   I   N   R   N   T   T   I   P   T   K   K
361 ttt acc aaa ctt att aat agg aat acc act att cca acc aag aag
      S   Q   V   F   S   T   A   A   D   G   Q   T   Q   V   E
406 agc cag cta ttc tct act gcc gct gat ggt caa acg caa gtg gaa
      I   K   V   C   Q   G   E   R   E   M   A   G   D   N   K
451 att aaa gtg tgt cag ggt gaa aga gag atg gct gga gac aac aaa
      L   L   G   Q   F   T   L   I   G   I   P   P   A   P   R
496 ctc ctt gga cag ttt act ttg att gga att cca cca gcc cct cgt
      G   V   P   Q   I   E   V   T   F   D   I   D   A   N   G
541 gga gtt cct cag att gaa gtt aca ttt gac att gat gcc aat ggg
      I   V   H   V   S   A   K   D   K   G   T   G   R   E   Q
586 ata gta cat gtt tct gct aaa gat aaa ggc aca gga cgt gag cag
      Q   I   V   I   Q   S   S   G   G   L   S   K   D   D   I
631 cag att gta atc cag tct tct ggt gga tta agc aaa gat gat att
      E   N   M   V   K   N   A   E   K   Y   A   E   E   D   R
676 gaa aat atg gtt aaa aat gca gag aaa tat gct gaa gaa gac cgg
      R   K   K   E   R   V   E   A   V   N   M   A   E   G   I
721 cga aag aag gaa cga gtt gaa gca gtt aat atg gct gaa gga atc
      I   H   D   T   E   T   K   M   E   E   F   K   D   Q   L
766 att cac gac aca gaa acc aag atg gaa gaa ttc aag gac caa tta
      P   A   D   E   C   N   K   L   K   E   E   I   S   K   M
811 cct gct gat gag tgc aac aag ctg aaa gaa gag att tcc aaa atg
      R   E   L   L   A   R   K   D   S   E   T   G   E   N   I
856 agg gag ctc ctg gct aga aaa gac agc gaa aca gga gaa aat att
      R   Q   A   A   S   S   L   Q   Q   A   S   L   K   L   F
901 aga cag gca gca tcc tct ctt cag cag gca tca ctg aag ctg ttc
      E   M   A   Y   K   K   M   A   S   E   R   E   G   S   G
946 gaa atg gca tac aaa aag atg gca tct gag cga gaa ggc tct gga
      S   S   G   T   G   E   Q   K   E   D   Q   K   E   E   K
991 agt tct ggc act ggg gaa caa aag gaa gat caa aag gag gaa aaa
      Q   *   *   *
1036 cag taa taa tag cag aaa ttt tga agc cag aag gac aac ata tga
1081 agc tta gga gtg aag aga ctt cct gag cag aaa tgg gcg aac ttc
1126 agt ctt ttt act gtg ttt ttg cag tat tct ata tat aat ttc ctt
1171 aat ttg taa att tag tga cca tta gct agt gat cat tta atg gac
1216 agt gat tct aac agt ata aag ttc aca ata ttc tat gtc cct agc
1261 ctg tca ttt ttc agc tgc atg taa aag gag gta gga tga att gat
1306 cat tat aaa gat tta act att tta tgc tga agt gac cat att ttc
1351 aag ggg tga aac cat ctc gca cac agc aat gaa ggt agt cat cca
1396 tag act tga aat gag acc aca tat ggg gat gag atc ctt cta gtt
1441 agc cta gta gtg ctg tac tgg cct gta tgt aca tgg ggt cct tca
1486 act gag gcc ttg caa gtc aag ctg gct gtg cca tgt ttg tag atg
1531 ggg cag agg aat cta gaa caa tgg gaa act tag cta ttt ata tta
1576 ggt aca gct att aaa aca agg tag gaa tga ggc tag acc ttt aac
1621 ttc cct aag gca tac ttt tct agc tac ctt ctg ccc tgt gtc tgg
1666 cac cta cat cct tga tga ttg ttc tct tac cca ttc tgg aat ttt
1711 ttt ttt ttt aaa taa ata cag aaa gca tct tga

```

Fig. 3 Cloning and expression of rhCRP40. **a** The 1,043-bp protein coding region of the human CRP40 mRNA transcript was amplified from human brain cDNA using specific primer sequences. Sequencing of the band indicated 100% sequence identity to mortalin. A parallel PCR lacking the initial cDNA template was included as a control to demonstrate the specificity of the priming sequences. The 100-bp DNA ladder (Std) has also been included to provide a visual reference point. **b** Coomassie Brilliant Blue staining of fractions obtained from a bacterial culture overexpressing the recombinant human CRP40 (rhCRP40) fusion protein. Prior to treatment with thrombin protease, the fusion protein possessed a molecular weight of 66 kDa. Following incubation with thrombin protease, the GST tag (26 kDa) was liberated from the rhCRP40 (40 kDa) fraction. Protein sequencing confirmed that rhCRP40 is a mortalin homolog. The molecular weight marker (Std) has also been included to provide a visual reference point

Alterations in human CRP40 expression and subcellular localization following antipsychotic treatment

Numerous findings from our laboratory have demonstrated the modulation of rat CRP40 by various antipsychotic drug regimens. In order to investigate whether antipsychotic drug treatment directly alters in human CRP40 expression, SH-SY5Y cells (stably transfected with the DA D₂ receptor) were cultured in the presence and absence of the DA D₂ receptor antagonist, haloperidol. Real-time PCR amplification indicated that DA D_{2L}-receptor-transfected SH-SY5Y cells cultured in the presence of haloperidol

Fig. 4 Human CRP40 displays chaperone and catecholamine binding functions. **a** Following 20 and 30 min of thermal denaturation at 42°C, incubation with rhCRP40 significantly inhibited firefly luciferase (Luc) aggregation (**p*<0.05). Data are presented as the mean relative absorbance ± SEM from five separate experiments. **b** Bar graph displaying results from an MTT assay of cell viability after incubation with H₂O₂/FeSO₄ (16 h) and transfection with human CRP40 or the empty expression vector. Overexpression of CRP40 significantly improved the viability of cells, as compared with empty vector-transfected cells (**p*<0.05) following H₂O₂/FeSO₄ treatment. Data are presented in triplicate as the mean percentage of viable cells ± SEM. **c** One-site competition curve showing binding of rhCRP40 with [³H]-DA after displacement with unlabeled DA. rhCRP40 binds DA with an IC₅₀ of approximately 25 μM. Data are represented as percentage of specific binding relative to total binding ± SEM from three separate experiments

exhibited a significant 32.6% increase in CRP40 mRNA transcript expression relative to untreated cells (2,860 ± 93 mRNA transcript copies per 5-ng total RNA versus 2,157 ± 144 mRNA transcript copies per 5-ng total RNA, respectively; Fig. 5b). In control experiments, no significant alteration in the mRNA expression of the human house-keeping gene, cyclophilin, was observed after haloperidol treatment (726,300 ± 27,330 mRNA transcript copies per 5-ng total RNA versus 732,900 ± 16,980 mRNA transcript copies per 5-ng total RNA, respectively; Fig. 5c).

In order to examine the subcellular distribution of CRP40 following antipsychotic drug treatment, murine midbrain neurons were isolated and a primary cell culture was established. Following exposure to haloperidol, mid-brain neurons appeared to display a significant upregulation of CRP40 protein expression, as evidenced by immunocytochemistry (Fig. 6b), as compared to untreated control cells (Fig. 6a). Interestingly, CRP40 primarily accumulated within the nucleus of these midbrain neurons after haloperidol exposure. Similarly, a significant increase in CRP40 immunoreactivity was observed following incubation with the DA D₁ receptor agonist, SKF38393 (Fig. 6c). In contrast, the addition of a DA-D₁ antagonist, SCH23390 (Fig. 6d), and also a specific inhibitor of protein kinase A (Fig. 6e) significantly suppressed CRP40 expression when compared to control cells, as evidenced by immunofluorescence intensity analysis.

Our laboratory has previously observed statistically significant reductions in CRP40 protein expression within the nucleus accumbens of schizophrenic patients as compared with age-matched control brain specimens from the Stanley Foundation Neuropathology Consortium (Gabriele et al. 2005). As such, in order to evaluate the *in vivo* localization of human CRP40, postmortem nucleus accumbens brain specimens were subjected to immunofluorescent analyses. In control patients, human CRP40 appeared to exhibit a distinct punctate immunofluorescent pattern throughout the cytoplasm of neurons within the

nucleus accumbens (Fig. 7a). In comparison to control subjects, the drug-naïve schizophrenic brain specimens also displayed a punctate cytoplasmic subcellular distribution of CRP40 expression (Fig. 7b). In contrast, nucleus accumbens brain specimens from schizophrenic patients treated with large doses of haloperidol demonstrated distinct alterations in CRP40 protein localization. CRP40 appeared to exclusively localize within the nucleus of these meso-

corticolimbic neurons after antipsychotic drug treatment (Fig. 7c).

Discussion

Previous reports from our laboratory have described the cloning and pharmacological properties of a brain-specific 40-kDa catecholamine-regulated protein (CRP40) in the bovine striatum (Gabriele et al. 2002, 2003; Modi et al. 1996; Sharan et al. 2001, 2003). Human, rat, and bovine CRP40 protein are known to bind DA and structurally

Fig. 5 Real-time PCR analysis of CRP40 mRNA expression. **a** Standard curve by real-time PCR using specific primers and varying concentrations of RNA. **b** Treatment of DA D_{2L}-transfected SH-SY5Y cells ($n=3$), with haloperidol, resulted in a statistically significant ($*p<0.05$) increase in human CRP40 mRNA expression as compared with untreated (PBS) control cells ($n=3$). **c** Treatment of DA D_{2L}-transfected SH-SY5Y cells ($n=3$), with haloperidol, failed to result in any significant alteration in human cyclophilin mRNA expression, when compared with untreated (PBS) control cells ($n=3$). Data are presented as mRNA copy number per 5 ng of total RNA $\pm$ SEM

Fig. 6 Altered CRP40 protein distribution following treatment with dopaminergic agents and kinase inhibitors. Increased immunofluorescent signals compared to the control (**a**) were observed following treatments with 10 μ M haloperidol (**HAL**; **b**) and 100 μ M SKF38393 (**SKF**; **c**). Conversely, a decrease in CRP40 immunofluorescence was observed following a treatment with 1 μ M SCH23390 (**SCH**; **d**). A

slight decrease was also found after treatment with 10 μ M of a protein kinase A inhibitor (**PKA-I**; **e**). A bar graph has also been included, depicting quantification of immunofluorescence intensity using ImageJ pixel quantitation, representative of $n=4$ image fields per experimental group and $*p<0.05$ (**f**)

related catecholamines. Furthermore, bovine CRP40 has significant amino acid sequence homology with the HSP70 family, which suggests CRP40 may possess chaperone-like functions. The present study was initiated based on the assumption that a human isoform of the bovine CRP40 may exist. Thus, a BLAST search of uncharacterized ESTs was performed using the bovine CRP40 protein sequence. The

results indicated that a specific EST, BQ224193, possessed high-sequence homology and gap similarities to the bovine CRP40 sequence. This comparison identified unique amino acid gaps and insertions present in bovine CRP40 and BQ224193 sequences that were not present in other members of the HSP70 family, indicating a structural relationship.

Fig. 7 Localization of human CRP40 on postmortem brain specimens. Human CRP40 is localized in distinct punctate structures within the ventral striatum of both the control (**CTL**) subjects (**a**) and drug-naïve schizophrenic patients (**SCZ (D-N)**; **b**). Interestingly, nuclear

translocation of human CRP40 was observed in a schizophrenic patient who underwent haloperidol treatment (**SCZ (HAL)**; **c**). In each case, immunolocalizations were carried out on tissue samples derived from three distinct patients

RLM-RACE PCR, using specific BQ224193 primer sets, was performed in order to determine the complete human CRP40 mRNA sequence. Sequencing of the 1,743-bp 3' RLM-RACE PCR product revealed 100% identity to the 3' half of the human mitochondrial HSP70, mortalin. Thus, we hypothesized that the human CRP40 protein was an alternative splice variant from the mortalin gene. Corroborating this finding, a Northern blot hybridization was also employed in order to probe large populations of polyA RNA for the expression of the BQ224193 sequence. Northern blot hybridization showed two distinct bands at approximately 2,850 and 1,750 bp. Interestingly, these two mRNA fragments correspond to the fragment sizes for mRNA transcript encoding mortalin (2,850 bp) and the 3' RLM-RACE human CRP40 PCR product (1,743 bp).

Mortalin was first discovered as a 66-kDa protein in mouse embryonic fibroblasts (Wadhwa et al. 1991). In order to trace the molecular mechanisms of cell immortalization, studies using mouse embryonic fibroblasts identified mortalin as a mortality marker. Mortalin is a ubiquitously expressed multifunctional protein, acting as a chaperone, an anchoring protein, and an active participant in signal transduction. In addition, it is also important in mitochondrial biogenesis, having the ability to shuttle proteins from the cytoplasm to the mitochondria (Kaul et al. 2007). Recent reports have also shown that the N-terminus of mortalin binds to the C-terminus of the tumor suppressor protein, p53 (Kaul et al. 2001, 2002; Wadhwa et al. 2002). This binding property inhibits the translocation of p53 to the nucleus, resulting in cell immortalization (Kaul et al. 2007). Consequently, mortalin overexpression is linked to cancer research and has been studied in this respect for many years. Human mortalin (HSPA9) is encoded on a single gene, which has been assigned to chromosome 5-band q31.1 (Xie et al. 2000), a gene locus previously implicated with various neurological disorders (Crowe and Vieland 1999; Hong et al. 2004; Lewis et al. 2003; Sklar et al. 2004). Similar reports have now implicated mortalin as a key protein in Parkinson's disease, specifically demonstrating that mortalin is significantly reduced in the early onset of the disease and worsens during the progression of the disease (Shi et al. 2008). In addition, mortalin has been shown to bind to alpha synuclein and DJ-1, proteins that are intimately associated with Parkinson's disease (Jin et al. 2005, 2006).

The mortalin mRNA transcript is spliced from 17 distinct intronic and exonic sequences. The data presented in this study suggest that the human CRP40 mRNA transcript is likely expressed from downstream exonic sequences, namely exons 10–17, indicating it is an alternative splice variant or overlapping mortalin gene product. Interestingly, the intron flanking the most 5' end of the human CRP40 open reading frame, intron 9, is

4,836 bp in length, making it mortalin's largest intron (Xie et al. 2000). Further bioinformatic analysis of intron 9 suggests that it likely contains a distinct promoter region and harbors multiple well-conserved transcription factor binding sites (SRY, CdxA, and C/EBP, among others), along with a distinct CpG island (data not shown). We hypothesize that human CRP40 is expressed from the HSPA9 gene via the activity of a consensus promoter region located with the ninth intron. Future studies are focused on identifying the key regulatory components positioned within this sequence and how they modify CRP40 expression.

In order to determine if human CRP40 has similar chaperone and catecholamine function properties as the bovine CRP40 protein, cloning experiments were designed and performed using a pGEX-2T bacterial expression vector system, which was transformed in BL-21 *E. coli* cells. Protein sequencing analysis confirmed the synthesis of rhCRP40 and its homology to mortalin. The Swiss-Prot ExPASy tool was used to obtain the human CRP40 translated protein sequence from the human CRP40 mRNA transcript sequence. The results showed the novel human protein to have a molecular weight of approximately 38 kDa and theoretical isoelectric point of 5.3. Preliminary functional studies demonstrated that rhCRP40 could prevent the thermal aggregation of firefly luciferase, while overexpression of human CRP40 resulted in a significant increase in cell viability. These data suggest that human CRP40 possesses distinct chaperone activity and can protect cells from the harmful effects of H₂O₂-induced oxidative stress. Additionally, radioligand binding assays were performed with [³H]-DA and rhCRP40 to assess the ability of human CRP40 to bind catecholamines. The findings from these experiments indicated that unlabeled DA was capable of competitively displacing [³H]-DA from rhCRP4, in a dose-dependent manner suggesting that rhCRP40 possesses distinct DA binding capabilities. Furthermore, given that rhCRP40 binds DA with an IC₅₀ of approximately 25 μM, the data suggest that it binds DA with low affinity and high capacity. Low-affinity and high-capacity binding parameters are a common feature of molecular chaperone proteins and have recently been suggested to function in the stabilization of the cytoplasmic architecture of the cell, specifically maintaining homeostasis in downstream signaling (Soti and Csermely 2002b). Not only have genetic linkage studies indicated that the mortalin gene (along with human CRP40) is positioned at locus 5q31, a putative schizophrenia and Parkinson's disease gene locus, but a recent postmortem study by our group has also implicated the CRP40 protein with schizophrenia. Ventral striatal brain specimens derived from schizophrenic patients possessed lower levels of CRP40 protein expression when compared to age-matched control subjects (Gabriele et al. 2005), suggesting an important association between CRP40 expression and DA dysregulation.

Additional functional studies were conducted at the transcriptional level with SH-SY5Y human neuroblastoma cells, a cell line that has been extensively characterized with regards to CNS function (Hillion et al. 2002; Nair et al. 1996; Verma et al. 2005). Real-time PCR using human-CRP40-specific primers revealed that this alternative splice variant of mortalin is differentially upregulated after exposure to the DA D₂ receptor antagonist, haloperidol, confirmed by absolute quantitation. Furthermore, normalization with the housekeeping gene, human cyclophilin, showed no significant change between the haloperidol-treated and untreated cells. Corroborating these findings, chronic treatment of rats with haloperidol, resulted in a significant increase in rat striatal CRP40 expression, suggesting that the CRP40 protein is directly modulated by DA receptor agonists, antagonists, and psychotropic drugs (Sharan et al. 2003).

Localization studies were also conducted using immunohistochemistry on postmortem brain samples of the ventral striatum region of the brain from the Stanley Foundation Neuropathology Consortium. The results demonstrated that the human CRP40 localizes to the perinuclear region of neurons within the ventral striatum of normal control brain specimens and brain tissue derived from drug-naïve schizophrenic patients. The punctate nature of its expression pattern suggests that CRP40 likely resides in a distinct subcellular compartment within the neuron. Given its relation to mortalin, we hypothesize that CRP40 likely associates with other mitochondrial-related proteins. Future studies are aimed at identifying these potential binding partners. Interestingly, immunofluorescent staining of brain sections derived from haloperidol-treated schizophrenia postmortem samples indicated a distinct nuclear CRP40 protein expression pattern. These immunolocalization studies correlate with immunohistochemical studies, in which mortalin translocates to the nucleus following induction of cell stress (Wadhwa et al. 1993b). The translocation of human CRP40 to the nucleus may be indicative of oxidative stress induced by increased DA concentrations caused by haloperidol treatment. Interestingly, bioinformatic analysis of the human CRP40 amino acid sequence indicates that human CRP40 possesses a conserved leucine zipper motif (data not shown), suggesting this novel protein likely possesses the ability to activate transcription.

The immunocytochemistry experiments in murine mid-brain primary cultures illustrated a significant increase in human CRP40 staining following antipsychotic treatment relative to controls, most likely through the disinhibition of adenylyl cyclase. In addition to an overall increase in the concentration of CRP40, antipsychotic treatment with haloperidol also induced similar translocation to the nucleus as seen in the human postmortem samples. The addition of SCH23390, a DA D₁ receptor antagonist, significantly suppressed CRP40 expression, while the DA D₁ receptor

agonist, SKF38393, induced a significant increase in human CRP40 protein expression. This further reinforces the hypothesis that human CRP40 expression is directly increased by DA D₁ receptor activation and reduced following DA D₁ receptor antagonism. Additionally, the significant reduction in CRP40 protein expression following incubation with a protein kinase A inhibitor strengthens the hypothesis that the DA receptor (and the related cAMP-dependent pathway) has a direct function in regulating cellular CRP40 concentrations.

In summary, the cloning, characterization, and localization of human CRP40 protein have demonstrated that this novel protein has catecholamine-regulated functions. Human CRP40 is transcribed as an alternative splice variant from the mortalin gene, which is known to be a putative schizophrenic and Parkinson's locus, implicating it in dopaminergic CNS disorders.

Acknowledgements This work was supported by the Canadian Institutes of Health Research and the Ontario Mental Health Foundation. RKM is a recipient of a senior fellowship from the Ontario Mental Health Foundation. We are grateful to the Stanley Neuropathology Consortium for providing the brain specimens.

References

- Bagchi M, Katar M, Maisel H (2001) A heat shock transcription factor like protein in the nuclear matrix compartment of the tissue cultured mammalian lens epithelial cell. *J Cell Biochem* 80:382–387 doi:10.1002/1097-4644(20010301)80:3<382::AID-JCB120>3.0.CO;2-D
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 72:248–254 doi:10.1016/0003-2697(76)90527-3
- Buck TM, Wright CM, Brodsky JL (2007) The activities and function of molecular chaperones in the endoplasmic reticulum. *Semin Cell Dev Biol* 18:751–761 doi:10.1016/j.semcdb.2007.09.001
- Calderwood SK, Mambula SS, Gray PJ Jr, Theriault JR (2007) Extracellular heat shock proteins in cell signaling. *FEBS Lett* 581:3689–3694 doi:10.1016/j.febslet.2007.04.044
- Crowe RR, Vieland V (1999) Report of the Chromosome 5 Workshop of the Sixth World Congress on Psychiatric Genetics. *Am J Med Genet* 88:229–232 doi:10.1002/(SICI)1096-8628(19990618)88:3<229::AID-AJMG4>3.0.CO;2-B
- Diano M, Le Bivic A, Hirn M (1987) A method for the production of highly specific polyclonal antibodies. *Anal Biochem* 166:224–229 doi:10.1016/0003-2697(87)90568-9
- Dobson CM (2004) Principles of protein folding, misfolding and aggregation. *Semin Cell Dev Biol* 15:3–16 doi:10.1016/j.semcdb.2003.12.008
- Gabriele J, Rajaram M, Zhang B, Sharma S, Mishra RK (2002) Modulation of a 40-kDa catecholamine-regulated protein following D-amphetamine treatment in discrete brain regions. *Eur J Pharmacol* 453:13–19 doi:10.1016/S0014-2999(02)02366-X
- Gabriele J, Culver K, Sharma S, Zhang B, Szechtman H, Mishra R (2003) Asymmetric modulation of a catecholamine-regulated protein in the rat brain, following quinpirole administration. *Synapse* 49:261–269 doi:10.1002/syn.10224

- Gabriele JP, Chong VZ, Pontoriero GF, Mishra RK (2005) Decreased expression of a 40-kDa catecholamine-regulated protein in the ventral striatum of schizophrenic brain specimens from the Stanley Foundation Neuropathology Consortium. *Schizophr Res* 74:111–119 doi:10.1016/j.schres.2004.03.025
- Gabriele J, Thomas N, N-Marandi S, Mishra R (2007) Differential modulation of a 40 kDa catecholamine regulated protein in the core and shell subcompartments of the nucleus accumbens following chronic quinpirole and haloperidol administration in the rat. *Synapse* 61:835–842 doi:10.1002/syn.20435
- Giffard RG, Xu L, Zhao H, Carrico W, Ouyang Y, Qiao Y, Sapolsky R, Steinberg G, Hu B, Yenari MA (2004) Chaperones, protein aggregation, and brain protection from hypoxic/ischemic injury. *J Exp Biol* 207:3213–3220 doi:10.1242/jeb.01034
- Hillion J, Canals M, Torvinen M, Casado V, Scott R, Terasmaa A, Hansson A, Watson S, Olah ME, Mallol J et al (2002) Coaggregation, cointernalization, and codesensitization of adenosine A2A receptors and dopamine D2 receptors. *J Biol Chem* 277:18091–18097 doi:10.1074/jbc.M107731200
- Hong KS, McInnes LA, Service SK, Song T, Lucas J, Silva S, Fournier E, Leon P et al (2004) Genetic mapping using haplotype and model-free linkage analysis supports previous evidence for a locus predisposing to severe bipolar disorder at 5q31–33. *Am J Med Genet B Neuropsychiatr Genet* 125B:83–86 doi:10.1002/ajmg.b.20091
- Irvine GB, El-Agnaf OM, Shankar GM, Walsh DM (2008) Protein aggregation in the brain: the molecular basis for Alzheimer's and Parkinson's diseases. *Mol Med* 14:451–464 doi:10.2119/2007-00100.Irvine
- Jin J, Meredith GE, Chen L, Zhou Y, Xu J, Shie FS, Lockhart P, Zhang J (2005) Quantitative proteomic analysis of mitochondrial proteins: relevance to Lewy body formation and Parkinson's disease. *Brain Res Mol Brain Res* 134:119–138 doi:10.1016/j.molbrainres.2004.10.003
- Jin J, Hulette C, Wang Y, Zhang T, Pan C, Wadhwa R, Zhang J (2006) Proteomic identification of a stress protein, mortalin/mthsp70/GRP75: relevance to Parkinson disease. *Mol Cell Proteomics* 5:1193–1204 doi:10.1074/mcp.M500382-MCP200
- Kaul SC, Reddel RR, Mitsui Y, Wadhwa R (2001) An N-terminal region of mot-2 binds to p53 in vitro. *Neoplasia* 3:110–114 doi:10.1038/sj.neo.7900139
- Kaul SC, Taira K, Pereira-Smith OM, Wadhwa R (2002) Mortalin: present and prospective. *Exp Gerontol* 37:1157–1164 doi:10.1016/S0531-5565(02)00135-3
- Kaul SC, Deocaris CC, Wadhwa R (2007) Three faces of mortalin: a housekeeper, guardian and killer. *Exp Gerontol* 42:263–274 doi:10.1016/j.exger.2006.10.020
- Lanneau D, Brunet M, Frisan E, Solary E, Fontenay M, Garrido C (2008) Heat shock proteins: essential proteins for apoptosis regulation. *J Cell Mol Med* 12:743–761 doi:10.1111/j.1582-4934.2008.00273.x
- Lewis CM, Levinson DF, Wise LH, DeLisi LE, Straub RE, Hovatta I, Williams NM, Schwab SG et al (2003) Genome scan meta-analysis of schizophrenia and bipolar disorder, part II: schizophrenia. *Am J Hum Genet* 73:34–48 doi:10.1086/376549
- Liberek K, Lewandowska A, Zietkiewicz S (2008) Chaperones in control of protein disaggregation. *EMBO J* 27:328–335 doi:10.1038/sj.emboj.7601970
- Min JN, Whaley RA, Sharpless NE, Lockyer P, Portbury AL, Patterson C (2008) CHIP deficiency decreases longevity, with accelerated aging phenotypes accompanied by altered protein quality control. *Mol Cell Biol* 28:4018–4025 doi:10.1128/MCB.00296-08
- Modi PI, Kashyap A, Nair VD, Ross GM, Fu M, Savelli JE, Marcotte ER, Barlas C et al (1996) Modulation of brain catecholamine absorbing proteins by dopaminergic agents. *Eur J Pharmacol* 299:213–220 doi:10.1016/0014-2999(95)00849-7
- Muchowski PJ, Wacker JL (2005) Modulation of neurodegeneration by molecular chaperones. *Nat Rev Neurosci* 6:11–22 doi:10.1038/nrn1587
- Nair VD, Mishra RK (2001) Molecular cloning, localization and characterization of a 40-kDa catecholamine-regulated protein. *J Neurochem* 76:1142–1152 doi:10.1046/j.1471-4159.2001.00117.x
- Nair VD, Niznik HB, Mishra RK (1996) Interaction of NMDA and dopamine D2L receptors in human neuroblastoma SH-SY5Y cells. *J Neurochem* 66:2390–2393
- Ohtsuka K, Suzuki T (2000) Roles of molecular chaperones in the nervous system. *Brain Res Bull* 53:141–146 doi:10.1016/S0361-9230(00)00325-7
- Ross GM, McCarry BE, Thakur S, Mishra RK (1993) Identification of novel catecholamine absorbing proteins in the central nervous system. *J Mol Neurosci* 4:141–148 doi:10.1007/BF02782497
- Ross GM, McCarry BE, Mishra RK (1995) Covalent affinity labeling of brain catecholamine-absorbing proteins using a high-specific-activity substituted tetrahydronaphthalene. *J Neurochem* 65:2783–2789
- Sharan N, Nair VD, Mishra RK (2001) Modulation of a 40-kDa catecholamine regulated protein by dopamine receptor antagonists. *Eur J Pharmacol* 413:73–79 doi:10.1016/S0014-2999(01)00736-1
- Sharan N, Chong VZ, Nair VD, Mishra RK, Hayes RJ, Gardner EL (2003) Cocaine treatment increases expression of a 40 kDa catecholamine-regulated protein in discrete brain regions. *Synapse* 47:33–44 doi:10.1002/syn.10140
- Sherman MY, Goldberg AL (2001) Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. *Neuron* 29:15–32 doi:10.1016/S0896-6273(01)00177-5
- Shi M, Jin J, Wang Y, Beyer RP, Kitsou E, Albin RL, Gearing M, Pan C, Zhang J (2008) Mortalin: a protein associated with progression of Parkinson disease? *J Neuropathol Exp Neurol* 67:117–124 doi:10.1097/nen.0b013e318163354a
- Sklar P, Pato MT, Kirby A, Petryshen TL, Medeiros H, Carvalho C, Macedo A, Dourado A et al (2004) Genome-wide scan in Portuguese Island families identifies 5q31–5q35 as a susceptibility locus for schizophrenia and psychosis. *Mol Psychiatry* 9:213–218 doi:10.1038/sj.mp.4001418
- Soti C, Csermely P (2002a) Chaperones and aging: role in neurodegeneration and in other civilizational diseases. *Neurochem Int* 41:383–389 doi:10.1016/S0197-0186(02)00043-8
- Soti C, Csermely P (2002b) Chaperones come of age. *Cell Stress Chaperones* 7:186–190 doi:10.1379/1466-1268(2002)007<0186:CCOA>2.0.CO;2
- Tomala K, Korona R (2008) Molecular chaperones and selection against mutations. *Biol Direct* 3:5 doi:10.1186/1745-6150-3-5
- van Noort JM (2008) Stress proteins in CNS inflammation. *J Pathol* 214:267–275 doi:10.1002/path.2273
- Verma V, Mann A, Costain W, Pontoriero G, Castellano JM, Skoblenick K, Gupta SK, Pristupa Z et al (2005) Modulation of agonist binding to human dopamine receptor subtypes by L-prolyl-L-leucyl-glycinamide and a peptidomimetic analog. *J Pharmacol Exp Ther* 315:1228–1236 doi:10.1124/jpet.105.091256
- Wadhwa R, Kaul SC, Ikawa Y, Sugimoto Y (1991) Protein markers for cellular mortality and immortality. *Mutat Res* 256:243–254
- Wadhwa R, Kaul SC, Ikawa Y, Sugimoto Y (1993a) Identification of a novel member of mouse hsp70 family. Its association with cellular mortal phenotype. *J Biol Chem* 268:6615–6621
- Wadhwa R, Kaul SC, Sugimoto Y, Mitsui Y (1993b) Spontaneous immortalization of mouse fibroblasts involves structural changes in senescence inducing protein, mortalin. *Biochem Biophys Res Commun* 197:202–206 doi:10.1006/bbrc.1993.2461
- Wadhwa R, Yaguchi T, Hasan MK, Mitsui Y, Reddel RR, Kaul SC (2002) Hsp70 family member, mot-2/mthsp70/GRP75, binds to the cytoplasmic sequestration domain of the p53 protein. *Exp Cell Res* 274:246–253 doi:10.1006/excr.2002.5468
- Xie H, Hu Z, Chyna B, Horigan SK, Westbrook CA (2000) Human mortalin (HSPA9): a candidate for the myeloid leukemia tumor suppressor gene on 5q31. *Leukemia* 14:2128–2134 doi:10.1038/sj.leu.2401935