

EXPERIMENTAL STUDY

Cell-specific expression of NADPH-dependent cytosolic 3,5,3'-triiodo-L-thyronine-binding protein (p38CTBP)

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Abstract

We have previously shown that cytosolic 3,5,3'-triiodo-L-thyronine (T3)-binding protein (CTBP) possesses a high affinity for T3 binding in the presence of nicotinamide adenine dinucleotide phosphate *in vitro*, and that p38CTBP increases intracellular content of T3, and suppresses T3-mediated transactivity. Screening of mRNA expression in 73 different human tissues has demonstrated that p38CTBP mRNA is expressed at high levels in brain and heart. We have examined the intracellular localization and tissue-specific distribution of this protein by using a specific antibody against human p38CTBP. Western blotting and immunoprecipitation studies have shown that the antibody recognizes human p38CTBP. Interaction of p38CTBP with the antibody did not affect the T3-binding activity of p38CTBP, and its dimer formation *in vitro*. Western blotting analysis has shown that p38CTBP is expressed in brain and heart predominantly, similar to the distribution of mRNA. Immunohistochemical studies have demonstrated p38CTBP in neural cells and cardiac muscle cells. p38CTBP localizes in cytoplasm rather than in nuclei in neural cells. The evidence for the presence of tissue-specific localization of p38CTBP has indicated that p38CTBP has a tissue-specific function, such as the regulation of T3 delivery from cytoplasm to nuclei.

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Introduction

Thyroid hormone regulates multiple cellular events, including energy expenditure and cellular differentiation (1). Although it is well known that the action of thyroid hormone is initiated through the activation of gene expression by binding to its nuclear receptor (2), the mechanism of the transport of the hormone to nuclei from cytoplasm is not certain (3–5).

We have previously demonstrated that nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cytosolic 3,5,3'-triiodo-L-thyronine (T3)-binding protein (CTBP) contributes to intracellular storage and translocation of T3 *in vitro* (6–10). We have identified two CTBPs from rat kidney and liver. One was 4.7S CTBP (p58CTBP) and the other was 5.1S CTBP, which consisted of a dimer of 38 kDa polypeptide (p38CTBP) (11). Each dimer or monomer was activated by NADPH. In the presence of NADPH, the affinity constant of T3 binding was 2×10^9 mol/l, which was similar to that of nuclear T3 receptors, indicating that CTBP may play a physiological role in thyroid hormone action in its target tissues.

Maximal binding capacity (MBC) also increased by activation with NADPH. In studies with rats, the MBC obtained in the presence of NADPH increased during the growth of animals, and a tissue-specific increase was observed (12, 13). Cellular levels of the protein could be increased by thyroid hormone, vitamin D3 and insulin *in vitro* (14–17). Dexamethasone increases the mRNA of CTBP in cultured human myeloblasts (18). Butyrate, which also induces deacetylation of histone, increases the activity of CTBP in primary cultures of rat hepatic cells (19). These results indicate that the activity of this protein is regulated by multiple factors (20).

It was recently demonstrated that p38CTBP was identical to μ -crystallin in studies with the determination of the amino acid sequence of the protein (21). The μ -crystallin protein, a taxon-specific 35 kDa protein that is highly expressed in lens (22), brain, muscle and kidney in the kangaroo (23), may be important in maintaining structural function in these tissues of the kangaroo. Kim *et al.* (23) also reported the cloning of μ -crystallin cDNA from human retina and showed that it was expressed at high levels in human neural tissue, muscle and kidney. The expression

of p38CTBP increased not only cytoplasmic but also nuclear content of T3, while it suppressed T3-mediated transactivation in CTBP-expressing stable GH3 cell lines, implying that p38CTBP regulates T3 content and T3-induced transactivation in tissues (24).

In this study, we have characterized the antibody against human p38CTBP, which was raised previously, *in vitro*. By using this antibody, intracellular localization of p38CTBP was assessed and we estimated the tissue-specific localization of this protein. We obtained results showing that the protein is distributed in various human tissues in a cell-specific manner.

Materials and methods

Cloning of human p38CTBP and construction of plasmids

Full-length human p38CTBP cDNA was amplified by PCR as described previously (24). The amplified fragment was cloned into the TA cloning site of pT7-Blue (Novagen, Madison, WI, USA). The BamHI–HindIII fragment was cloned into pQBI-25-fc2 (Quantam Biotechnologies Inc, Quebec, Canada) vector which induces green fluorescent protein (GFP)-tagged CTBP in mammalian cells. The BamHI–SalI fragment of pT7-CTBP was ligated into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA), which is available for the expression in mammalian cells and *in vitro* transcription and translation. The EcoRI–SalI fragment was inserted into the same sites of pGEX-6p-3 (Amersham Pharmacia Biotech, Amersham, Bucks, UK). pGEX-CTBP is expressed as GST-tagged CTBP in *E. coli* strain BL21.

Establishment of p38CTBP-expressing CHO cell lines (CPC45)

CPC45, in which GFP-tagged p38CTBP is expressed, was established as previously described (24).

Preparation of polyclonal antibodies to p38CTBP (CTBP168)

Synthetic peptides containing a part of the human p38CTBP amino acid sequence were used in the immunization as described previously (24). The amino acid sequence (CNRTKENAEKFADTV) was chosen because of its high antigenicity index, determined by Epitope Adviser (Fujitsu, Shizuoka, Japan). Antibodies were raised in rabbits obtained from Takara Inc, Ohtsu, Japan. The antibody was purified by affinity column chromatography with the immunized peptide.

Detection of p38CTBP with antibody

Immunoprecipitation of p38CTBP expressed in reticulocyte lysate was performed as follows. [³⁵S]Methionine-labeled p38CTBP was expressed in the TNT Coupled

Reticulocyte Lysate System (Promega, Madison, WI, USA). An aliquot (1 µl) containing radioactive p38CTBP translated *in vitro* was incubated for 2 h at 4 °C with 10 µl (9 µg) CTBP168 or rabbit polyclonal anti-human Fas antibody as a negative control antibody in 1 ml TBSD buffer (10 mmol/l Tris–HCl, pH 7.6, 100 mmol/l NaCl and 1 mmol/l dithiothreitol (DTT)), containing 1% bovine serum albumin and 0.05% Triton X-100. Incubation was continued further for 30 min at 4 °C after adding 10 µl protein A (recombinant)-agarose (Upstate Biotechnology, Lake Placid, NY, USA). The mixture was centrifuged at 1500 *g* for 5 min, and the resultant pellet was washed twice with 1 ml of the same buffer. The final pellet obtained was applied to SDS-PAGE, and autoradiography was performed by the bio-imaging analyzer BAS-1500 (Fuji, Chiryuh, Japan).

Western blotting of p38CTBP was performed as previously described (24). Briefly, cells were washed twice in ice-cold phosphate-buffered saline (PBS) and lysed by adding lysis (RIPA) buffer (0.05% SDS, 1% Nonidet P-40, 150 mmol/l NaCl, 50 mmol/l Tris–HCl, pH 7.2, containing 1 mmol/l phenylmethylsulfonyl fluoride). Proteins were resuspended in lysis buffer containing 2% SDS, and samples were separated in 10% SDS-PAGE gels and transferred to immobilon-P membranes (Millipore, Bedford, MA, USA). The membranes were blocked with TBS-T (100 mmol/l NaCl, 10 mmol/l Tris–HCl, pH 7.5 and 0.1% Tween 20) containing 1% skim milk. Prior to blocking experiments, CTBP168 antibody (10 µl) was incubated with 0.5 mg of the blocking peptide in 5 ml TBS-T containing 1% skim milk at 4 °C for 18 h. Detection was carried out by measuring the enhanced chemiluminescence using a horseradish peroxidase (HRP)-coupled mouse-anti-goat IgG antibody (Amersham, Arlington Heights, IL, USA).

Estimation of effect of antibody on the function of p38CTBP

Estimation of the effect of antibody on the dimerization of p38CTBP was performed by using a GST pull-down assay as previously described (25). Briefly, the bacterial pellet was resuspended in 600 µl ice-cold PBS containing 0.05% Triton X-100 and 1 mmol/l DTT (PBS-TD). After sonication and centrifugation, the supernatant was incubated with 10 µl GST-Sepharose beads (Amersham Pharmacia Biotech) for 30 min at 4 °C. Coated beads were washed twice with PBS-TD, and incubated with [³⁵S]methionine-labeled p38CTBP with or without various concentrations of anti-p38CTBP antibody (CTBP168) in 200 µl TEND (50 mmol/l Tris–HCl, pH 7.6, 100 mmol/l NaCl, 1 mmol/l EDTA and 1 mmol/l DTT) for 6 h at 4 °C. The mixtures were centrifuged, and the pellet was washed three times with TEND, and proteins were separated by SDS-PAGE. Radioactivity was quantified by using the BAS-1500.

The effect of antibody on T3 binding was estimated as follows. Fractions (5 μ l) containing p38CTBP, expressed in the TNT Coupled Reticulocyte Lysate System, were incubated for 24 h at 4 °C with various amounts of CTBP168 or with rabbit polyclonal anti-human FAS antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a negative control antibody in a final volume of 200 μ l with TBSD buffer, containing [¹²⁵I]T3 (2250–3300 μ Ci/ μ g) and 100 μ mol/l NADPH. After appropriate incubation times, bound and free hormones were separated by dextran-coated charcoal as described (6).

Detection of p38CTBP RNA in human tissues

The cDNA fragments of human p38CTBP were isolated from pT7-CTBP. Hybridization with a ³²P-labeled fragment of CTBP cDNA was performed by using Human RNA Master Blot membrane, purchased from Clontech Inc. (Palo Alto, CA, USA), according to the manufacturer's protocol. This commercial dot blot contains polyA⁺ mRNA, and has been normalized by the manufacturer using a range of housekeeping genes. The autoradiographic signals were quantitated by the BAS-1500.

Immunohistochemistry

Human tissue sections were purchased from Novagen (Madison, WI, USA). Sections were dewaxed, rehydrated, and endogenous peroxidase was blocked by using 0.3% H₂O₂ in methanol. After washing with PBS, sections were reacted with primary antibody (CTBP168; 1:100 dilution) overnight at 4 °C. For competition studies, CTBP168 was preabsorbed by the immunizing peptide (100 μ g/ml in final concentration) overnight at 4 °C. As a control, the same concentration of non-specific peptide was added. After washing with PBS, sections were incubated with HRP-labeled-goat anti-rabbit IgG (1:100 dilution) for 2 h at room temperature. After washing with PBS, the HRP sites were visualized by 3-3' diaminobenzidine, H₂O₂, Co²⁺ and Ni⁺. Rodamine-labeled-goat anti-rabbit IgG (DAKO, Carpinteria, CA, USA) was used as the second antibody in some experiments. The nuclei were stained with 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) in methanol. After washing with PBS, the sections were mounted with Slow-Fade (Molecular Probes, Eugene, OR, USA). Photographs were taken at \times 100 or \times 400 magnification.

Results

Tissue-specific expression of p38CTBP mRNA in the human

mRNA of p38CTBP was expressed in various tissues in the human (Fig. 1). Brain, heart and pituitary were tissues which expressed mRNA highly. Relatively high

expression was observed in kidney, prostate and salivary glands. However, minimal expression was detected in the gastrointestinal tract, immune system (including spleen), lung, placenta, thyroid glands and pancreas. Although high expression was observed in the brain, it was heterogeneous. Cerebral cortex, cerebellum, amygdala, caudate nucleus, hippocampus, putamen and accumbens were the sites of highest expression. In contrast to these regions, expression was not high in pons, corpus callosum, medulla oblongata, substantia nigra, thalamus and spinal cord. Expression in the fetus was low even in heart and brain. The human cancer cells studied did not show expression.

Characterization of anti-p38CTBP antibody (CTBP168)

As shown in Fig. 2A (left panel), fluoroscopic examination detected the fluorescent signals in the cytoplasm of CPC45 cells which permanently express the GFP-p38CTBP fusion protein in CHO-K1 cells. The calculated molecular mass of GFP-fused p38CTBP is 66 kDa. Western blotting demonstrated that CTBP168, an anti-p38CTBP antibody, recognized a 66 kDa protein in the CPC45 but not in CHO-K1 cells. Pre-absorption of the antibody with the immunized peptide caused the recognizing activity to disappear (Fig. 2A, right panel). The antibody (CTBP168), in addition to recognition of the fusion protein, was able to immunoprecipitate [³⁵S]-labeled p38CTBP expressed in the reticulocyte lysate (Fig. 2B). However, the radioactive protein was not detected by protein agarose A alone or rabbit anti-Fas polyclonal antibody which was chosen as one of the negative control antibodies (Fig. 2B). Since we observed NADPH-dependent T3-binding activity in the reticulocyte lysate, the antibody (CTBP168) raised by our system recognized not only GFP-fused p38CTBP but also native (non-denatured) p38CTBP.

CTBP168, an anti-p38CTBP antibody, did not inhibit the NADPH-dependent T3 binding activity of the p38CTBP expressed in reticulocyte lysate (Fig. 2C). Further, we observed that the p38CTBP–[³⁵S]-p38CTBP interaction was not modified by the antibody in studies with the GST pull-down assay (Fig. 2D). These results indicated that the antibody influenced neither T3 binding nor dimerizing activity of the p38CTBP.

Tissue-specific expression of p38CTBP in the human

Expression of p38CTBP was estimated by Western blotting. As shown in Fig. 3, the protein was predominantly expressed in brain and heart. Tissue-specific distribution of p38CTBP was evaluated by an immunohistochemical technique, using CTBP168 as an anti-p38CTBP antibody. p38CTBP was preferentially expressed in neurons in the cerebral cortex (Fig. 4a). In order to evaluate the intracellular localization of

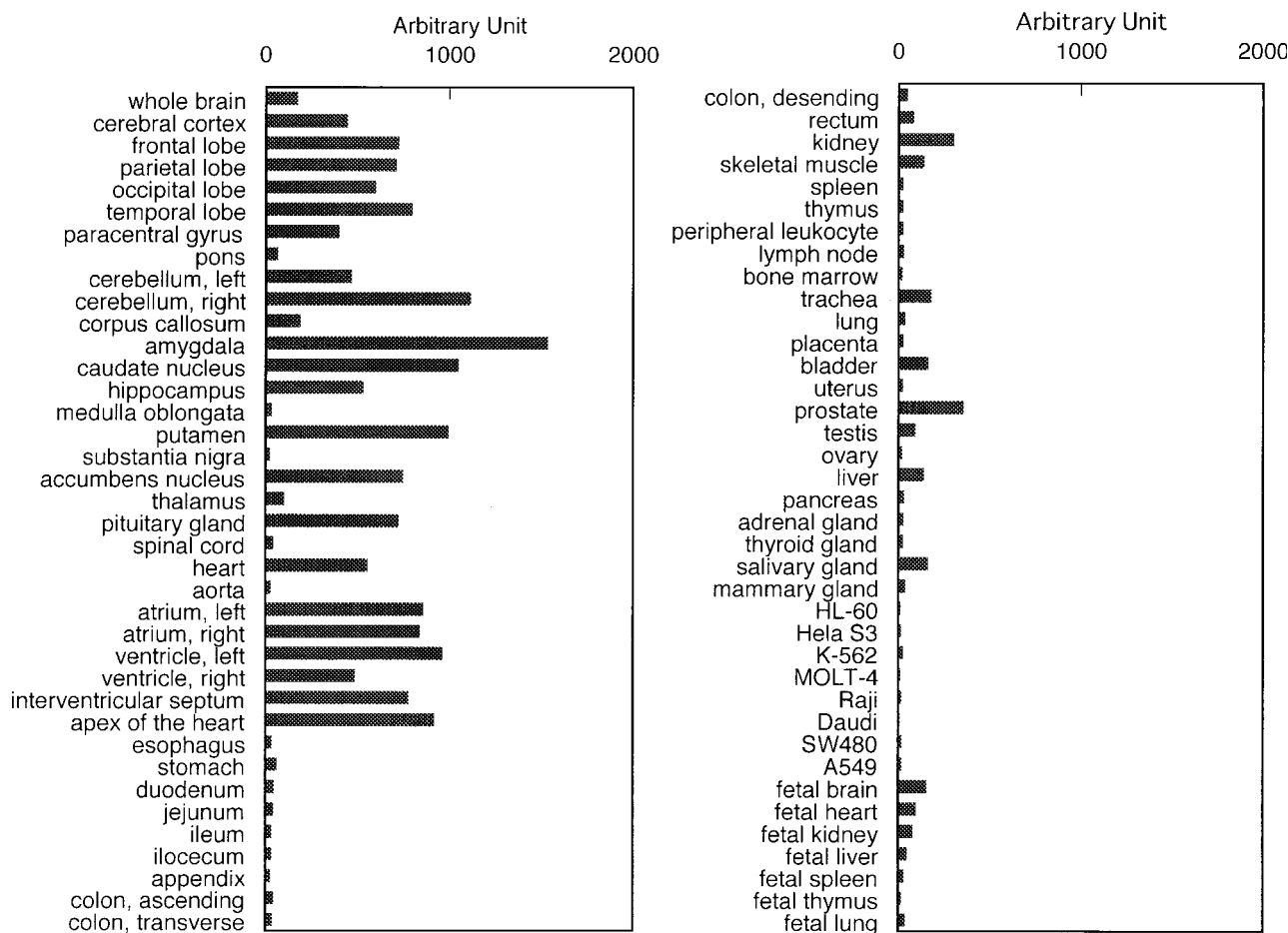


Figure 1 p38CTBP mRNA expression in human tissues. ^{32}P -labeled p38CTBP cDNA was hybridized with the Human RNA Master Blot. Autoradiographic signals were quantitated by BAS-1500. Expression levels are expressed as arbitrary units. The expression in eight cancer cell lines is also shown.

p38CTBP in neural cells, we examined the staining of nuclei and p38CTBP-antibody complex with DAPI and rodamine-labeled anti-rabbit IgG respectively. Nuclei in neural and glial cells were stained by DAPI. The p38CTBP-antibody (CTBP168) complexes were also recognized by rodamine-labeled IgG. p38CTBP predominantly localized in cytoplasm in these cells (Fig. 4c and d).

Expression was mosaic in the internal layer and weak in Purkinje cells in cerebellum (Fig. 5a). Cytoplasmic staining was dominant in heart (Fig. 5c). In kidney, the expression was observed in renal tubules but not in glomerulus (Fig. 6a). Only a weak signal was observed in liver (Fig. 6c). The pancreatic islet cell was one of the p38CTBP-positive cells studied (Fig. 6e). By treatment of antibodies with 100 $\mu\text{g}/\text{ml}$ of the peptide at 4°C for 24 h, the staining reaction was strongly reduced in each tissue (Figs 4b, 5b and d, 6b, d and f). However, the staining reaction was not affected by the treatment of the antibody with a non-specific peptide (data not shown).

Discussion

We previously identified CTBP in rat kidney and liver, and suggest that the CTBP plays roles in cellular storage and in nuclear transport of T3 *in vitro* (26). Expression of p38CTBP increased T3 content and suppressed T3-mediated transactivation in p38CTBP-expressing GH3 cells, suggesting that p38CTBP stores T3 and controls the T3-inducible transactivation in tissues (24). From these data, expression of p38CTBP probably plays crucial roles in stabilizing the T3-induced transactivity when extracellular T3 concentration alters physiologically or pathologically.

We prepared an anti-p38CTBP antibody which was obtained by immunization of a part of the amino acid sequence of p38CTBP. The antibody (CTBP168), which reacted with the GFP-coupled p38CTBP, specifically recognized radiolabeled native p38CTBP as demonstrated in the Results. We observed that the recognizing activity was inactivated by immunoadsorption with native p38CTBP prepared by the transfection

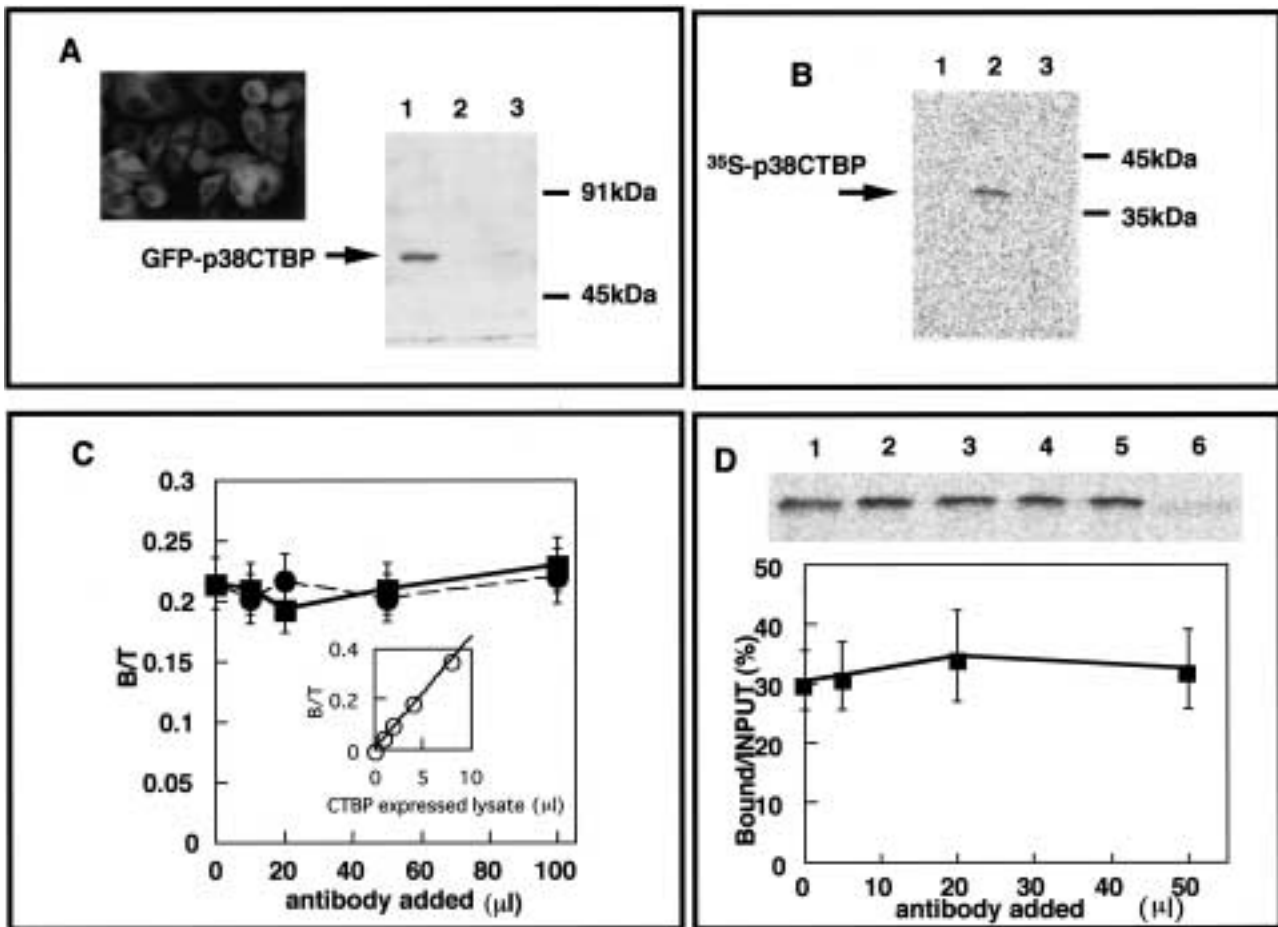


Figure 2 (A) Characterization of anti-p38CTBP antibody (CTBP168). Left panel: detection of GFP-tagged p38CTBP expressed in CPC45 cells. The fluoroscopic examination of GFP-tagged p38CTBP in CPC45 cells which were established from CHO-K1 cells as a cell line permanently expressing the fusion protein. CPC45 possessed NADPH-dependent T3-binding activity, while parental CHO-K1 did not. Cytoplasmic green fluorescent signal was detected in CPC45 but not in CHO-K1. Right panel: Western blotting analysis of GFP-p38CTBP. CPC45 or CHO-K1 was harvested with RIPA buffer. After freezing and thawing twice, the sample was centrifuged and the supernatant was boiled in SDS buffer. Aliquots (10 μg) were applied onto a 10% SDS-PAGE, and transferred to polyvinylidene difluoride membrane. CTBP168 (1:200 dilution) recognizes a 66 kDa protein corresponding to GFP-p38CTBP expressed in CPC45 (lane 1), but the protein could not be identified in CHO-K1 cell extract (lane 2). Antibody preadsorbed with the peptide did not recognize the protein which was prepared from CPC45 (lane 3). (B) Autoradiography of [³⁵S]p38CTBP expressed in reticulocyte lysate. One microliter of the [³⁵S]methionine-labeled translated product was incubated without antibody (lane 1), with 5 μg CTBP168 (lane 2) or with the 5 μg anti-Fas polyclonal antibody (lane 3) for 2 h at 4 °C in 1 ml TBSD buffer. Mixtures were further incubated with 10 μl protein A agarose for 1 h. The resultant pellets were washed with TBSD and applied onto 12% SDS-PAGE. Autoradiographic visualization was carried out by BAS-1500. (C) Effect of CTBP168 on NADPH-dependent T3 binding. The inset shows the correlation between the amount of p38CTBP detected by antibody in reticulocyte lysate and [¹²⁵I]T3-specific binding, and a linear correlation was obtained. Five microliters of *in vitro* translated product were incubated for 2 h at 4 °C with various amounts of CTBP168 (■) or rabbit polyclonal anti-human Fas antibody (●) as a negative control antibody in TBSD containing [¹²⁵I]T3 and 100 μM NADPH. The concentration of CTBP168 was 0.9 mg/ml estimated by photometer. Two hours after the beginning of incubation, bound and free hormones were separated by dextran-coated charcoal. Each value indicates the mean ± s.d. of triplicate determinations. B/T, bound/total radioactive ratio. (D) Effect of CTBP168 on homodimerizing activity of p38CTBP. Upper panel: GST-p38CTBP (6 μg protein) immobilized on glutathione beads and ³⁵S-labeled p38CTBP synthesized *in vitro* were incubated without (lane 2) or with 5 μl (lane 3), 20 μl (lane 4) or 50 μl (lane 5) CTBP168 for 6 h at 4 °C. The concentration of CTBP168 was 0.9 mg/ml estimated by photometer. GST immobilized on glutathione beads was incubated with ³⁵S-labeled p38CTBP as a negative control for 6 h at 4 °C (lane 6). Proteins bound to the washed beads were subjected to 12% SDS-PAGE, and followed by autoradiography. In the first lane, 20% of ³⁵S-labeled p38CTBP alone was electrophoresed. Lower panel: results obtained in the upper panel were quantified by using an imaging analyzer (Fuji BAS-1500). The results are presented as the means ± s.d. of each construct relative to the total labeled p38CTBP. An additional five independent experiments gave similar results.

of the cDNA of p38CTBP which has a T3-binding activity in the presence of NADPH. These results indicate that the antibody is available for the detection of native p38CTBP in Western blotting analysis and immunohistochemical studies.

p38CTBP has a high affinity for T3 binding in the presence of NADPH, and the protein has a sedimentation coefficient of 5.1S, a Stokes radius of 35 Å and 76 kDa (p76) of calculated molecular weight (11). The molecular mass estimated by SDS-PAGE, however, is

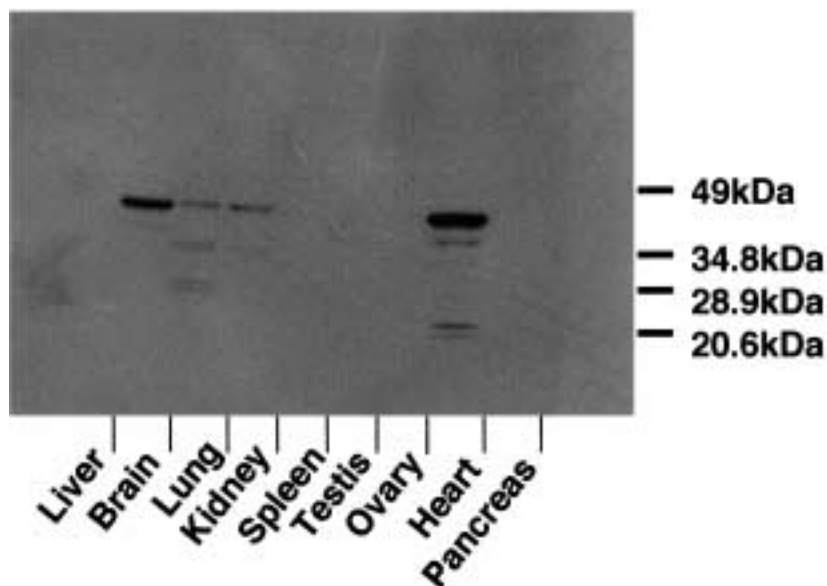


Figure 3 p38CTBP expression in adult human tissues. Multiple Tissue Blot Membrane (Geno Technology, St Louis, MO, USA) was blocked with TBS-T containing 5% skim milk, and probed with anti-p38CTBP antibody (CTBP168) (diluted 1:200) in TBS-T containing 1% skim milk. Detection was performed by enhanced chemiluminescence using an HRP-conjugated mouse-anti-goat IgG antibody.

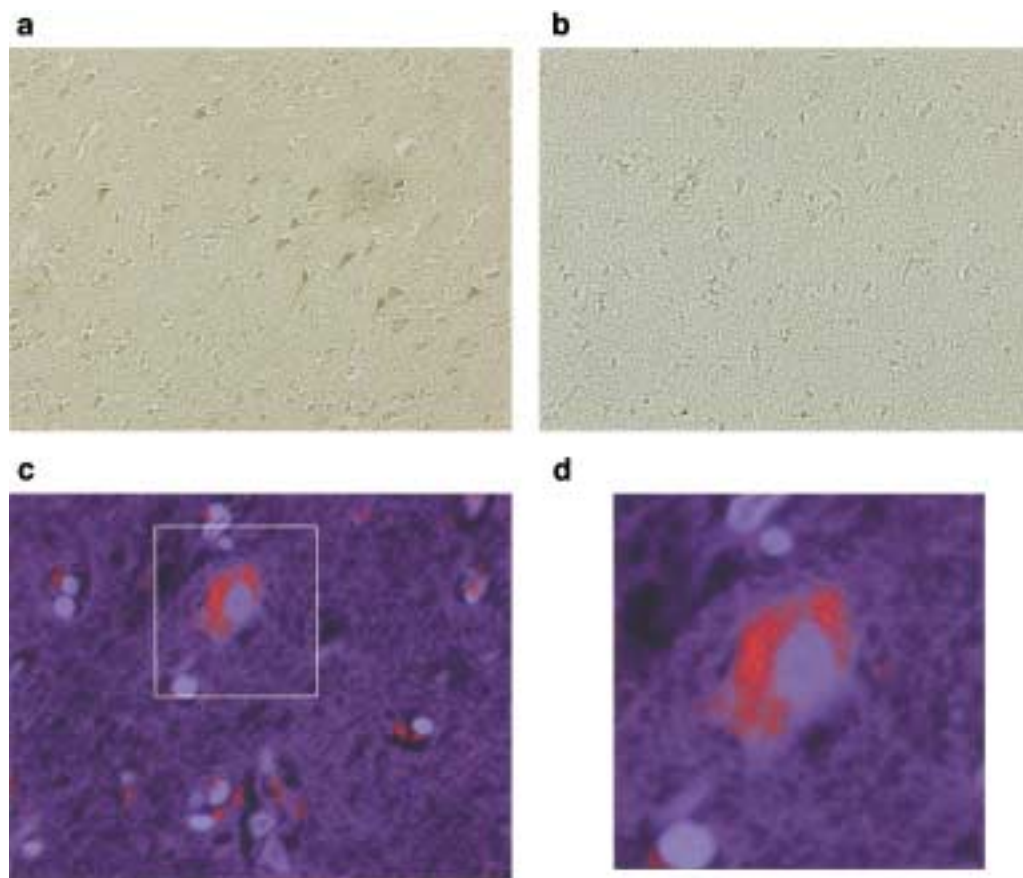


Figure 4 Immunostaining of p38CTBP in adult human cerebrum. Cerebrum prepared from the human was immunostained as described in Materials and methods. (a) Neural and some glia cells are stained. (b) Immunostainings using antibody fraction which was preadsorbed with the peptide. A section of cerebrum stained with DAPI and rodamine-antibody complex which recognized p38CTBP-CTBP168. (c) DAPI and rodamine were visualized with fluoroscopy. The boxed area is magnified in (d).

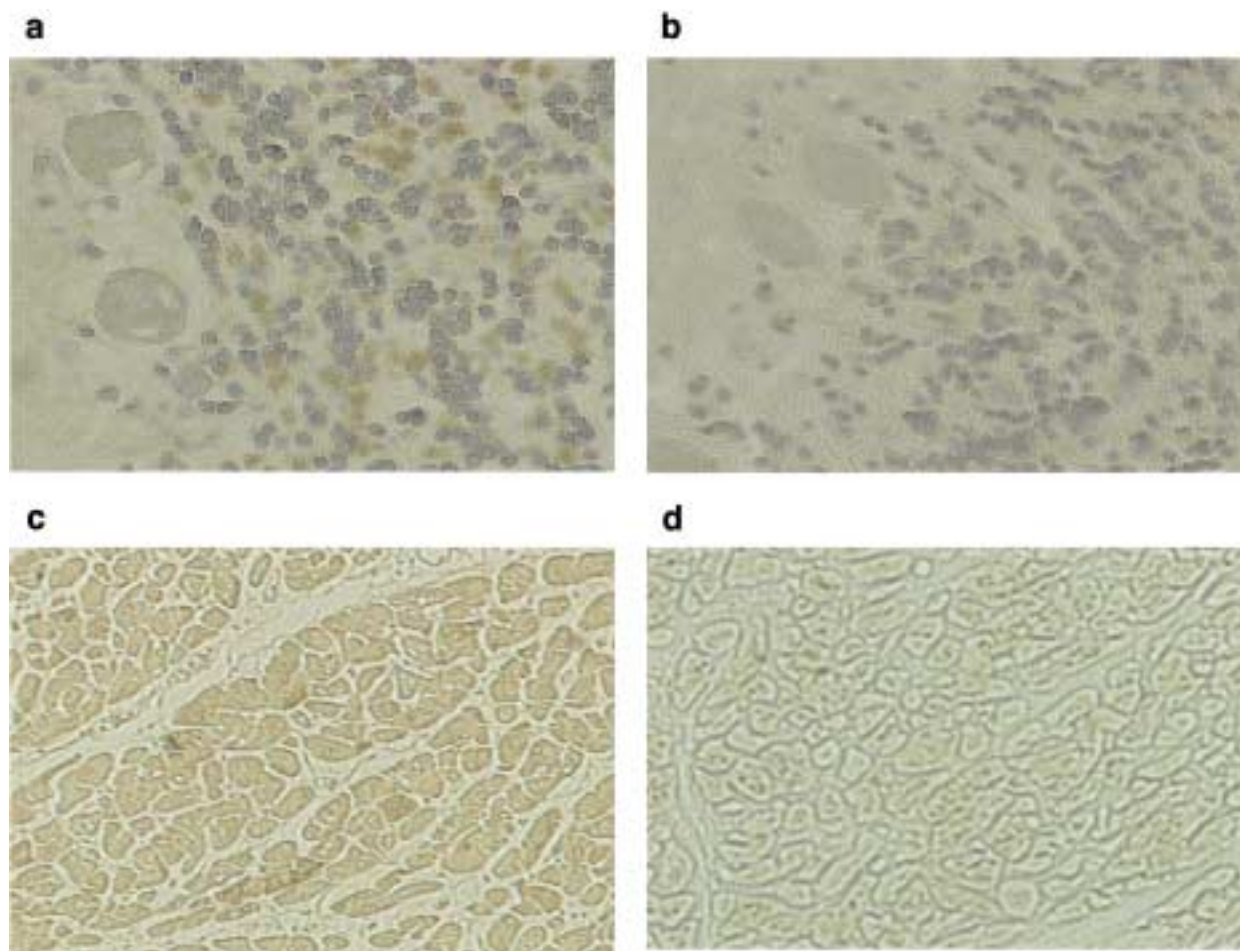


Figure 5 Immunostaining of p38CTBP in adult human cerebellum and heart. Cerebellum (a and b) and heart (c and d), were immunostained as described in Materials and methods. The internal layer is stained with a mosaic pattern, and Purkinje cells are weakly stained with the antibody (a). Homogeneous staining was observed in heart (c). (b and d) Immunostaining using an antibody fraction which was preadsorbed with the peptide. Nuclei were stained with hematoxylin in each preparation.

38 kDa (p38), suggesting that p76CTBP is a homodimer of p38CTBP (11). In order to evaluate whether the antibody interferes with NADPH-dependent T3 binding and dimerization, we examined the effect of CTBP168 on these activities. CTBP168 affected neither the T3 binding nor homodimerizing activities. These results indicate that the antibody can react with p38CTBP without modification of these important functions of this protein.

We examined the localization of p38CTBP in human tissues. Northern blotting in eight human tissues demonstrated that p38CTBP is a single mRNA species (23). As shown in the Results, dot hybridization demonstrated that there is heterogeneous expression of p38CTBP in the central nervous system. Expression is high in cerebral cortex, cerebellum, amygdala, cauda nucleus, hippocampus, putamen and accumbens nucleus. However, expression in pons, corpus callosum, medulla oblongata, substantia nigra, thalamus and spinal cord is low. The expression of thyroid hormone

receptors (TRs) is known to be heterogeneous in the central nervous system (27, 28). TR α 1 is abundant in brain, heart, skeletal muscle and brown adipose cells (29, 30), whereas TR β 1 expression is more abundant (31), suggesting that p38CTBP appears to be expressed in tissues where TR α 1 is abundantly expressed. However, the precise relationship between expressions of p38CTBP and TRs could not be resolved in this study.

p38CTBP was not expressed in the human cancer cell lines studied. The NADPH-dependent T3 binding, however, was measurable in rat hepatoma cell lines, dRLh (19), suggesting that the pathological role of p38CTBP expression remains to be characterized.

As has been observed, abundant p38CTBP was detected in brain and heart by immunohistochemical studies. In cerebrum, p38CTBP was predominantly expressed in the cytoplasm of both neural and glial cells. The nuclear expression level of p38CTBP may be too low to detect the immunofluorescence of the

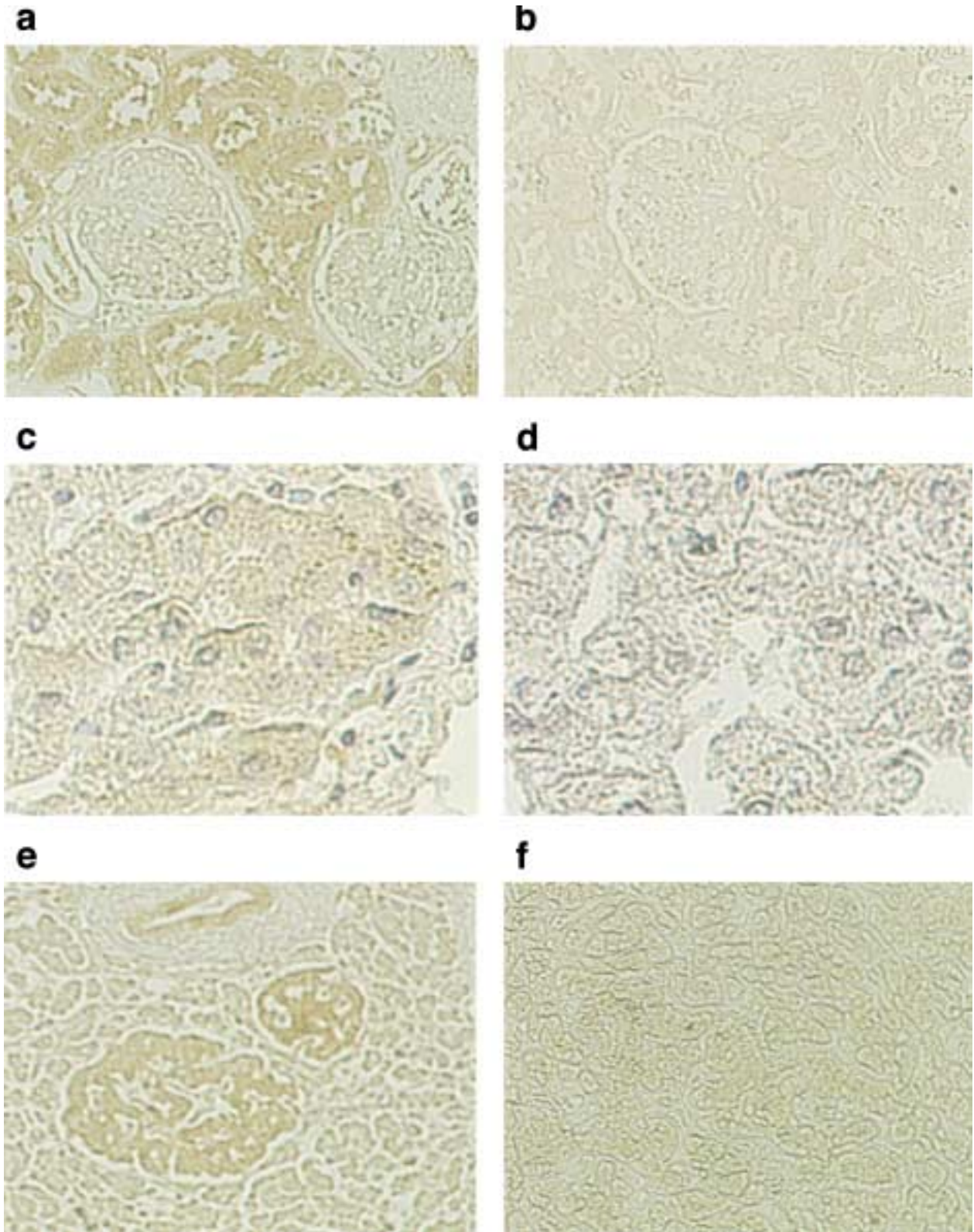


Figure 6 Immunostaining of p38CTBP in adult human kidney, liver and pancreas. Kidney (a and b), liver (c and d), and pancreas (e and f) were immunostained as described in Materials and methods. (a) Renal tubules but not glomerular cells are stained. (c) Weak staining is observed in liver. (e) Islet but not exocrine cells are stained with the antibody. Immunostainings using antibody fraction which was preadsorbed with the peptide are shown in b, d and f.

antibody in nuclei, since we observed that a part of the CTBP could be transported into nuclei and that acceptor protein for CTBP is present in nuclei as previously reported (7, 9). The study showed cell-specific localization of p38CTBP in the inner layer of cerebellum. Granule cells were specifically, but not homogeneously, stained with the antibody. This mosaic staining of granule cells may reflect the presence of a sub-population of granule cells (32). Cell-specific localization of p38CTBP was also observed in other tissues. Tubular but not glomerular cells in kidney, and islet but not exocrine cells in pancreas were stained with the antibody, suggesting that strictly controlled cell-specific expression must be present.

In this study, by estimating the expression of p38CTBP, we have demonstrated that p38CTBP is preferentially expressed in human brain and heart, and that cell-specific p38CTBP expression is present. While the cell-specific distribution of p38CTBP may play important roles in the cell-specific regulation of thyroid hormone action, the precise physiological function of p38CTBP remains to be elucidated.

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