A Serine/Threonine Kinase Which Causes Apoptosis-Like Cell Death Interacts with a Calcineurin B–Like Protein Capable of Binding Na⁺/H⁺ Exchanger[1]

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Received March 30, 2001, accepted May 10, 2001

We surveyed proteins capable of binding to the cytoplasmic domain of Na^+/H^+ exchanger (NHE)1 in a rat brain cDNA library with the yeast two-hybrid system. One clone obtained coded for a protein reported previously as a human calcineurin homologous protein (CHP). Since CHP is homologous to the regulatory subunit B of calcineurin, we expected a possible interacting partner of CHP like the catalytic subunit of calcineurin (calcineurin A), and surveyed this putative partner again with the yeast two-hybrid system. A clone thus obtained coded for a kinase, which is basically the same as that reported for human DRAK2. Overexpression of the rat homologue of DRAK2 caused apoptosis-like cell death of NIH3T3 cells, which was dependent on the kinase activity, confirming the previous result for DRAK2. The purified CHP and rat DRAK2 proteins synthesized in *Escherichia coli* could bind *in vitro*. CHP and rat DRAK2 expressed in COS-7 cells were found to be localized in the Golgi apparatus and nucleus, respectively. Some of them was also found in the membrane peripheral region. When they were co-expressed in the same cells, most of CHP moved to the nucleus where rat DRAK2 is located, suggesting *in vivo* interaction of these proteins. However, minor but significant fractions of both proteins were also found in the membrane peripheral region. Rat DRAK2 is expressed highly in thymus, spleen, and testis, where the apoptosis plays an important role in physiology.

Key words: apoptosis, calcineurin B–like protein, NHE, serine/threonine kinase.

Na^+/H^+ exchangers (NHEs) comprise a family of plasma membrane proteins of mammalian cells that mediate the electroneutral exchange of intracellular H^+ and extracellular Na^+. The NHEs participate in a number of cellular functions, including regulation of pH, cell volume, cell proliferation, differentiation, and neoplastic transformation (*1–3*). Although the C-terminal cytoplasmic domains of NHEs are thought to be involved in regulation of the exchanger activity in response to extracellular signals (*4, 5*), little is known about the mechanism of this regulation. Several regulatory factors, calmodulin (*6*), human calcineurin B homologous protein (CHP) (*7*), and NHERF (*8*), and E3KAP (*9*), which bind to the cytoplasmic domains of NHEs, were reported recently for NHE1 and NHE 3, respectively. However, the detailed mechanisms of the regulation with these factors remained unknown.

Using the yeast two hybrid-system (*10*), we obtained a rat cDNA which codes for CHP capable of binding the cytoplasmic region of rat NHE1 in the present study. Human CHP has been shown to interact with the cytoplasmic region of NHE1 previously by a survey based on direct protein-protein interaction (*7*). CHP has a primary structure that is extensively similar to that of the regulatory subunit of protein phosphatase 2B (calcineurin B) (*11*) and belongs to the EF-hand superfamily of Ca^{2+} binding proteins (*12*). Calcineurin (CN) is a heterodimer of catalytic (calcineurin A, CNA) and regulatory (calcineurin B, CNB) subunits (*13*). CNA exhibits very low phosphatase activity by itself, but binding of Ca^{2+}/CaM to the A subunit and/or binding of Ca^{2+} to the B subunit induces noticeable CN activation (*13, 14*). Because of the extensive homology of CHP to CNB, we expected that CHP might function as a regulatory protein in response to the Ca^{2+} signal with a putative CNA-like catalytic subunit protein.

In the present study, we attempted to isolate this putative protein which interacts with CHP by using the yeast two-hybrid system (*10*). As a result, one positive clone was obtained. The complete nucleotide sequence of this clone

[1] The present study was supported partly by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan to H.K., and also by a grant from CREST of the Japan Science and Technology Corporation to H.K.

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Abbreviations: NHE1, Na^+/H^+ exchanger 1, CHP, calcineulin homologous protein, DRAK, DAP kinase related apoptosis inducing protein kinase, DAP, death associated protein, rDRAK2, rat homologue of DRAK2; GST, glutathione S-transferase, GAPDH, glycerol aldehyde-3-phosphate dehydrogenase; DMEM, Dulbecco's Modified Eagle Medium; PCR, polymerase chain reaction, SD, synthetic medium containing dextrose

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contained an open reading frame encoding 371 amino acids. This protein turned out to be a rat homologue of human apoptotic kinase DRAK2 (*15*) We found that CHP and rat DRAK2 produced in *Escherichia coli* bound *in vitro* and colocalized within cultured mammalian cells, suggesting significant interaction of the two proteins *in vivo*.

MATERIALS AND METHODS

E. coli Strains and Cultures—E. coli JM109 (*16*) cells were cultured to propagate plasmid DNAs in L broth medium (*17*) with vigorous shaking at 37°C or incubated on L broth agar containing 50 μg/ml ampicillin to select plasmids. For the production of fusion proteins in *E coli*, M9ZB medium (*18*) was used. For cloning with the yeast two-hybrid system, yeast strains HF7c (His⁻), for His⁺ selection, and SFY526, for expression of β-galactosidase, were used (*19*). Clones were selected on SD medium agar with or without appropriate supplements. To reduce the background of His⁺ clones, 10 mM 3-amino-triazol was added to selecting plates (*19*).

Construction of Bait Plasmids and Selection of Desired Clones with the Yeast Two-Hybrid System—DNA fragments coding for the region between residues 504 and 670 of rat NHE1 (*20*) were produced by PCR (*21*) and then introduced into pGBT9 (*19*). The entire coding region of rat CHP (195 amino acid residues) was amplified with PCR and then integrated between the unique *Bam*HI and *Sal*I sites in pGBT9. These bait plasmids were introduced into yeast HF7c. For selection of the desired clones, a cDNA library (Clontech) of rat brain fused to the activation domain of *GAL4* in the pGAD10 expression plasmid was introduced into the clones with a bait plasmid. Then, His⁺ clones were selected as described previously (*19*). Transformants of *E. coli* HB101 cells with the isolated clones were selected as to the Leu⁺ phenotype to isolate pGAD10 derivative plasmids. These plasmids were introduced into yeast SFY526 carrying the reporter β-galactosidase gene. Positive clones which showed β-galactosidase activity were selected on SD plates (*19*) after incubation for 1 day at 30°C.

Cloning of the Entire Rat DRAK2 Gene—The entire coding region of rDRAK2 was cloned from a cDNA library of rat brain (Clontech, rat brain 5′-stretch cDNA library, with λgt11 as the vector). By using the coding region of the carboxy-terminal half of rDRAK2 (*Eco*RI fragment between nucleotide numbers 679 and 1375 of the coding frame of rDRAK2) as a probe for hybridization, 3×10^{-6} independent clones were surveyed. Six independent clones which carry different portions of the rDRAK2 coding region were isolated. *E. coli* was infected with the obtained phages and phage DNAs were recovered as described previously (*22*).

Overexpression, Purification and Polyclonal Antibodies for CHP and the CHP Binding Region of rDRAK2—The entire region of DNA encoding CHP derived from the clone obtained on the two-hybrid screening in this study was fused to a 6 × Histidine tag in expression vector pET21b (*23*). The CHP-His fusion protein was overexpressed in *E. coli* and affinity-purified as described previously (*23*) for an antigen.

DNA fragments corresponding to amino acid residues between 227 and 371 (rDRAK2-C), and the entire region of rDRAK2 (rDRAK2-FL) were produced by PCR with primer oligonucleotides, 11-FW, 5′-CTAGCTAGCACGTTGTTGACTCATAC-3′, and 11-RV, 5′-CCGCTCGAGACAGAGACAGAACAAATCTGGAACG-3′, for rDRAK2-C, and rDRAK2-FW2, 5′-GGAATTCCATATGTCTCGGAGGAGATTG-3′, and 11-RV for rDRAK2-FL. The amplified DNAs were integrated between unique restriction sites, *Nhe*I and *Xho*I, for rDRAK2-C, or between *Nde*I and *Xho*I for rDRAK2-FL, respectively, in an expression plasmid, pET21a(+) (Novagen) (*23*), which includes 6 × histidines as a tag sequence at the carboxy-terminal end. The primers have sequences for *Nhe*I, *Nde*I, or *Xho*I sites. The resulting plasmids were introduced into *E. coli* BL21 and then the CHP binding region in rDRAK2 (rDRAK2-C) or the entire region of rDRAK2 with 6 × His-tag (rDRAK2-C-His or rDRAK2-FL-His) was overproduced These peptides were purified by affinity chromatography in agarose resin with nickel NTA (*23*) (Qiagen) as the ligand.

Purified CHP-His and rDRAK2-C-His were injected into rabbits to induce polyclonal antibodies. The polyclonal antibodies were purified by conventional chromatography as described previously (*18*), and further subjected to affinity chromatography using purified CHP fused to GST (glutathione S-transferase) (*18*) or rDRAK2-C fused to GST as the ligand. A single band corresponding to the expected molecular size for CHP or rDRAK2 was observed on Western blotting analyses with the purified antibodies for extracts from several rat tissues. These results suggested that the antibodies used are specifically reactive to CHP or rDRAK2.

Assaying of Binding between CHP and rDRAK2 with the Yeast Two-Hybrid System—DNA encoding the entire CHP fused to the binding domain of *GAL4* in expression vector pGBT9 was used to detect interactions with various portions of rDRAK2. DNAs corresponding to various portions of rDRAK2 shown in Fig. 3 were amplified by PCR with the cloned rDRAK2 DNA as the template and the following primers for each segment: FL, 5′-TCGGATCCATGTCTCGGAGGAGATTG-3′ (CBK-FW primer) and 5′-AGAGGTCGACTAACAGAACAAATCTGG-3′ (11-RV primer); KD, rDRAK2-FW primer and 5′-GTCCGTCGACTACAGCCATGAGTGGGAAAG-3′ (KD-RV primer); Cbk-C1, 5′-TAGGATCCATGTTGACTCATAC-3′ (rDRAK2-C1-FW primer) and 11-RV primer; rDRAK2-C2, 5′-CAGGATCCCAGCAGTGGGACTTTGG-3′ (rDRAK2-C2-FW primer) and rDRAK2-RV primer; rDRAK2-C3, rDRAK2-C1-FW primer and KD-RV primer; and rDRAK2-C4, 5′-TGGGATCCTCAGTTTCACAACTGG –3′ (rDRAK2-C4-FW Primer) and KD-RV primer. Amplified DNAs were integrated into expression plasmid pGAD10. These plasmids and the pGBT9 derivative carrying CHP were introduced into yeast SFY526, and then expression of the reporter β-galactosidase was assayed as described previously (*19*).

Detection of Protein–Protein Interaction—An expression vector of a GST (*18*) fusion of CHP was constructed as follows: the coding sequence of rat CHP was amplified by PCR with primers corresponding to the 5′ and 3′ end portions (17 nucleotides) of CHP (primer 18-3, 5′-CGTCGGATCCATGGGGTCTCGGGCGTCCAC-3′ and primer 18-4, 5′-TCTCGTCGACTTAGTGAAFAAATCGGATGCT-3′), and the entire sequence of CHP as the template. The primers have unique restriction sites, *Bam*HI and *Sal*I, and the amplified DNAs with the restriction sites after digestion with these enzymes were integrated between the two sites in expression vector pGEX-4T-1 (*24*). The GST-CHP fusion

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overproduced in *E. coli* was purified by affinity chromatography on glutathione Sepharose 4B (Pharmacia Biotech) as described previously (*18*). rDRAK2-C-His and rDRAK2-FL-His, which were also purified by affinity chromatography, were used as probes for protein-protein interaction. Purified GST-CHP (8 μg) or GST (4 μg) was subjected to SDS-PAGE and then blotted onto a nylon filter, followed by incubation in phosphate-buffered saline (PBS^-) containing 5% skimmed milk to block the filter. The filter was incubated with purified rDRAK2-C-His (15 μg/ml) for 12 hr at 4°C as described previously (*18*). Then the bound rDRAK2 was visualized with rDRAK2 specific antibodies and by using an ABC vectastain kit as described previously (*18*).

Detection of Expression of rDRAK2 on Northern Blot and Western Blot Analyses—Various rat tissues were subjected to acid guanidinium/phenol/chloroform extraction to prepare total RNA as described in Ref. *22*. Frozen tissues were homogenized in a solution containing guanidinium-isothiocyante (ISOGEN) purchased from Nippon Gene. The homogenates were centrifuged at 15,000 ×*g* for 10 min to remove tissue debris. The resulting supernatants were mixed with solution containing phenol and chloroform. Then total RNA (20 μg) recovered by ethanol precipitation was subjected to agarose gel electrophoresis with formaldehyde (*22*). After electrophoresis, RNAs were blotted onto a transfer membrane filter (Hybond N^+, Amersham), fixed under UV, and then hybridized with a ^{32}P labeled probe. For detecting rDRAK2 expression, an *Eco*RI fragment (nucleotides 679–1375) derived from the 3′ terminal region including the 3′ non-coding region of rDRAK2, which was labeled with α-^{32}P-dCTP according to the published procedure was used (*22*). For hybridization and washing, the procedures described in Ref. *22* were used. The membrane filters were exposed to an imaging plate of BAS 1000 (Fuji Film) and the labeled bands were visualized.

Proteins (40 μg) from various rat tissues were subjected to SDS-PAGE and the separated proteins were transferred to nylon membranes as described previously (*18*). Affinity-purified antibodies against CHP and rDRAK2 were added to the blotted membrane filter. Immuno-reactive materials were visualized by means of an ABC vectastain kit or the ECL chemiluminesence method, as described previously (*25*)

Assaying of Apoptosis by rDRAK2—For the analysis of apoptosis-like cell death caused by expression of rDRAK2 in NIH3T3 cells, the following expression plasmids were constructed. A 1.5 kbp *Eco*RI DNA fragment derived from a cloned lambda phage which covers 167 bp DNA from the initiation codon (5′ non-coding region), the entire coding region and 246 bp of the 3′ non-coding region of rDRAK2 coding frame was integrated into the *Eco*RI site in plasmid vector pEF-BOS-EX, which contains the promoter for elongation factor 1, the SV 40 ori sequence and the polyadenylation signal of human G-CSF (*26*), and named myc-rDRAK2/pEF-BOS-EX. As a negative control, the same expression vector except for substitution of Ala for Lys-62, which is possibly essential for the kinase activity, was constructed by substituting GCA (Ala) for AAA (Lys). These expression vectors were introduced into NIH3T3 cells by means of the lipofection method, as described previously (*27*), together with pRSV-neo (*22*), which confers neomycin resistance. For lipofection, Trans IT (LT-1) polyamine Transfection reagents (Mirus) were used. After three weeks transfection and incubation in DMEM (10% fetal calf serum) with the addition of G418 (600 μg/ml, Gibco), the numbers of surviving cells were determined after staining with a dye (0.5% toluizine blue in boric acid solution).

Expression of CHP and rDRAK2 in COS-7 Cells—The entire coding sequence of CHP was amplified by PCR with the primer oligonucleotides (primers 18-3 and 18-4) carrying *Bam*HI and *Sal*I sites, respectively. After digestion of these restriction sites, the amplified DNA was integrated between the *Bam*HI and *Sal*I sites in pEF-BOS-EX (named rDRAK2/pEF-BOS-EX). A Myc-tag sequence was added at the 5′ end of the coding sequence of rDRAK2 or rDRAK2 with the K62A mutation in rDRAK2/pEF-BOS-EX (named myc-rDRAK2/pEF-BOS-EX and myc-rDRAK2K62A/pEF-BOS-EX, respectively). These plasmid DNAs purified by CsCl density gradient centrifugation were introduced into COS-7 cells by means of the lipofection method (*25*) Transfected cells were incubated in DMEM supplemented with 10% fetal calf serum for 48 h at 37°C in a CO_2 incubator (5% CO_2). Expression of CHP and rDRAK2 tagged with a myc sequence in COS-7 cells was detected by immunological staining with affinity purified anti-CHP antibodies or anti-myc monoclonal antibodies, and then anti-rabbit IgG labeled with FITC (American Qualex Antibodies) or anti-mouse IgG labeled with Alexa 546 (Molecular Probes). For identification of the Golgi apparatus, wheat germ agglutinine labeled with tetramethylrhodamine (Molecular Probes) was used.

DNA Sequencing and Other Methods—The nucleotide sequences of various DNA segments used in this study were determined by the protocol for cycle sequencing recommended by Perkin-Elmer for an ABI 373S or ABI 377 prism DNA sequencer. Preparation of plasmids, digestion with restriction endonucleases, ligation with T4 DNA ligase of the DNA fragments and other techniques related to the handling of DNA were performed according to the published procedures (*22*)

Materials Used—Restriction endonucleases, T4 DNA ligase, *Tth* and *Pfu* DNA polymerases, and T7 DNA polymerase were purchased from Toyobo, New England Biolabs, and Takara. Oligonucleotides were synthesized by Pharmacia Biotech. Other reagents and materials were of the highest grade commercially available.

RESULTS

Cloning of a New Protein Gene Capable of Binding to Rat Calcineurin Homologous Protein (CHP)—We have obtained a rat homologue of calcineurin homologous protein, CHP, in the present study by means of yeast two-hybrid screening as the binding partner of NHE1. CHP was reported found to be a human NHE1 binding protein with an assay based on direct protein–protein interaction (West-Western method) (*7*). Thus we confirmed the previous results for the case of rat by means of a different approach. Since CHP has a sequence homologous to that of the calcineurin B subunit, we thought that this protein could bind to calcineurin A or a protein similar to calcineurin A. To obtain such a putative binding partner of CHP, we have surveyed a cDNA library of rat brain with the yeast two-hybrid system. As shown in Table I, 162 clones which grew on selecting SD plates without histidine were obtained from 1.65×10^{-6} transformants with the rat brain cDNA library with the HF7c strain

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(His^-) as a host. After repeated selection with the same plate, 116 clones were isolated as His^+. These clones were further subjected to selection under the same conditions but with 10 mM 3-amino-triazol, a histidine analogue which reduces the background of the selection. Three clones were selected and plasmids were isolated from these clones. These plasmids were introduced again into another yeast strain, SFY526, which carries a reporter gene, β-galactosidase Finally, one clone was obtained as a candidate which contained a gene encoding a peptide capable of binding to CHP. Expression of the reporter β-galactosidase was much higher than that in the case of the combination of the α and β subunits of *E. coli* F_1 ATPase as a positive control of protein–protein interaction (*19*). This suggested that the binding of the putative peptide cloned here with CHP is significantly tight.

Cloning of the Whole Sequence of the Putative CHP Binding Protein—The candidate clone contained an insert DNA in a derivative plasmid of pGAD10. Then we sequenced the DNA encoding the CHP binding peptide and found a potential open reading frame encoding 145 amino acid residues (Fig. 1). Since no initiation codon was found in this open reading frame, we further cloned the entire coding region of the putative CHP binding peptide from a rat brain cDNA library. The isolated clone contained a potential initiation codon lying in the same reading frame upstream of the stop codon. The predicted protein sequence consists of 371 amino acid residues and has a calculated molecular weight of 42,132 (Fig. 1). The original sequence found in the two-hybrid system corresponded to the residues between 227 and 371. The peptide has a domain highly homologous to that found for a kinase domain derived from a death-associated protein kinase (Fig. 1).

Apoptotic and Protein Kinase Activities of the Cloned Peptide—After we had cloned the gene encoding the CHP binding protein, Sanjo *et al.* found human DRAK2 (*15*) by surveying clones carrying the sequence homologous to the kinase domain derived from the death associated protein kinase (*15*). Human DRAK2 showed the protein kinase activity and also the ability to cause apoptosis-like cell death (*15*). The CHP binding protein found in the present study was essentially the same as human DRAK2 (96% homology in the amino acid sequence) and named rDRAK2 (rat homologue of DRAK2). Here we prepared COS-7 cells

TABLE I **Screening of CHP binding clones with the yeast two-hybrid system.**

Screening	Number of screened clones
original	1.65×10^5
1st His^+	162
2nd His^+	116
3rd His^+ with 3-AT	3
4th β-galactosidase$^+$	1

The details of the screening of positive clones are given under "MATERIALS AND METHODS" For the 1st and 2nd screening, clones with the His^+ phenotype of HF7c (His^-) carrying the expression plasmid of CHP (derivative of the pGBTK plasmid) were selected after introduction of a cDNA library of rat brain that was integrated into the pGAD10 vector. For the 3rd screening, 10 mM 3- amino-triazol was added to the plates used for screening to reduce the His^+ background After the 3rd screening, plasmids were recovered from the candidate clones and introduced again into yeast SFY526 to test the expression of β-galactosidase

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CGCCGCTGCACTTTTCAAACCTCAACTGTAAGAAGCGTCGGTCAGCGTCTGTGCGGTCGCCGCCGGGAGTCTCCTCATAGGGGTCTGGCT 90
GACGGCGACCAGCCGCTGTGGGGAAGAGTGCGAGGTGAAAGTCAGTGCCTAGAGAAGCAGGCCTGGTAGTCATCAACATGTCTCGGAGGA 180
                                                                              M  S  R  R
GATTTGATTGCCGAAGTATCTCGGGCTTGCTAACTACAACCCCTCAAACGCCGATGAAAACAGAGAATTTTAATAATTTCTATACGCTTA 270
R  F  D  C  R  S  I  S  G  L  L  T  T  T  P  Q  T  P  M  K  T  E  N  F  N  N  F  Y  T  L
CCCCGAAAGAACTTGGGAGAGGAAAATTTGCAGTAGTTAGACAGTGTATATCAAAGTCAACTGGACAGGAGTATGCTGCCAAATTCCTGA 360
T  P  K  E  L  G  R  G  K  F  A  V  V  R  Q  C  I  S  K  S  T  G  Q  E  Y  A  A  ■  F  L
AGAAGAGGAGAAGAGGGCAGGACTGCCGGGCAGAGATTCTGCACGAGATAGCGGTGCTGGAGCTGGCCAGGTCTTGTCCCCACGTGATTA 450
K  K  R  R  R  G  Q  D  C  R  A  E  I  L  H  E  I  A  V  L  E  L  A  R  S  C  P  H  V  I
ATCTGCACGAGGTCTATGAAACGGCAACGGAAATCATTTTGGTGTTAGAATATGCTGCAGGTGGAGAAATTTTCAACCTGTGTTTACCTG 540
N  L  H  E  V  Y  E  T  A  T  E  I  I  L  V  L  E  Y  A  A  G  G  E  I  F  N  L  C  L  P
AGTTGGCTGAAATGGTGTCTGAAAATGATGTTATCCGACTCATTAAACAAATACTTGAAGGAGTTCATTATCTACATCAGAATAACATTG 630
E  L  A  E  M  V  S  E  N  D  V  I  R  L  I  K  Q  I  L  E  G  V  H  Y  L  H  Q  N  N  I
TTCATCTTGATTTAAAGCCACAGAATATACTTTTGAGCAGTATATACCCACTTGGGGACATAAAAATTGTAGATTTTGGAATGTCTCGAA 720
V  H  L  D  L  K  P  Q  N  I  L  L  S  S  I  Y  P  L  G  D  I  K  I  V  D  F  G  M  S  R
AAATTGGGAATGCAAGTGAGCTTCGGGAAATCATGGGAACGCCTGAATACTTAGCTCCAGAAATCCTCAACTATGATCCCATTACCACAG 810
K  I  G  N  A  S  E  L  R  E  I  M  G  T  P  E  Y  L  A  P  E  I  L  N  Y  D  P  I  T  T
CAACAGATATGTGGAATATTGGCATAATAGCATATATGTTGTTGACTCATACATCACCATTTGTAGGAGAAGATAACCAAGAAACATATC 900
A  T  D  M  W  N  I  G  I  I  A  Y  M  L  L  T  H  T  S  P  F  V  G  E  D  N  Q  E  T  Y
TGAATATTTCTCAAGTGAATGTAGATTATTCAGAAGAAATGTTTTCATCAGTTTCACAACTGGCCACAGACTTCATCCAGAGCCTTCTAG 990
L  N  I  S  Q  V  N  V  D  Y  S  E  E  M  F  S  S  V  S  Q  L  A  T  D  F  I  Q  S  L  L
TAAAGAATCCAGAGAAAAGACCAACAGCAGAATCCTGCCTTTCCCACTCATGGCTGCAGCAGTGGGACTTTGGAAGCTTGTTTCATCCTG 1080
V  K  N  P  E  K  R  P  T  A  E  S  C  L  S  H  S  W  L  Q  Q  W  D  F  G  S  L  F  H  P
AGGAAACTTCAGAGTCCTCTCAAACTCAGGATCTGAGCCTCAGGTCCTCTGAAGATAAGACCCCCAAGTCCTGTAATGGAAGCTGTGGAG 1170
E  E  T  S  E  S  S  Q  T  Q  D  L  S  L  R  S  S  E  D  K  T  P  K  S  C  N  G  S  C  G
ACCGAGAGGACAAGGAGAACATCCCTGAGGATGACAGCTTACTTTCTAAAAGATTTCGATTCGATGACTCCTTGCCCAGCCCCCACGAAC 1260
D  R  E  D  K  E  N  I  P  E  D  D  S  L  L  S  K  R  F  R  F  D  D  S  L  P  S  P  H  E
TCGTTCCAGATTTGTTCTGTTAGCATTTCTCTCTGTCATTCATCTGGACTGACTTGGAAATTTGAAATCTTCTCTGGTGTCAGATTGTGT 1350
L  V  P  D  L  F  C
CTAGCATATCATTTCCTGTCCTGAAATTGTTTTATAGAGAAAAATATTTAAATATATGACAAAACTGTAAATTTTGCATGTGAGTTCATA 1530
TGCAACTGAAAGAATTCAAGTTCAGGTGAAGTTATAAAATGGGTTATGTTATGTTTAGTAAATATTGAACAAAATGAAAAAATAGGAATT 1620
TTGTAGCTTCATATATAATATGTTTATATTATAAGTGCACTTTTGCTTAGAAGAACTTAAGGAACAGTTCAAATGCTAGCCTTCTATTAG 1440
TAGTAGGTCCTCTCTAAGGTAAGCCCTATACCAGAACCCTATTAGCAGAGAATTTTGTTTGGCTGTCAAGGGTAAACCACAATATATACT 1710
GCTTTATACATACTCCAGAGATATTTTATAACTGAAAATGCTTCCCAGTTACCAATAGCTTGAAATTGTACGATTCTTTCTGTGCCATGT 1800
GCTCTGTGAGAAGGCACAGTCAACCAGAGCAGGGGAAACCAGATTGGTCCAGGCTGTTTCTCTGTAAACTGACATCATTTCCAACTCACA 1890
TATCTTGCACATGCATCCTATTTCAGATCTAGTAAGATCTGGAAGACGGATATGCATGGTATGCAAATAAAAGAGGACTTCATCCTCTAG 1980
AATTGTTAGAGTAGAGGAGAATTGGGGAGTAGCAAATGTGTTCTAATTTCAGTCAGACATATTCATAAAGGGAAATGTTAAGTCCAGGCA 2070
GCTGACTGAGTGTTGGGTGTCTAAGTCTCAGGATGCAAGCCCATCGTTTAGAGCATAGGCACTTCTGGAGTTCTTGTGTGAAATTTTAG 2159
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Fig. 1 **Nucleotide sequence and deduced amino acid sequence of rDRAK2.** The nucleotide sequence (2,159 bp) around the rDRAK2 coding region derived from the rat brain cDNA library is shown together with a putative amino acid sequence, the latter being shown under the nucleotide sequence as capital letters as abbreviations of amino acids. A putative kinase domain which is highly homologous to human and rat DAP kinase (*29*), and human and mouse ZIP kinase (*30, 31*) is underlined Lys-62 essential for the kinase activity is shown by a black box The coding region that was originally cloned with the yeast two-hybrid system was underlined with hatched lines

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transfected with the expression vector of rDRAK2 with a myc-tag. We found that materials immunologically reactive with anti-rDRAK2 antibodies from extract of the transfected COS-7 cells showed the protein kinase activity (data not shown). We also observed that overexpression of rDRAK2 in NIH3T3 caused apoptosis like cell death, as described for human DRAK2 (*15*). A significant number of cells exhibited morphological changes found typically for apoptotic cells during transient expression of the gene (data not shown). As shown in Table II, after 3 weeks culture of transfected cells, the surviving cell number had decreased compared to those for the control without transfection and also a mutant rDRAK2 with an amino acid substitution (Lys62Ala) in the putative catalytic domain. Since the mutant rDRAK2 (Lys62Ala) did not show the kinase activity (data not shown), the apoptotic activity was found to be dependent on the kinase activity, as reported previously for human DRAK2 (*15*).

Binding of CHP and rDRAK2—To test the binding of CHP and rDRAK2 by means of *in vitro* assaying, we overexpressed in *E. coli*, and purified the peptide corresponding to the region between residues 227 and 371 of rDRAK2 (rDRAK2-C1) and the entire rDRAK2 sequence with a 6 × histidine-tag. CHP fused to glutathione S-transferase produced in *E. coli* was subjected to polyacrylamide gel electrophoresis, blotted onto a nylon filter, and then reacted with the purified rDRAK2-C1 and the entire rDRAK2 As shown in Fig. 2, the peptide and the entire rDRAK2 bound to CHP but not to GST. The binding was slightly weaker for the entire rDRAK2 than the peptide (Fig. 2)

TABLE II **Assaying of apoptosis-like cell death with rDRAK2 in NIH 3T3 cells.**

Plasmid	Clones
Vector	203
Wild-type rDRAK2	44
Mutant rDRAK2(K62A)	144

An expression vector, myc-rDRAK2/pEF-BOS-Ex or myc-rDRAK2-(K62A)/pEFBOS-EX (8 μg), together with pRSV-neo (0 8 μg) was introduced into NIH3T3 cells (8 × 10^3 cells) After 3 weeks incubation in DMEM with G418 (600 μg/ml), the numbers of neomycin-resistant clones were determined by staining cells with toluizine blue

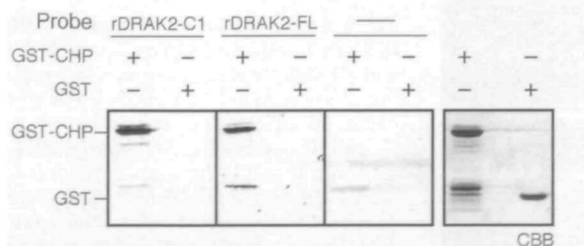

Fig 2 **Interaction of rDRAK2 and CHP.** The CHP-GST fusion, which was synthesized in *E coli* cells and then purified, was subjected to SDS gel electrophoresis and then transferred to a nylon membrane The blotted membrane was incubated with rDRAK2-C1 or rDRAK2-FL as shown in the legend to Fig. 3 rDRAK2 bound to CHP was detected by means of specific binding of anti-rDRAK2 polyclonal antibodies and visualized with an ABC vectastain kit (in the left three columns) As a negative control, GST alone was applied In the most right column, CHP-GST or GST applied is stained with Coomasie Brilliant Blue

As shown in Figs. 1 and 2, the original clone selected from the two-hybrid library carried a peptide corresponding to the region between residues 227 and 371 in rDRAK2 We mapped the binding domain within a more limited area with the two-hybrid system by constructing a series of truncated CHP binding kinases as shown in Fig. 3. The results indicated that a shorter region between residues 227 and 293 could bind to CHP, suggesting that at least 67 residues of the carboxy-terminal end region of the kinase domain are responsible for the binding to CHP.

Expression of rDRAK2 in Rat Tissues and Cell Lines—Expression of rDRAK2 was surveyed in various rat tissues by analyzing mRNA and also protein. As shown in Fig. 4, mRNA (approximately 4 5 kbp) was found for spleen and testis, suggesting higher expression of rDRAK2 in these tis-

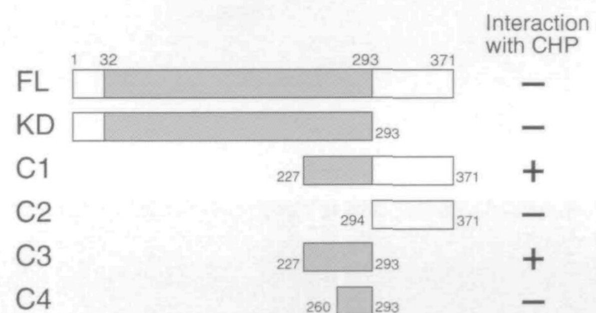

Fig 3 **Mapping of the binding domain of rDRAK2 as to CHP with the yeast two-hybrid system.** Portions of rDRAK2 which are required for binding to CHP were examined with the yeast two-hybrid system (*10*) Expression plasmids carrying various portions of rDRAK2 (FL, full length of the coding region; KD, putative kinase domain, C1-C4, carboxy-terminal half region, amino acid residues are shown at the ends of each domain) fused to pGAD10 were introduced into yeast SFY526 cells carrying the expression vector of the entire sequence of CHP fused to pGBT9 Expression of the reporter β-galactosidase was assayed for yeast cells carrying the expression plasmids of portions of both rDRAK2 and CHP as described under "MATERIALS AND METHODS" As a positive control for the system, expression of the α and β subunits of *E coli* F_1-ATPase fused to pGAD10 and pGBT9, respectively, was examined to confirm the two-hybrid system worked as described previously (*19*) When pGBT9 instead of the CHP fusion plasmid was introduced, no significant expression of the β-galactosidase was observed (data not shown)

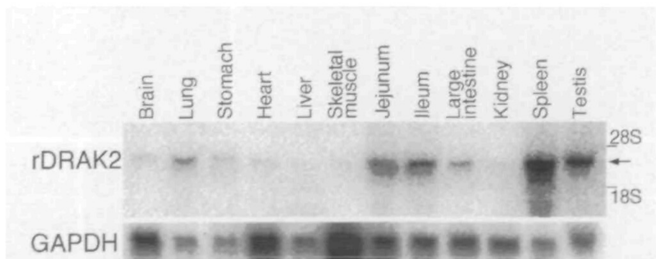

Fig 4 **Northern blot analysis of rDRAK2 expression in various rat tissues.** Total RNA from the various rat tissues shown at the top of figure was subject to agarose gel electropohoresis and then blotted onto a nylon membrane. The filter was incubated with probe DNA (700 bp *Eco*RI fragment of the carboxy terminal end region of rDRAK2) labeled with ^{32}P and hybridized bands were visualized with Fuji BAS 1000. As a control, expression of GAPDH was analyzed with the entire sequence of rat cDNA of GAPDH as the probe. A hybridized band was observed between those of 28S and 18S ribosomal RNA, as indicated by an arrow.

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sues. Then we analyzed the expression of this protein in various organs by means of immunological detection, and found significantly high levels of expression in thymus, spleen, and testis (Fig. 5). In brain relatively low but significant expression was observed in a relatively slow migration area. In cultured cell line NRK (normal rat kidney cell), expression of rDRAK2 was detected, but not in COS-7 cells.

Colocalization of CHP and rDRAK2 in COS Cells—To examine the interaction of CHP and rDRAK2 within mammalian cells, we determined the location of these proteins by means of an immuno-cytochemical procedure in COS-7 cells, in which both proteins were expressed due to the introduction of the expression vectors. Expression of CHP

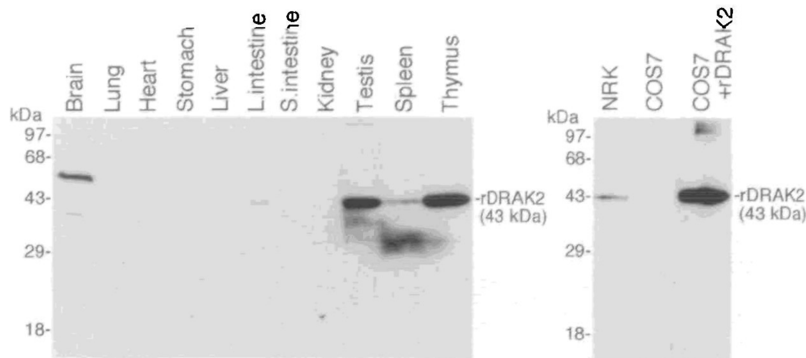

Fig 5 **Western blotting analyses of rDRAK2 in various rat tissues and cultured cells.** Proteins prepared from various rat tissues (40 μg) or cultured cells (NRK and COS-7, 10 μg for each lane, COS-7 with rDRAK2, 1 μg) were separated by SDS gel electrophoresis and then blotted onto a nylon membrane. The membrane was incubated with anti-rDRAK2 polyclonal antibodies and the reacted bands were visualized by means of a chemiluminescence method (*25*)

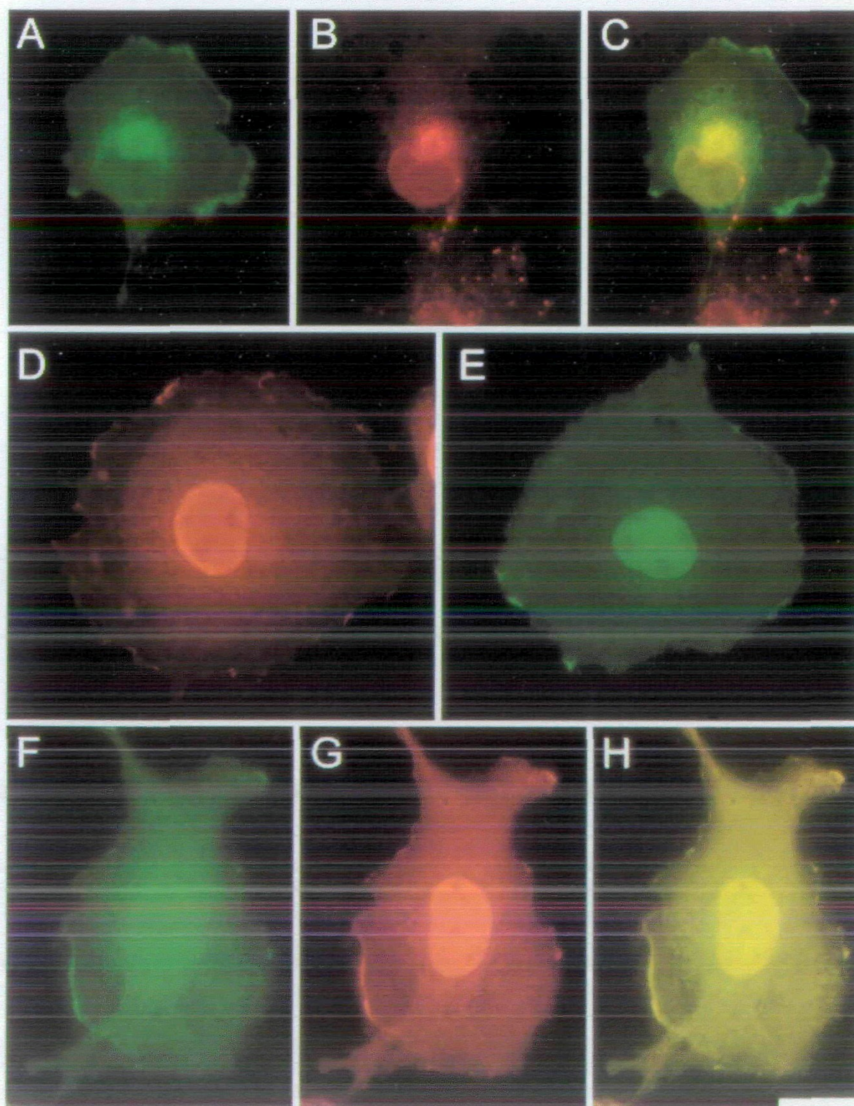

Fig 6 **Localization of CHP and rDRAK2 in COS-7 cells.** Expression plasmids of CHP and rDRAK2 with or without a myc-tag were introduced into COS-7 cells, followed by incubation for 48 h at 37°C (expression of CHP, A, B, and C, expression of myc-rDRAK2 and rDRAK2, D and E, respectively; expression of both CHP and myc-rDRAK2, F, G, and H) The cells were reacted with affinity-purified anti-CHP (A) or anti-rDRAK2 (E) rabbit polyclonal antibodies, and/or anti-myc mouse monoclonal antibodies (D and G). The reacted materials were visualized with secondary anti-rabbit IgG of goat labeled with FITC (panels A, E, and F) or Alexa-546 (panels D and G). Panel B, stained with wheat germ agglutinin labeled with tetramethylrhodamine (Golgi marker), panel C, A and B superpositioned, panel H, F and G superpositioned Bar, 25 μm.

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alone was found to be localized mainly in the Golgi apparatus (Fig. 6, A and C), while expression of rDRAK2 alone was found mainly in the nucleus (Fig. 6, D and E) Endogenous CHP and rDRAK2 were also found in the Golgi apparatus and nucleus, respectively, but the expression level of endogenous rDRAK was very low, as expected from the results in Fig. 5 (data not shown). Thus, the major locations of CHP and rDRAK2 within COS-7 cells were different for each other. However, expression of both proteins due to the introdution of the expression vectors within the same cells caused a drastic change in the location of CHP. A significant fraction of CHP moved to the nucleus without a basic change in the location of rDRAK2 in the nucleus. This shift of the location of CHP was thought to be due to the increased expression of rDRAK2 in COS-7 on the introduction of the expression vector of rDRAK2. These results suggested that *in vivo* interaction of the two proteins caused the transfer of CHP from its original location to the nucleus (Fig. 6, F and H). It should be noted that minor but significant fractions of both proteins were found in the same region in the membrane surface.

DISCUSSION

Here we found with the yeast two-hybrid system that calcineurin homologous protein (CHP) in rat bound to the cytoplasmic domain of Na^+/H^+ exchanger 1 and confirmed the previous observation for human CHP (*7*). It has been reported that CHP inhibited the Na^+/H^+ exchange activity stimulated by GTPase (*7*). We did not observe a significant effect of this protein on the Na^+/H^+ exchange activity on over-expression in opossum kidney cells (*28*) (data not shown) Since CHP is highly homologous to the B subunit (CNB) of calcineurin, which has a catalytic subunit (calcineurin A) besides CNB, the question arose as to whether or not CHP has a putative catalytic subunit. Therefore we surveyed this putative protein with the yeast two-hybrid system. As a result we found a protein kinase capable of binding CHP, instead of a putative protein phosphatase like calcineurin A. This kinase has a domain specifically found for a certain family of protein kinases (*29*). It has been reported that this family of kinases shows apoptotic activity (death-associated kinase activity) (*29*). We have confirmed that this novel CHP binding protein has the protein kinase activity (data not shown).

The protein kinase capable of binding CHP was the same as human DRAK2, which had been found in a homology search of the kinase domain conserved in DAP (*29*) and ZIP(Dlk) (*30, 31*) kinases, and shown to possess the apoptotic kinase activity (*15*). We confirmed the activity causing apoptosis-like cell death for rDRAK2 in this study The binding of CHP and rDRAK2 was demonstrated by *in vitro* binding assaying and the CHP binding region in rDRAK2 localized within 67 amino acid residues in the C-terminal region of the kinase domain. It should be noted that this binding region was in a relatively conserved region of the kinase domain rather than in the unconserved C-terminal region. Analyses of CHP binding to other kinases including DAP and ZIP (Dlk) (*30, 31*) would be of interest to clarify the biological significance of the binding. Here we did not observe binding of the entire rDRAK2 (rDRAK2-FL) to CHP in the two-hybrid assay. A false negative result for the protein-protein interaction in the two-hybrid assay, as reported previously (*19*), might also be the case for this result

The major locations of CHP and rDRAK2 are the Golgi apparatus and nucleus, respectively, in COS-7 cells. However, expression of endogenous rDRAK2 was very low. Higher expression of rDRAK2 on the introduction of the expression vector caused a shift of the CHP location from the Golgi to the nucleus, suggesting *in vivo* interaction of the two proteins. Since rDRAK2 was found to possess a nuclear localizing signal sequence, the location of rDRAK2 in the nucleus seems to be reasonable. However, it is not clear at present why the major portion of CHP, probably bound to rDRAK2, moved to the nucleus. There are two possibilities for this question (i) the CHP and rDRAK2 complex could be moved to the nucleus by the nuclear localizing signal of rDRAK2. (ii) CHP might be capable of being cycled between the nucleus and the Golgi apparatus, and trapped in the nucleus by rDRAK2. Analyses of these possibilities are now in progress.

We found that small but significant fractions of CHP and rDRAK2 colocalized on the membrane surface. Since the presence of NHE1 on the cytoplasmic membrane of COS-7 cells was confirmed preliminarily by detecting the antiporter activity and by immunological staining (data no shown), portions of rDRAK2, CHP and NHE1 might interact in the membrane. Further study is required to obtain direct evidence of the interaction of these proteins in the membrane. It would also be of great interest to clarify the physiological significance of the colocalization of these proteins. In this connection, it should be noted that an essential role of CHP in the function of NHE1 was reported during preparation of this manuscript (*32*)

rDRAK2 belongs to an emerging family of apoptotic kinases originally found for DAP kinase, which mediates apoptosis induced by γ-interferon in HeLa cells (*29*). New members of this family, DAPK2 (DRP1) (*33, 34*), ZIP (Dlk) kinase (*30, 31*), Duet (*35*), and Trio (*36*), have also been reported. The function of DAP kinase has been extensively characterized (*37*) and its action as a tumor suppressor was found (*38*) However, the physiological significance of the apoptosis caused by these serine/threonine protein kinases has not been clarified yet (*39*). In this connection identification of regulatory factor(s) of these kinases would be of interest for clarifying their roles *in vivo*. DAP and DAPK2 kinases have been shown to bind calmodulin (*31, 37*), and the peptides of these proteins without the calmodulin binding domain enhanced the apoptosis (*31, 37*). ZIP(Dlk) has been shown to bind Par-4, which caused retention of ZIP (Dlk) in the cytoplasm and enhanced the apoptosis (*40*). ZIP (Dlk) also binds a novel transcription factor, AATF, leading to suppression of the apoptosis caused by ZIP (Dlk) (*41*). These results raised the possibility that rDRAK2 might be regulated by CHP *in vivo*. In a preliminary experiment the addition of CHP with rDRAK2 did not have a significant effect on the apoptosis of NIH3T3 caused by rDRAK2. Overexpression of rDRAK2 in rat fibroblast and COS-7 cells did not affect the Na^+/H^+ exchange activity (data not shown). Therefore, although interaction of rDRAK2 and CHP is evident *in vivo*, determination of the exact physiological significance of the binding of these proteins *in vivo* awaits further study.

CHP has been reported to be a protein associated with intracellular vesicles and also microtubules (*42, 43*), and

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suggested to be required for membrane traffic (*42*). It has also been reported that CHP inhibits the phosphatase activity of calcineurin (*44*). We found that a major portion of CHP localized in the Golgi apparatus in COS-7 cells, which is different from the previous observation for other cell lines (*43*). Although the reason for such differences in the localization of CHP among different cell lines is not clear, the location of CHP in the Golgi apparatus in COS-7 cells seemed to agree well with the notion that CHP is related to membrane traffic in cells (*43*), because the Golgi apparatus plays a central role in the membrane traffic (*45*). CHP might be involved in the transfer of NHE1 from the Golgi apparatus to the cytoplasmic membrane, and rDRAK2 might play some roles in this process. Another possibility for the rDRAK2 function is that rDRAK2 together with CHP and/or NHE 1 is involved in an as yet unidentified apoptotic process. In this connection it should be noted that materials immunologically cross-reacting with anti-rDRAK2 polyclonal antibodies were found in spleen, thymus, testis and brain. Since it was shown that apoptotic events are essential for the physiology in these organs, rDRAK2, CHP and/or NHE1 might be involved in the putative apoptotic events in these organs. The identification of the target proteins of phosphorylation by rDRAK2 would provide an insight into the physiological significance of the interaction of CHP, rDRAK2, and NHE1.

The expression of DRAK2 in human tissues was found as several bands corresponding to different sizes on Northern-blot analyses (*15*). The sizes of these mRNAs are different from that of rDRAK2. Although expression of rDRAK2 was found in brain, thymus, spleen and testis in rat, high levels of expression of DRAK2 in these tissues of man have not been described previously (*15*). These results suggest that the expression of rDRAK2 and human DRAK2 exhibits different features in terms of tissue specificities and transcription mechanisms.

The authors thank Dr S Ogihara for his help in the analysis involving microscopy

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