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The Apoptosis-Inducing Protein Kinase DRAK2 Is Inhibited in a Calcium-Dependent Manner by the Calcium-Binding Protein CHP

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Calcineurin homologous protein (CHP) is an EF-hand Ca^{2+}-binding protein capable of interacting with various cellular proteins including Na^+/H^+ exchangers, kinesin-related proteins, and apoptosis-inducing protein kinase DRAK2. We investigated the role of CHP on the DRAK2 protein kinase *in vitro*. CHP significantly reduced (~85% inhibition) the kinase activity of DRAK2 for both autophosphorylation and phosphorylation of exogenous substrate (myosin light chain). The inhibitory effect of CHP was dependent on the presence of Ca^{2+}, whereas the interaction between CHP and DRAK2 was not Ca^{2+}-dependent. These observations suggest that CHP negatively regulates the apoptosis-inducing protein kinase DRAK2 in a manner that depends on intracellular Ca^{2+}-concentration.

Key words: apoptosis, apoptosis-inducing protein kinase, calcium-binding protein, calcium signalling.

Abbreviations: CaM, calmodulin; CHP, calcineurin-homologous protein; DAPK, death-associated protein kinase; DMEM, Dulbecco's modified Eagles' medium; DRAK, DAP-kinase-related apoptosis-inducing protein kinase; MLC, myosin light chain.

DRAK2 (death-associated protein kinase [DAP-kinase]–related apoptosis-inducing kinase) is a serine/threonine protein kinase belonging to the DAP kinase family (for reviews, see Refs. *1* and *2*). DRAK2 and other members of the DAP kinase family are thought to play important roles in apoptotic signal transduction. Recent studies have suggested that DAP-kinase, the first member of this family, is critically involved in apoptosis induced by interferon-γ, TNF-α, Fas ligand, and matrix detachment (*3–5*). The death receptor-mediated apoptotic signal and intrinsic signal from the mitochondria increase both the expression and the enzyme activity of DAP-kinase (*3–6*). Consequently, by acting as a positive mediator of the pro-apoptotic signals, DAP-kinase is thought to cause cell death. While the function of DAP-kinase has been extensively studied, the physiological role of the family member DRAK2 remains unclear. Since increasing the DRAK2 kinase activity by overexpression causes apoptotic morphological changes in the cell and subsequently results in cell death (*7*, *8*), DRAK2 is suggested to be involved in executing the apoptotic process.

Recently, we found that the rat homologue of DRAK2 associates with calcineurin B-homologous protein (CHP) using a yeast two-hybrid system (*8*). CHP is an EF-hand Ca^{2+}-binding protein capable of interacting with various cellular proteins including Na^+/H^+ exchangers (*8–10*), kinesin-related proteins (*11*), the calcineurin complex (*12*), and cytoskeletal microtubules (*13*). The primary sequence of CHP is highly similar to that of the regulatory subunit (subunit B) of calcineurin, a heterodimeric protein phosphatase involved in a number of Ca^{2+}-dependent cellular processes (*14*). The similarity to the calcineurin regulatory subunit suggests that CHP also acts as a regulator for cellular catalytic proteins that depend on Ca^{2+} levels. Supporting this notion, CHP has been shown to enhance the ion-transporting activity of Na^+/H^+ exchangers by associating with the cytoplasmic domain of the exchanger proteins (*8–10*).

The finding that CHP interacts with DRAK2 suggests that DRAK2 is regulated by CHP in a Ca^{2+}-dependent manner. In this study, we examined the kinase activity of DRAK2 *in vitro* and found that it is negatively regulated by CHP depending on Ca^{2+} concentration.

MATERIALS AND METHODS

Purification of Recombinant DRAK2 and CHP Proteins—The bacterial expression plasmids for rat CHP-6xHis and DRAK2-6xHis proteins have been described previously (*8*). The recombinant proteins were expressed in *E. coli* strain BL21(DE3). The cells were lysed in lysis buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 20 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride [PMSF]). The 6×histidine-tagged proteins were adsorbed to nickel-NTA–conjugated agarose resin (Qiagen) and then eluted with lysis buffer containing 250 mM imidazole. The protein purities of the CHP-6xHis and DRAK2-6xHis were estimated to be at least 95% and 90%, respectively, by SDS-PAGE and Coomassie Brilliant Blue staining (data not shown). The fraction containing CHP-6xHis protein was filtrated using an Ultrafree filtration unit (10,000 NMWL, Millipore) with 10 mM Tris-HCl (pH 7.5) containing 20 mM NaCl to reduce the concentrations of

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imidazole and NaCl. For the amino-terminal myristoylation of the CHP-6xHis, yeast *N*-myristoyl transferase was co-expressed with CHP-6xHis in *E. coli* cells (*15*). The expression plasmid for yeast *N*-myristoyl transferase was kindly provided by Dr. Jeffrey I. Gordon (University of Texas). The *N*-myristoylation of CHP-6xHis was confirmed by sequencing the amino-terminal residues.

Immunoprecipitation of DRAK2—COS7 cells were cultured in DMEM containing 10% fetal calf serum (FCS), non-essential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 and 95% air. The cells (1×10^6 cells) transfected with myc-rDRAK2/pEF-BOS-EX, myc-rDRAK2(K62A)/pEF-BOS-EX, or pEF-BOS-EX (*8*, *16*) were cultured for 48 h, and washed with ice-cold phosphate-buffered saline (PBS), and then incubated for 10 min at 4°C in PBS containing 0.02% ethylenediamine-tetraacetic acid (EDTA). The cells were harvested by a brief centrifugation at 350 ×*g* and lysed in 50 mM Tris-HCl (pH 7.6) containing 150 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, and Complete™ protease inhibitor mixture (EDTA-free, Roche Diagnostics). After centrifugation at 15 k ×*g* for 15 min at 4°C, the resulting supernatants were incubated with anti-myc antibody (6 μg) for 1 h at 4°C. The myc-DRAK2/antibody complex was adsorbed to protein G-conjugated agarose resin (30 μl, Santa Cruz) by incubation for 2 h at 4°C. The resins were washed with 50 mM Tris-HCl (pH 7.6) containing 150 mM NaCl, 1 mM PMSF, and Complete™ protease inhibitor mixture (EDTA-free). The resulting immunoprecipitates (5 μl aliquots of resin) were subjected to *in vitro* kinase assay and immunoblotted with the anti-myc antibody.

In Vitro Kinase Assay—*In vitro* kinase reactions were performed by incubating 0.3 μg (350 nM) of DRAK2-6xHis protein and 5 μg of bovine muscle myosin light chain (Sigma) in kinase buffer (10 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 3 mM $MnCl_2$, 0.1 mM [γ-^{32}P]ATP [111 GBq/mmol, Amersham Pharmacia]) and either 0.5 mM $CaCl_2$ or 1 mM ethyleneglycol-*O,O′*-bis(2-aminoethyl)-*N,N,N′,N′*-tetraacetic acid (EGTA) in a total volume of 20 μl at 30°C for 15 min. To assay the myc-DRAK2 proteins immunopurified from COS7 cells, the DRAK2-6xHis was replaced with the immunoprecipitates bound on protein G-agarose. The CHP-6xHis, bovine calmodulin (Sigma), and frog S-modulin proteins were pre-mixed with DRAK2-6xHis in buffer containing either 2 mM $CaCl_2$ or 4 mM EGTA, and incubated at 30°C for 20 min before starting the phosphorylation reaction. The various free Ca^{2+} concentrations in the pre-incubation and kinase reaction mixtures were adjusted by adding 2 mM EGTA and appropriate amounts of $CaCl_2$ to the solution (*17*). The kinase reactions were terminated by adding 10 μl of 3× SDS-PAGE loading buffer, and then the proteins were resolved by SDS-polyacrylamide gel electrophoresis. The gels were stained with Coomassie Brilliant Blue, dried, and then autoradiographed. The radioactive phosphoprotein bands were visualized with an auto-imaging analyzer (BAS1800, Fuji Film) and quantified using Image-Gauge software (version 3.41, Fuji Film). Phosphate incorporation into MLC was ~1×10^{-3} mol phosphate/mol MLC protein.

Binding Assay of CHP and DRAK2—DRAK2-coupled agarose was prepared as follows. *N*-hydroxysuccinimidyl-agarose (0.1 ml, Sigma) was washed with 1 mM HCl, and then incubated with 80 μg of DRAK2-6xHis protein in 0.2 M $NaHCO_3$ (pH 8.3) and 0.5 M NaCl for 30 min at room temperature. The resin was washed with 0.5 M ethanolamine (pH 8.3) and 0.5 M NaCl, incubated for 30 min, and suspended in 10 mM Tris-HCl (pH 7.5) and 20 mM NaCl. The DRAK2-6xHis protein was conjugated with 0.64 μg/μl agarose. To prepare the control resin, DRAK2-6xHis protein was omitted from the reaction mixture. The DRAK2-coupled agarose was washed with binding buffer (10 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 3 mM $MnCl_2$, 10 mM NaCl, and either 0.5 mM $CaCl_2$ or 0.5 mM EGTA), and resuspended in binding buffer containing 1 mg/ml bovine serum albumin at a final concentration of 20 μl resin/ml. An aliquot (6.6 μl) of the resin was incubated with CHP-6xHis (6.6 μg) for 30 min at 30°C, and then washed with the binding buffer. The proteins bound to the resin were eluted by heating in 20 μl of SDS-PAGE loading buffer, and then visualized by SDS-PAGE with Coomassie Brilliant Blue staining.

Antibodies and Other Materials—The rabbit polyclonal anti-DRAK2 antibody has been described previously (*8*). The mouse monoclonal anti-myc antibody (clone 9E10) was purchased from Santa Cruz. Horseradish peroxidase–conjugated secondary antibody against rabbit IgG was purchased from Vector Co. Recombinant frog S-modulin was kindly provided by Dr. S. Kawamura (Osaka University). Cell culture reagents were obtained from Sigma and GIBCO BRL. Other chemicals were from Sigma, unless otherwise specified.

RESULTS

DRAK2 Phosphorylates Both Itself and Myosin Light Chain (MLC) In Vitro—We expressed rat DRAK2 with a six histidine tag in *E. coli* and purified it by affinity absorption on nickel-NTA-conjugated resin. The purified protein was approximately 45 kDa on SDS-PAGE, which coincides with the predicted molecular mass of the DRAK2-6xHis protein (Fig. 1A, lane 3). To examine the kinase activity of DRAK2, the DRAK2-6xHis protein was incubated with MLC as an exogenous phosphorylation substrate and [γ-^{32}P]-labeled ATP, and then subjected to SDS-PAGE and autoradiography. DRAK2-6xHis caused the phosphorylation of both itself and MLC (Fig. 1A, lane 1). When a catalytically inactive mutant DRAK2, with a mutation at its ATP-binding site [Lys-62→Ala (K62A), Fig. 1A, lane 4], was used instead of wild type DRAK2, phosphorylation was not observed (Fig. 1A, lane 2). This indicates that phosphate incorporation with wild type DRAK2 is due to an enzymatic reaction, excluding the possibility that the DRAK2 preparation was contaminated with other kinases. Phosphate incorporation into MLC and DRAK2 increased at a rate proportional to incubation time, indicating that the kinase reaction was not saturated. We confirmed the auto- and substrate-phosphorylation activity of DRAK2 using protein immunopurified from mammalian cells (Fig. 1B). A myc-epitope tagged DRAK2 was expressed in COS7 cells, immunoprecipitated using anti-myc antibody, and subjected to kinase assay. The myc-DRAK2 protein showed

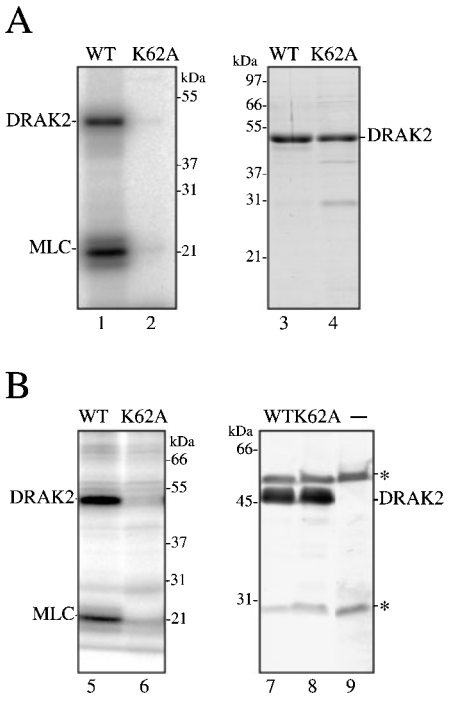

Fig. 1. **DRAK2 phosphorylates itself and MLC *in vitro*.** (A) The wild-type DRAK2-6xHis (WT, lanes 1, 3) and its catalytically inactive mutant (K62A, lanes 2, 4) were affinity-purified from *E. coli* lysates and subjected to *in vitro* kinase assay (0.3 μg protein each, lanes 1, 2). The proteins were also subjected to SDS-PAGE and visualized by Coomassie Brilliant Blue (2 μg protein each, lanes 3, 4). (B) The wild-type (lanes 5, 7) and K62A mutant (lanes 6, 8) proteins with a myc-tag were expressed in COS7 cells and immunoprecipitated using anti-myc antibody. The immunoprecipitates were used in the *in vitro* kinase assay (lanes 5, 6). The immunoprecipitates were also immunoblotted with the anti-myc antibody (lanes 7–9). The lysate from COS7 cells transfected with the vector plasmid was used as a control in the immunoprecipitation (lane 9). The positions of the DRAK2 proteins and MLC are indicated. Asterisks indicate the positions of the heavy and light chains of the immunoglobulins used in the immunoprecipitation.

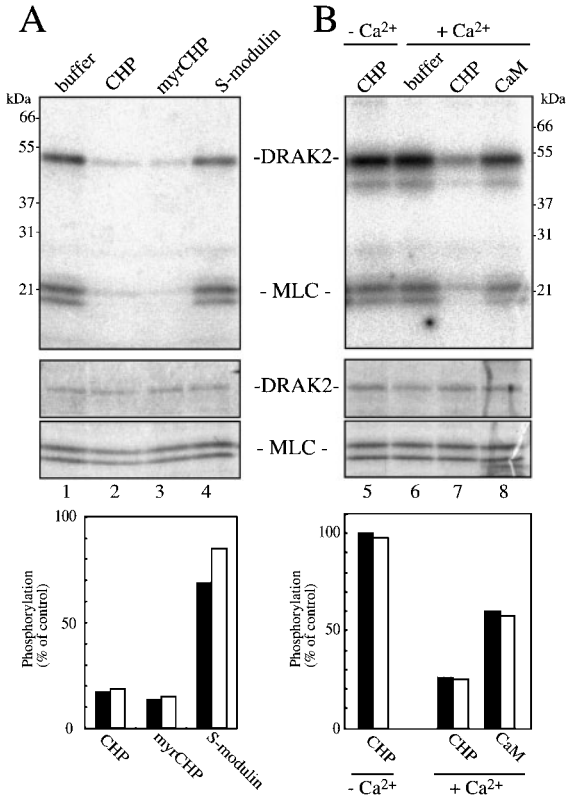

Fig. 2. **DRAK2 kinase activity is inhibited by CHP in the presence of calcium.** (A) DRAK2-6xHis purified from *E. coli* was assayed for autophosphorylation and phosphorylation of MLC in the presence of $CaCl_2$ (0.5 mM) and either 6.8 μM CHP-6xHis (lane 2), *N*-myristoylated CHP (lane 3) or frog S-modulin (lane 4). (B) The inhibition of kinase activity by CHP-6xHis (lanes 5, 7) and calmodulin (lane 8) was examined in the presence of 1 mM EGTA (lane 5) or 0.5 mM $CaCl_2$ (lanes 6–8). Top and middle panels show autoradiograms and Coomassie Brilliant Blue staining, respectively. Bottom graphs show the percentages of auto- (closed bar) and MLC- (open bar) phosphorylation to buffer alone (lane 1 for A and lane 7 for B).

phosphorylation of both itself and MLC (Fig. 1B), as observed using the bacterially expressed DRAK2-6xHis protein.

CHP Reduces DRAK2 Kinase Activity—We have previously shown that CHP interacts with the carboxyl-terminal region of the kinase domain of DRAK2 (*8*). This suggests that CHP might regulate the kinase activity of DRAK2. To test this possibility, we examined the auto- and substrate-phosphorylation activities of DRAK2 in the presence of CHP. The addition of CHP-6xHis protein to the kinase reaction mixture containing 0.5 mM Ca^{2+} significantly reduced both the auto- and substrate-phosphorylation activities of DRAK2 (83% and 81%, respectively, Fig. 2A). Similar CHP-mediated inhibition of DRAK2 kinase activity was observed when myc-DRAK2 immunopurified from COS7 cells was used instead of DRAK2-6xHis (data not shown). Frog S-modulin is another member of the EF-hand Ca^{2+}-binding protein family and acts as an inhibitor for rhodopsin-kinase at high Ca^{2+} concentrations (*18*). When an equivalent molar concentration of recombinant S-modulin was used instead of CHP, DRAK2 auto- and substrate-phosphorylation was only weakly inhibited (32% and 15%, respectively, Fig. 2A), indicating that CHP specifically inactivates DRAK2 kinase activity. We examined DRAK2 kinase activity in the presence of various amounts of CHP. The auto- and substrate-phosphorylations by 0.35 μM DRAK2-6xHis were substantially inhibited at a concentration of 0.68 μM CHP-6xHis, and the IC_{50} values for the auto- and substrate-phosphorylation were approximately 1.5 μM (Fig. 3). We note that the inhibition of kinase activity was enhanced by pre-incubating DRAK2 and CHP at 30°C before the start of the phosphorylation reaction (see "MATERIALS AND METHODS"). We next examined the effects of another member of the EF-hand Ca^{2+}-binding protein family, calmodulin, on the kinase activity of DRAK2. Interestingly, calmodulin weakly reduced the kinase activity of DRAK2 (43% and 63% for auto- and substrate-phosphorylation, respectively, Fig. 2B, lanes 6–8), although the effect was less than that of CHP, suggesting that calmodulin may also be involved in the regulation of DRAK2.

The inhibitory effect of CHP does not require N-myristoylation, but is Ca^{2+}-dependent—CHP contains a covalently attached fatty acyl group at its amino-terminus

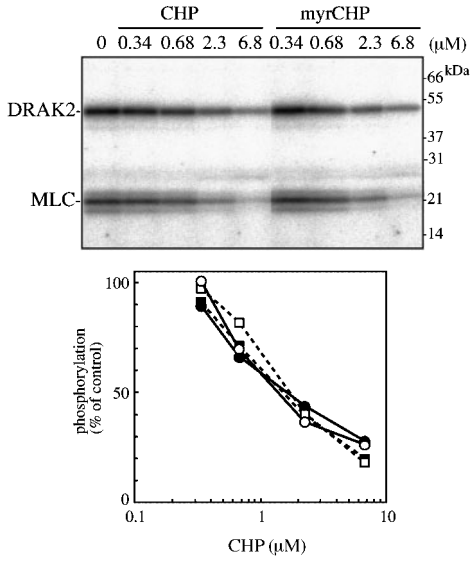

Fig. 3. **Dose-dependent inhibition of DRAK2 kinase activity by CHP.** DRAK2-6xHis (0.35 μM) was subjected to *in vitro* kinase assay in the presence of Ca^{2+} (0.5 mM) and variable concentrations (0–6.8 μM) of CHP-6xHis with (myrCHP) or without (CHP) N-myristoylation. Top panel, autoradiogram; bottom panel, the autoradiogram was quantified by phosphoimage scanning and the data are presented as percentages of the amount of phosphorylation in the absence of CHP-6xHis. Open and closed symbols show CHP and myrCHP, respectively, and circles and squares show auto- and MLC-phosphorylation, respectively.

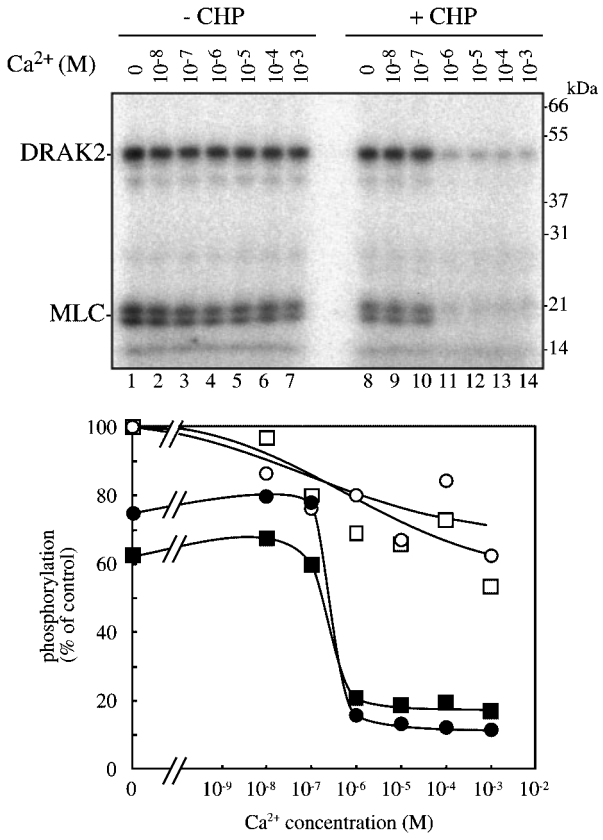

Fig. 4. **Ca^{2+}-dependent inhibition of DRAK2 kinase activity by CHP.** DRAK2-6xHis (0.35 μM) was subjected to *in vitro* kinase assay in the presence (lanes 8–14, closed symbols) or absence (lanes 1–7, open symbols) of CHP-6xHis (6.8 μM) under variable concentrations (0–1 mM) of free Ca^{2+}. Top panel, autoradiogram; bottom panel, the autoradiogram was quantified by phosphoimage scanning and the data are presented as percentages of the amount of phosphorylation in the absence of CHP and free Ca^{2+} (lane 1). Circles and squares show auto- and MLC-phosphorylation, respectively.

(Ref. *19* and our unpublished data). The physiological function of myristoyl groups is still unknown, however, it appears to be involved in anchoring proteins to the cell membrane and facilitating interactions with target proteins (*20*, *21*). We assessed whether the myristoylation of CHP affects its ability to inhibit DRAK2 kinase activity by using N-myristoylated CHP-6xHis purified from *E. coli* cells expressing yeast N-myristoyl transferase. The myristoylated CHP inhibited the DRAK2 kinase activity to a similar extent as the unmyristoylated form (Fig. 2A). The IC_{50} of the myristoylated CHP was approximately 1.5 μM, and 6.8 μM CHP-6xHis inhibited the auto- and substrate-phoshorylation by 89% and 87%, respectively (Fig. 3). Thus, myristoylation of CHP is not crucial for the inhibition of DRAK2 kinase activity.

As CHP is an EF-hand Ca^{2+}-binding protein, we examined the Ca^{2+}-dependence of the inhibition by CHP. The inhibition observed at high Ca^{2+} concentrations (0.5 mM) was significantly diminished by the depletion of free Ca^{2+} (Fig. 2B, lanes 5–7). The presence of 1 μM free Ca^{2+} was sufficient for almost full inhibition of the kinase activity by CHP (Fig. 4, lanes 8–14). This suggests that CHP regulates DRAK2 activity depending on the intracellular Ca^{2+}-concentration. The DRAK2 kinase activity was slightly reduced in the absence of CHP when the Ca^{2+}-concentration increased (Fig. 4, lanes 1–7). DRAK2 kinase itself may also be regulated by Ca^{2+}-concentration.

The Interaction between DRAK2 and CHP Is Not Dependent on Ca^{2+} Concentration—We examined the Ca^{2+}-dependence of the binding between DRAK2 and CHP (Fig. 5). CHP-6xHis was incubated with DRAK2-6xHis coupled to agarose resin in the presence or absence of free Ca^{2+}, and then the CHP bound to the resin was analyzed by SDS-PAGE. A substantial but slightly smaller amount of CHP was bound to DRAK2 in the absence of Ca^{2+} (85% of that in the presence of 0.5 mM Ca^{2+}), indicating that the binding between DRAK2 and CHP is essentially independent on Ca^{2+} concentration

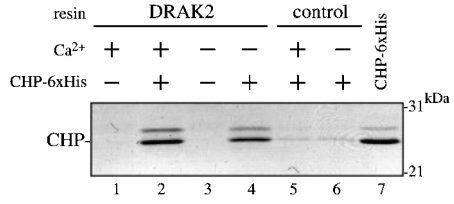

Fig. 5. **Interaction between DRAK2 and CHP is not dependent on Ca^{2+} concentration.** DRAK2-conjugated resin (lanes 1–4) and control resin (lanes 5, 6) were incubated with either CHP-6xHis (lanes 2, 4–6) or buffer alone (lanes 1, 3) in the presence of 0.5 mM $CaCl_2$ (lanes 1, 2, 5) or 0.5 mM EGTA (lanes 3, 4, 6) for 30 min at 30°C. The CHP-6xHis bound to the resin was eluted by heating in SDS-PAGE sample buffer, electrophoresed, and then visualized by Coomassie Brilliant Blue staining. The CHP-6xHis (0.5 μg) used in this binding assay is shown in lane 7.

(Fig. 5, lanes 1–4). The amount of CHP-6xHis eluted from the control resin was negligible (Fig. 5, lanes 5, 6), suggesting the specific binding of CHP-6xHis to DRAK2. These observations suggest that the inhibition of kinase activity by CHP is not due to the simple binding of CHP to DRAK2, and the association and dissociation of Ca^{2+} with CHP bound to DRAK2 may cause a conformational change in the DRAK2/CHP complex between inactive and active states.

DISCUSSION

In conclusion, we showed that the Ca^{2+}-binding protein CHP negatively regulates the kinase activity of the apoptosis-inducing kinase DRAK2 in a Ca^{2+}-dependent manner. CHP inhibited both auto- (83%) and substrate- (81%) phosphorylations by DRAK2 in the presence of Ca^{2+}, and two-fold molar excess of CHP caused substantial inhibition of the phosphorylations by DRAK2. The inhibition by CHP is dependent on free Ca^{2+} concentration. The presence of 1 μM free Ca^{2+} was sufficient for almost full inhibition of the kinase activity. Myristoylation of CHP, which appears to be involved in membrane-anchoring and facilitating protein interactions, is not crucial for the inhibition of DRAK2 kinase activity. The binding of CHP to DRAK2 was essentially independent of Ca^{2+} concentration, suggesting that the inhibition of DRAK2-kinase activity by CHP is not due to the simple binding of CHP to DRAK2, and that the association of Ca^{2+} with CHP bound on DRAK2 causes a conformational change in the DRAK2/CHP complex to a kinase-inactive state.

The repressive regulation of protein kinase by a Ca^{2+}-binding protein has been found in the light-adaptation mechanism of vertebrate rod photoreceptors (*18*). S-modulin/recoverin, a Ca^{2+}-binding protein has been shown to inhibit rhodopsin kinase activity under high (>1 μM) Ca^{2+}-concentrations (*22*). When the intracellular Ca^{2+}-concentration is decreased to the nanomolar range under light conditions, the repression of rhodopsin kinase by S-modulin is lifted, and the subsequent phosphorylation of light-activated rhodopsin results in the termination of transducin activation. The effect of CHP on DRAK2 seems to be similar to that of S-modulin on rhodopsin-kinase. However the interaction between S-modulin and rhodopsin-kinase is Ca^{2+}-dependent, and the association of Ca^{2+}/S-modulin with rhodopsin-kinase is thought to result in the inhibition of the kinase activity (*23*). The finding that the binding of Ca^{2+} to the CHP/DRAK2 complex reduces its kinase activity indicates that the regulatory molecular mechanism is different from that of the regulation of rhodopsin-kinase by S-modulin. This suggests a novel molecular mechanism for the regulation of a protein kinase by a Ca^{2+}-binding protein, although we have the issue of the physiological role of CHP on the induction of apoptosis by DRAK2.

It has been shown that several Ca^{2+}-binding proteins are involved in the regulation of the apoptotic process. These proteins respond positively to increases in cytosolic Ca^{2+} levels evoked by a variety of apoptosis-inducing stimuli, and trigger the apoptotic process by transducing the calcium signal. For example, ALG-2, a member of the EF-hand family, promotes the dexamethasone- and Fas-induced apoptosis of T-cell hybridomas by associating with the possible signalling protein AIP1/SETA (*24*, *25*). Another example is calmodulin, which interacts with DAPK1 and DAPK2, members of the same family to which DRAK2 belongs (*26–29*). The interaction of Ca^{2+}/calmodulin with these DAPKs enhances their kinase activities and thereby elevates their apoptosis-inducing activities. Given these examples of positive activity in Ca^{2+}-driven apoptotic signal transduction, it is interesting that Ca^{2+} and CHP negatively regulate the kinase activity of DRAK2 required for apoptotic induction. Matsumoto *et al.* (2001) suggested that CHP and DRAK2 are mainly localized in cytoplasm and nucleus, respectively (*8*). Immunoprecipitation and gel filtration analyses of the CHP/DRAK2 complex suggested that a large portion of the cellular DRAK2 is not assembled in the complex (our unpublished data). These observations imply that CHP may play a role in assuring that DRAK2 operates within its own apoptotic pathways under condition of high Ca^{2+} concentration. Alternatively, it is possible that DRAK2 is involved in Ca^{2+}-independent apoptosis and/or events during the apoptotic process (*30–32*), and that CHP may function to repress DRAK2 kinase activity at high Ca^{2+} levels. Further studies are presently under way to investigate these possibilities and to uncover the physiological functions of CHP and DRAK2 in apoptotic cell death.

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REFERENCES

1. Kimchi, A. (1998) DAP genes: novel apoptotic genes isolated by a functional approach to gene cloning. *Biochim. Biophys Acta* **1377**, F13–F33
2. Kogel, D., Prehn, J.H., and Scheidtmann, K.H. (2001) The DAP kinase family of pro-apoptotic proteins: novel players in the apoptotic game. *Bioessays* **23**, 352–358
3. Deiss, L.P., Feinstein, E., Berissi, H., Cohen, O., and Kimchi, A. (1995) Identification of a novel serine/threonine kinase and a novel 15-kD protein as potential mediators of the gamma interferon-induced cell death. *Genes Dev.* **9**, 15–30
4. Inbal, B., Cohen, O., Polak-Charcon, S., Kopolovic, J., Vadai, E., Eisenbach, L., and Kimchi, A. (1997) DAP kinase links the control of apoptosis to metastasis. *Nature* **390**, 180–184
5. Cohen, O., Inbal, B., Kissil, J.L., Raveh, T., Berissi, H., Spivak-Kroizaman, T., Feinstein, E., and Kimchi, A. (1999) DAP-kinase participates in TNF-α- and Fas-induced apoptosis and its function requires the death domain. *J. Cell Biol.* **146**, 141–148
6. Raveh, T., Droguett, G., Horwitz, M.S., DePinho, R.A., and Kimchi, A. (2001) DAP kinase activates a p19ARF/p53-mediated apoptotic checkpoint to suppress oncogenic transformation. *Nat. Cell Biol.* **3**, 1–7
7. Sanjo, H., Kawai, T., and Akira, S. (1998) DRAKs, novel serine/threonine kinases related to death-associated protein kinase that trigger apoptosis. *J. Biol. Chem.* **273**, 29066–29071
8. Matsumoto, M., Miyake, Y., Nagita, M., Inoue, H., Shitakubo, D., Takemoto, K., Ohtsuka, C., Murakami, H., Nakamura, N., and Kanazawa, H. (2001) A serine/threonine kinase which causes apoptosis-like cell death interacts with a calcineurin B-like protein capable of binding Na^+/H^+ exchanger. *J. Biochem.* **130**, 217–225

9. Lin, X. and Barber, D.L. (1996) A calcineurin homologous protein inhibits GTPase-stimulated Na-H exchange. *Proc. Natl Acad. Sci. USA* **93**, 12631–12636
10. Pang, T., Su, X., Wakabayashi, S., and Shigekawa, M. (2001) Calcineurin Homologous Protein as an Essential Cofactor for Na^+/H^+ Exchangers. *J. Biol. Chem.* **276**, 17367–17372
11. Nakamura, N., Miyake, Y., Matsushita, M., Tanaka, S., Inoue, H., and Kanazawa, H. (2002) KIF1Bβ2, capable of interacting with CHP, is localized to synaptic vesicles. *J. Biochem.* **132**, 483–491
12. Lin, X., Sikkink, R.A., Rusnak, F., and Barber, D.L. (1999) Inhibition of calcineurin phosphatase activity by a calcineurin B homologous protein. *J. Biol. Chem.* **274**, 36125–36131
13. Timm, S., Titus, B., Bernd, K., and Barroso, M. (1999) The EF-hand Ca^{2+}-binding protein p22 associates with microtubules in an N-myristoylation-dependent manner. *Mol. Biol. Cell* **10**, 3473–3488
14. Rusnak, F. and Mertz, P. (2000) Calcineurin: form and function. *Physiol. Rev.* **80**, 1483–1521
15. Hisatomi, O., Ishino, T., Matsuda, S., Yamaguchi, K., Kobayashi, Y., Kawamura, S., and Tokunaga, F. (1997) Functional expression and characterization of frog photoreceptor- specific calcium-binding proteins. *Biochem. Biophys. Res. Commun.* **234**, 173–177
16. Murai, K., Murakami, H., and Nagata, S. (1998) Myeloid-specific transcriptional activation by murine myeloid zinc- finger protein 2. *Proc. Natl Acad. Sci. USA* **95**, 3461–3466
17. Tsien, R. and Pozzan, T. (1989) Measurement of cytosolic free Ca^{2+} with quin2 in *Methods Enzymol.* (Simon, M.I. and Abelson, J.N., eds.) Vol. **172**, pp. 230–262, Academic Press, New York
18. Kawamura, S. (1993) Rhodopsin phosphorylation as a mechanism of cyclic GMP phosphodiesterase regulation by S-modulin. *Nature* **362**, 855–857
19. Barroso, M.R., Bernd, K.K., DeWitt, N.D., Chang, A., Mills, K., and Sztul, E.S. (1996) A novel Ca^{2+}-binding protein, p22, is required for constitutive membrane traffic. *J. Biol. Chem.* **271**, 10183–10187
20. Braunewell, K.H. and Gundelfinger, E.D. (1999) Intracellular neuronal calcium sensor proteins: a family of EF-hand calcium-binding proteins in search of a function. *Cell Tissue Res.* **295**, 1–12
21. Resh, M.D. (1999) Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochim. Biophys Acta* **1451**, 1–16
22. Klenchin, V.A., Calvert, P.D., and Bownds, M.D. (1995) Inhibition of rhodopsin kinase by recoverin. Further evidence for a negative feedback system in phototransduction. *J. Biol. Chem.* **270**, 16147–16152
23. Chen, C.K., Inglese, J., Lefkowitz, R.J., and Hurley, J.B. (1995) Ca^{2+}-dependent interaction of recoverin with rhodopsin kinase. *J. Biol. Chem.* **270**, 18060–18066
24. Vito, P., Lacana, E., and D'Adamio, L. (1996) Interfering with apoptosis: Ca^{2+}-binding protein ALG-2 and Alzheimer's disease gene ALG-3. *Science* **271**, 521–525
25. Chen, B., Borinstein, S.C., Gillis, J., Sykes, V.W., and Bogler, O. (2000) The glioma-associated protein SETA interacts with AIP1/Alix and ALG-2 and modulates apoptosis in astrocytes. *J. Biol. Chem.* **275**, 19275–19281
26. Cohen, O., Feinstein, E., and Kimchi, A. (1997) DAP-kinase is a Ca^{2+}/calmodulin-dependent, cytoskeletal-associated protein kinase, with cell death-inducing functions that depend on its catalytic activity. *EMBO J.* **16**, 998–1008
27. Kawai, T., Nomura, F., Hoshino, K., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., and Akira, S. (1999) Death-associated protein kinase 2 is a new calcium/calmodulin-dependent protein kinase that signals apoptosis through its catalytic activity. *Oncogene* **18**, 3471–3480
28. Inbal, B., Shani, G., Cohen, O., Kissil, J.L., and Kimchi, A. (2000) Death-associated protein kinase-related protein 1, a novel serine/threonine kinase involved in apoptosis. *Mol. Cell. Biol.* **20**, 1044–1054
29. Shani, G., Henis-Korenblit, S., Jona, G., Gileadi, O., Eisenstein, M., Ziv, T., Admon, A., and Kimchi, A. (2001) Autophosphorylation restrains the apoptotic activity of DRP-1 kinase by controlling dimerization and calmodulin binding. *EMBO J.* **20**, 1099–1113
30. Krebs, J. (1998) The role of calcium in apoptosis. *Biometals* **11**, 375–382
31. Nicotera, P. and Orrenius, S. (1998) The role of calcium in apoptosis. *Cell Calcium* **23**, 173–180
32. McConkey, D.J. and Orrenius, S. (1997) The role of calcium in the regulation of apoptosis. *Biochem. Biophys. Res. Commun.* **239**, 357–366