CHP2 Activates the Calcineurin/Nuclear Factor of Activated T Cells Signaling Pathway and Enhances the Oncogenic Potential of HEK293 Cells*

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CHP2 (calcineurin B homologous protein 2) was initially identified as a tumor-associated antigen highly expressed in hepatocellular carcinoma. Its biological function remains largely unknown except for a potential role in transmembrane Na+/H+ exchange. In the present study, we observed that ectopic expression of CHP2 promoted the proliferation of HEK293 cells, whereas knockdown of endogenous CHP2 expression in HepG2 inhibited cell proliferation. When inoculated into nude mice, CHP2 transfected HEK293 cells displayed markedly increased oncogenic potential. In analysis of the underlying molecular mechanisms, we found that like calcineurin B, CHP2 was able to bind to and stimulate the phosphatase activity of calcineurin A. In accord with this, CHP2-transfected cells showed increased nuclear presence of NFATc3 (nuclear factor of activated T cells) and enhanced NFAT activity. Finally, both accelerated cell proliferation and NFAT activation following CHP2 transfection could be suppressed by the calcineurin inhibitor cyclosporine A, suggesting an intrinsic connection between these events. Taken together, our results highlighted a potential role of CHP2 in tumorigenesis and revealed a novel function of CHP2 as an activator of the calcineurin/NFAT signaling pathway.

Calcineurin, a serine/threonine-specific protein phosphatase, is an important mediator in the calcium signaling pathways. It is normally composed of catalytic subunit calcineurin A (CnA)4 and regulatory subunit calcineurin B (CnB). In response to elevated levels of intracellular calcium, the phosphatase activity of calcineurin is fully activated upon calcium-dependent binding of calmodulin (CaM) to the CnA-CnB complex (1, 2). The transcription factor NFAT (nuclear factor of activated T cells) is a major substrate of calcineurin. Among the five known members of the NFAT family, four contain docking sites for and are regulated by calcineurin. In resting cells, the NFAT proteins are hyperphosphorylated and reside within the cytoplasm. Calcineurin activation induced by a number of extracellular stimuli results in rapid dephosphorylation of NFAT molecules and their translocation to the nucleus. Once located in the nucleus, NFAT binds to and induces the transcription of target genes, either by itself or in combination with other factors (3–5). The best known targets are those involved in the activation and effector functions of immune cells (5). More recently, NFAT have also been shown to participate in the transcriptional control of many other genes related to cell cycle progression, differentiation, and apoptosis (3, 4). Given the fundamental nature of the cellular processes it regulates, it comes as no surprise that deregulation of calcineurin/NFAT signaling and/or abnormal expression of its components are reported to be associated with a number of human malignancies (6–8).

The activity of calcineurin is tightly regulated. In the past few years, several endogenous inhibitors have been discovered, which include calcineurin-binding protein 1 (Cabin1/Cain) (9, 10), protein kinase A anchor protein AKAP79 (11), members of the calcipressin family (12, 13), FK506-binding protein 38 (FKBP38) (14), calcineurin homologous protein 1 (CHP1) (15), and tescalcin (also known as CHP3) (16). The last two are of particular interest for their sequence similarity to CnB. As a ubiquitously expressed molecule, CHP1 demonstrates diversified biological activities. In addition to being a cofactor for Na+/H+ exchangers (NHEs) (17) and a modulator for the organization and assembly of microtubule cytoskeleton and endoplasmic reticulum (18), CHP1 serves as a negative regulator in calcineurin/NFAT signaling (15). In cells overexpressing CHP1, both the phosphatase activity of calcineurin and the transcriptional activity of NFAT are significantly reduced, probably due to impaired assembly of CnA-CnB-CaM heterotrimers in the presence of CHP1. CHP3/tescalcin was initially

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The abbreviations used are: CnA, calcineurin A; CnB, calcineurin B; CaM, calmodulin; CHP, calcineurin homologous protein; CsA, cyclosporine A; HEK293, human embryonic kidney 293; NFAT, nuclear factor of activated T cells; NHE, Na+/H+ exchanger; pNPP, p-nitrophenyl phosphate; GFP, green fluorescent protein; siRNA, small interfering RNA; RT, reverse transcriptase; PBS, phosphate-buffered saline.

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identified as a transcript preferentially expressed in testis, but subsequent studies demonstrated abundant expression of CHP3 in several other tissues, including heart, brain, and stomach (16). Similar to CHP1, recombinant CHP3 inhibits CaM-stimulated CnA activity in reconstituted assays (16). Moreover, ectopic expression of CHP3 up-regulates the cell surface activity of NHE1 (19).

In the search for tumor-associated antigens expressed in hepatocellular carcinoma, we identified another calcineurin homologous protein. This protein was initially designated as HCA520 (hepatocellular carcinoma antigen 520), but renamed CHP2 on the basis of a high degree of sequence identity to CnB (54%) and CHP1 (61%) (20, 21). In contrast to the wide distribution of CHP1, CHP2 expression in normal tissues is, if any, extremely low. On the other hand, high levels of CHP2 mRNA are frequently detected in malignant tissues. Such a pattern implies a potential role for CHP2 in tumorigenesis. In fact, an earlier study by Pang et al. (21) indicated that CHP2 may increase the viability of malignant cells by maintaining high intracellular pH through serum-independent activation of NHE1. In view of the multiple biological functions of CHP1 that have been identified, we wonder whether CHP2 is involved in the regulation of cellular functions other than Na+/H+ exchange, which in turn may contribute to its oncogenic potential.

In the present study, we examined alterations in cell behavior following CHP2 overexpression, and sought to determine the molecular mechanisms by which CHP2 exerts its function, with special attention to the influence on phosphatase activity of calcineurin and the downstream signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—CHP2, CnA, CnB, and CaM recombinant proteins were prepared in *Escherichia coli* as previously described (22–24). Anti-CHP2 antibody was generated in rabbit by immunizing with recombinant CHP2. Other antibodies used in the study were obtained from various commercial sources: anti-β-actin from Beijing Ding Guo Chang Sheng Biotechnology Corporation (Beijing, China); anti-FLAG from Sigma; anti-Histone H3.1 from Signalway Antibody Company (Pearland, TX); anti-α-tubulin 1 from ProteinTech Group Incorporation (Chicago, IL); anti-CnA from Cell Signaling Technology (Danvers, MA); and anti-pan-NFAT (sc-1149), NFATc1 (sc-7294), NFATc2 (sc-7295), NFATc3 (sc-8405), and NFATc4 (sc-1153A) from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell Cultures**—Human embryonic kidney cell line HEK293, liver immortalized cell line LO2, hepatocellular carcinoma cell line HepG2, and cervical carcinoma cell line HeLa were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% newborn calf serum supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and sodium bicarbonate (2.2 g/liter). T cell leukemia cell line Jurkat was cultured in RPMI 1640 (Invitrogen) medium containing 10% newborn calf serum supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml).

**Construction and Transfection**—pcDNA3-Flag-CHP2 and pEGFP-CHP2 expression vectors were constructed using standard molecular biology techniques. Transfection was performed using Lipofectamine™ 2000 transfection reagent (Invitrogen) or calcium phosphate precipitation. Stable transfectants were obtained by selection in G418 (2 mg/ml), and subcloned by limiting dilution. For transient transfection, cells were co-transfected with 8 μg of pcDNA3-Flag-CHP2 and 1 μg of pEGFP plasmid in 60-mm dishes. The green fluorescent protein (GFP) positive population was sorted out for further analysis 24 h after transfection. Cells transfected with empty pcDNA3 vector served as a mock control.

**RNA Interference**—Three putative siRNA oligos (siRNAi#1, GGG AGA CCG AAU AUA AGA A; siRNA#2, GAA GGA ACA AAC UUC ACU A; siRNA#3, CAG AAG AGC AGG AGA A) and a non-silencing control (UUC UCC GAA CGU GUC ACG U) were synthesized at GenePharma Co. Ltd. (Shanghai, China), and tested for their capacity to quench fluorescence in co-transfection with pEGFP-CHP2. To inhibit endogenous CHP2 expression, siRNA#2 (20 nM) were transfected into HepG2 cells using the INTERFERin reagent (Polyplus-transfection Inc., Illkirch, France) according to the manufacturer’s protocol.

**RNA Isolation and RT–PCR**—Total RNA was isolated using TRIzol reagent (Invitrogen). CDNA was synthesized using the Reverse Transcription System (Promega, Madison, WI). PCR amplifications were performed using the following primers: 5'-GGA CAG TAT TCG GGA GAC C-3' (forward) and 5'-CAT TTT ATT GTC GCC CTC GTG C-3' (reverse) for CHP2; 5'-ACC ACA GTC CAT GCC ATC AC-3' (forward) and 5'-TCC ACC ACC CTG TTG CTG TA-3' (reverse) for glycer-aldehyde-3-phosphate dehydrogenase, PCR products were resolved on 1% agarose gels.

**Cell Proliferation Analysis**—To determine the cell growth rate, CHP2 stable transfectants and the mock control were seeded into 24-well plates at a density of 1 × 10⁶ cells/well. At each time point, cells from three independent wells were collected by trypsinization and counted with a hemocytometer. For proliferation assay by [³H]Tdr incorporation, stable or transient transfectants (24 h after transfection) were seeded in 96-well culture plates in triplicate at a density of 1 × 10⁴ cells/well and cultured for 24, 48, or 72 h. [³H]Tdr (Beijing Atomic Energy Institute, China) was added at 0.5 μCi (18.5 KBq)/well 12 h before the end of culture. Cells were collected with Harvester 96 Mach II (Tomtec Inc., Hamden, CT) and the counts/ min value for each well was determined with scintillation fluid on a β-counter.

**Inoculation of Nude Mice**—HEK293 transfectants were tested for their tumorigenic potential in vivo using nude mice. Five 6-week-old male BALB/c-nu/nu mice were included in each group. In subcutaneous models, 2 × 10⁶ cells suspended in 0.2 ml of PBS were injected into the right flank of each mouse at a single site. Tumor length and width were measured every 3 days after injection. Volume was calculated as length × width²/2. For intraperitoneal inoculation, 2 × 10⁶ cells suspended in 1 ml of PBS were injected into the lower right abdomen. All mice were kept in aseptic cages and sacrificed 2 months after inoculation by cervical dislocation. Immunohistochemical staining was performed to inspect tumor cell invasion into host tissues using anti-CHP2 antibody.

**Co-immunoprecipitation of Calcineurin and CHP2 in Vivo**—1 × 10⁶ CHP2 stable transfectants or mock controls were lysed
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in 20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were incubated with anti-FLAG or anti-CNα antibodies or control IgG. The immune complex was precipitated using Protein-A-agarose beads and resolved by SDS-PAGE. The blot was then probed with anti-CNα or anti-FLAG antibodies, respectively.

**Pull-down Assay**—CNα was incubated with CHP2 and CNB at different molar ratios in a reaction buffer (50 mM Tris, pH 7.4, 5 mM CaCl₂) for 2 h at 4 °C. The protein mixture was then further incubated with CaM-Sepharose pre-equilibrated with the reaction buffer on a rotator for 2 h at 4 °C. After washing 3 times with the reaction buffer, the protein was eluted from the Sepharose beads into 50 mM Tris, pH 7.4, 5 mM EGTA. Proteins in the supernatant were resolved by SDS-PAGE and then visualized by Coomassie Brilliant Blue staining.

**Extraction of Cytoplasmic and Nuclear Protein and Western Blot**—CHP2 stable transfectants or mock controls were collected, washed with PBS, and resuspended in buffer A (2 mM HEPES, pH 7.8, 10 mM NaCl, 0.5 mM EDTA, 1 mM EGTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin and aprotinin). The thoroughly mixed samples were incubated on ice for 10 min and followed by addition of Nonidet P-40 to a final concentration of 1.2% (buffer B). The cells were centrifuged at 3,000 × g for 5 min. The supernatant was collected as the cytosolic extract. The nuclear pellet was extracted with 50 μl of buffer C (2 mM HEPES, pH 7.8, 500 mM NaCl, 0.5 mM EDTA, 1 mM EGTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin and aprotinin, 10% glycerol) for 30 min on a rocking platform. After centrifugation at 14,000 × g for 10 min, the supernatant was collected as nuclear extract. Proteins from equal numbers of cells were fractionated by SDS-PAGE, transferred onto membrane, and probed with appropriate antibodies.

**Measurement of Calcineurin Phosphatase Activity**—Protein phosphatase activity of CNα was determined using either p-nitrophenyl phosphate (pNPP) (Sigma) or γ-32P-labeled RII peptide (BIOMOL Research Laboratories, Plymouth Meeting, PA) as substrate as previously described (25). The regulatory potential of CHP2 was evaluated by the relative activity of CNα at different doses of CHP2 with or without CaM.

**Immunofluorescence Staining**—Cell suspension was dropped on sterile glass slides at the bottom of 6-well plate. After overnight culture at 37 °C, slides were fixed with cold acetone, air-dried, washed in PBS, and blocked with 10% normal goat serum in PBS, 0.1% bovine serum albumin. Then, HEK293 transfectants were incubated with rabbit anti-pan-NFAT antibodies for 30 min at 25 °C. Wild type HEK293 cells were incubated with isotype-matched control antibodies. After washing, fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) was added for a further 30 min incubation at 25 °C. Nucleus was visualized by Hoechst 33342 staining. Single color images (fluorescein isothiocyanate or Hoechst) were acquired on a LEICA Q550CW microscope and analyzed using the software Qwin.

**Luciferase Reporter Assay**—0.5 μg of NFAT luciferase reporter plasmid and 0.5 μg of pRL-SV40 plasmid were co-transfected into a HEK293 stable transfectant and a mock control. pRL-SV40 Renilla luciferase reporter plasmid was added as an internal control to normalize the transfection efficiency. Luciferase reporter assays were performed using Dual Luciferase reporter assay system (Promega). To test whether the increased NFAT activity resulted from calcineurin activation, CsA (Sigma) was added 6 h before cell harvesting to block calcineurin function.

**Statistical Analysis**—Data were presented as mean ± S.D. and significant differences were defined as p < 0.05, which was determined by the software SPSS 11.0 for Windows. One-way analysis of variance with LSD test was performed for multiple comparisons and t test was used for paired samples.

**RESULTS**

CHP2 Accelerates Cell Proliferation—Previous studies have documented much increased expression of CHP2 in a variety of tumor tissues (20, 21). Using RT-PCR, we further examined its expression in established cell lines. Fig. 1A showed the results of a few representative lines HEK293, LO2, HepG2, Jurkat, and HeLa, which were used in subsequent studies. To explore the potential contribution of CHP2 to tumorigenesis, we attempted to enforce CHP2 expression in an otherwise non-expressing cell line HEK293. Among the multiple stable transfectants we obtained, clone 4G9 expressed the transgene at a level similar to that in hepatocellular carcinoma tissues, and was thus chosen as a representative in most of the subsequent studies. Fig. 1B showed CHP2 mRNA and protein expression in this specific clone in comparison with a mock control.

At each time point examined over the course of an 8-day culture, the CHP2 stable transfectant constantly produced more cells than the mock control (Fig. 1C). Given that there was no obvious difference in cell death between the two cultures (<5% in both), the cell number increase is most likely to be the result of accelerated proliferation. In support, 3H]Tdr incorporation assays demonstrated that thymidine uptake by the CHP2 transfectant was about 3-fold more than that by the mock control (p < 0.01) (Fig. 1D). To exclude the possibility that the observed effect might be due to some unknown genetic events unique for clone 4G9, two additional clones (4B7 and 4D10) were tested. Similar results were obtained. More intriguingly, the magnitude of the proliferative response seemed to be correlated with the expression levels of the transgene (Fig. 1E).

As further evidence, enhanced cell proliferation was also observed with transiently transfected HEK293 cells in bulk cultures (p < 0.01) (Fig. 1F). Moreover, the proliferation-enhancing effect was not restricted to HEK293 cells. LO2, a human liver cell line, showed the same trend following transfection with CHP2 (p < 0.01) (Fig. 1F).

CHP2 effect on cell proliferation was further investigated using the technique of RNA interference to knockdown endogenous CHP2 expression in HepG2 cells. Three siRNA oligos were thus synthesized, and tested for their capacity to inhibit the expression of GFP-CHP2 fusion proteins by co-transfecting HEK293 cells with pEGFP-CHP2 and the oligos. siRNA#2 (GAA GGA ACA AAC UUC ACU A) showed the most potent inhibition, and was therefore used in subsequent experiments (Fig. 2A). In HepG2 cells, the mRNA level of endogenous CHP2 was substantially reduced following transfection with siRNA#2 (Fig. 2B). At the same time, the proliferation of siRNA#2 trans-
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We next examined whether CHP2 overexpression altered the tumorigenic capacity of HEK293 cells in vivo. When cells were inoculated subcutaneously, tumors formed in the CHP2 group were consistently larger in size than those in the mock control group (Fig. 3, A and B). Nevertheless, no tumor metastasis was detected in either group by immunohistochemistry or hematoxylin-eosin staining (data not shown). In intraperitoneal inoculation, palpable tumors formed within a week with the CHP2 transfectant, whereas it took about 2 weeks with the mock control. At the end, the total mass of the tumors harvested from the CHP2 group was about 3 times more than that of the control group ($p < 0.01$) (Fig. 3C). Furthermore, the CHP2 transfectant appeared to be more aggressive, invading into many abdominal organs, such as spleen, liver, and kidney (Fig. 3D), whereas no HEK293 cells were found in any of these organs in the controls (data not shown). These data indicate that CHP2 overexpression significantly increased the tumorigenic capacity of HEK293 cells.

CHP2 Binds to CnA in Vivo and in Vitro—CHPs share a high degree of homology with the regulatory subunit of calcineurin, CnB. Previous studies have reported that CHP1 is able to bind to the catalytic subunit, CnA, and exert a substantial influence on the enzymatic activity (15). This, together with the accumulating evidence for a critical role of calcineurin signaling in the regulation of cell growth (26), prompted us to investigate the possibility that CHP2 may also bind to CnA and affect the proliferation of HEK293 cells by regulating calcineurin activity. Using immunoprecipitation, we demonstrated that CHP2 protein was co-precipitated with CnA in CHP2-transfected cells. Similarly, CnA was detected in the immune complex precipitated from cells transfected with CHP2 or empty vectors, together with pEGFP plasmid. GFP positive cells were sorted 24 h after transfection and cultured for an additional 48 h before being tested for thymidine incorporation. Data shown are averages of three independent experiments. G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

CHP2 activates the transcription of the endogenous CnA gene in a dose-dependent manner (Fig. 4A). CHP2 increased the thymidine incorporation by transiently transfected cells. HEK293 and LO2 cells were transfected with CHP2 or empty vectors, together with pEGFP plasmid. GFP positive cells were sorted 24 h after transfection and cultured for an additional 48 h before being tested for thymidine incorporation. Data shown are averages of three independent experiments. G3PDH, glyceraldehyde-3-phosphate dehydrogenase.
Using this assay, we further analyzed the competitive binding to CnA by CHP2 and CnB. The two proteins were thus mixed at a molar ratio of 0.5–8, and incubated with CnA. The relative amount of CHP2 and CnB precipitated by CaM-Sepharose was then compared. With the increase of input CHP2, CnB proteins recovered progressively decreased while increasingly more CHP2 proteins were recovered (Fig. 4C). Specifically, almost equal amounts of CHP2 and CnB were pulled down at a molar ratio of 1 (Fig. 4B), suggesting that CHP2 and CnB may have a similar affinity for CnA.

**CHP2 Enhances the Phosphatase Activity of CnA**—In reconstitution assays with purified recombinant proteins, the impact of CHP2 on the phosphatase activity of CnA was directly evaluated. As expected, CnA by itself had limited efficacy in dephosphorylating the substrate pNPP, but its activity was dramatically increased with the addition of CnB and CaM. Interestingly, comparable levels of CnA activation could also be achieved when CnB was substituted by CHP2. In both cases, calcium appeared to be required as CnA activation was being suppressed with increasing concentrations of EGTA (Fig. 5A). Further analysis revealed a dose-dependent response. In the range of 0.5–4 μM, CHP2 caused a steady increase of CnA activity (p < 0.01) (Fig. 5B, left). Moreover, CHP2 was found to act synergistically with CnA. Although CnA activity was doubled with 2 μM CaM alone, a maximum 6-fold increase was achieved in the presence of both CaM and CHP2 (Fig. 5B, right). Subsequently, we tested γ-32P-labeled R11 peptide, a more physiologically relevant substrate of CnA, in such assays. Similar results were obtained. A nearly 3-fold increase in CnA activity was achieved with 1.6 μM CHP2 alone (Fig. 5C, left), whereas the combination of CHP2 and CaM caused a maximum of 8-fold increase in CnA activity (Fig. 5C, right).

**CHP2 Promotes NFAT Translocation and Activation**—The results described above demonstrated that CHP2 may directly interact with CnA and result in the activation of calcineurin. The next question is how this would affect the activity of NFAT, a well-known substrate of calcineurin. We first examined the subcellular localization of NFAT following CHP2 overexpression in HEK293 cells. Immunofluorescent staining with pan-NFAT antibodies demonstrated that the NFAT protein was primarily localized in the cytoplasm of the parent HEK293 cells, but mainly detected in the nuclei of CHP2-transfected cells (Fig. 6A). Competitive binding to CaM by CHP2 and CnB. Calmodulin-Sepharose beads was incubated with recombinant CnB or CHP2 in the presence or absence of CnA at an equal molar ratio. The eluate was fractionated on SDS-PAGE and visualized by Coomassie Brilliant Blue staining. C, competitive binding to CnA by CHP2 and CnB. Calmodulin-Sepharose beads was incubated with CHP2 and CnB mixed at different ratios in the presence of CnA. The relative amounts of CHP2 and CnB bound to CnA were revealed by Coomassie Brilliant Blue staining. WB, Western blot.
Surprisingly, whereas NFAT proteins were exclusively found in the cytoplasm of unstimulated Jurkat cells, significant amounts of NFAT proteins were detected in the nuclear fraction of HEK293 cells, suggesting a low level of constitutive activation. Nevertheless, in comparison with the mock control, the CHP2 transfectant showed much increased nuclear accumulation of NFATc3. The partition of NFATc1, on the other hand, was largely unaffected (Fig. 6B).

In view of the complexity introduced by the basal level of NFAT activation in HEK293 cells, the nuclear translocation of NFATc3 induced by CHP2 was further examined in HeLa cells with no noticeable endogenous NFAT activity. When cells were transfected with pEGFP-NFATc3 and mock control, the fusion protein was mainly seen in the cytoplasm. Co-transfection with CHP2, however, led to an apparent shift to the nuclei (Fig. 6C).

To explore the functional consequences of NFAT nuclear translocation, a luciferase reporter assay was performed. As shown in Fig. 6D, NFAT-driven luciferase activity was increased by ~3-fold in CHP2 transfectants in comparison with the mock control (p < 0.01). Moreover, this increase was completely abolished following pretreatment of the cells with CsA, a potent inhibitor of calcineurin. Therefore, CHP2-induced calcineurin activation was very likely responsible for the enhanced NFAT activity.

Up to this point, we showed that CHP2 could bind to and activate the phosphatase activity of CnA, which in turn led to
the nuclear translocation and activation of NFAT. But a direct link of these biochemical changes with altered cell proliferation remains to be established. Circumstantial evidence for such a link came from the observation that CsA was able to counteract the growth-promoting effect of CHP2 in a dose-dependent way (Fig. 6E).

DISCUSSION

The present study focused on the functional characterization of CHP2, a newly identified small calcium-binding protein that shares significant homology with CnB. It was found that, similar to CnB, CHP2 was able to bind to and stimulate the phosphatase activity of CnA. As a result, CHP2-transfected cells showed the increased nuclear presence of NFATc3 and enhanced NFAT activity. In addition to these biochemical changes, CHP2 had a profound influence on cell behavior. As such, ectopic expression of CHP2 enhanced the proliferation and oncogenic potential of HEK293 cells, whereas knockdown of endogenous CHP2 expression inhibited the proliferation of HepG2 cells.

CHP2 has previously been shown to be implicated in the regulation of transmembrane Na\(^+\)/H\(^+\) exchange (21). Our studies reveal a novel function of CHP2 in calcineurin activation. An increasing number of calcineurin regulators have been identified in recent years, many of which down-regulate calcineurin activity (9–16). CHP2, on the other hand, primarily acts as an activator of calcineurin. Immunoprecipitation demonstrated that CHP2 was physically associated with CnA in CHP2-transfected cells. CnA contains at least four functionally distinct domains, including the catalytic domain, the CnB-binding and CaM-binding domains, and the C-terminal autoinhibitory domain (2). The competitive binding to CnA by CHP2 and CnB as shown in pull-down assay suggests that they may share the same binding site. Reconstitution assays indicated that the assembly of the CHP2-CnA complex enabled some activation of the enzyme, but the activity was low compared with that attained in the presence of CaM. The mechanism whereby CHP2 executes its activating potential remains elusive. A simple model would be that CHP2 functions as a substitute for CnB and its binding to CnA induces a conformational change that increases the affinity of the catalytic domain for substrate or weakens the interaction of the catalytic domain with the autoinhibitory domain.

Our in vitro assay demonstrated that the CHP2-mediated calcineurin activity was calcium-dependent. It is thus puzzling how CHP2 activates endogenous calcineurin without calcium influx. Using Fura-2 as an indicator, we measured the intracellular calcium concentration in CHP2-transfected HEK293 cells. It was estimated to be in the range of 150–250 nm, similar to that in the mock control (data not shown). This concentration is far below the calcium requirement for enzyme activation by CaM (20 \(\mu\)M for half-maximal change) (27). On the other hand, it is close to the submicromolar concentration required for enzyme activation induced by CnB in the absence of CaM (28). In consideration of the functional similarity between CHP2 and CnB, we speculate that some enzyme activation may also be induced by CHP2 under such a condition.

It is interesting to note that, although closely related in structure, CHP1 and CHP2 display significant divergence in function. CHP1 have been previously described as negative regulators of the calcineurin/NFAT signaling pathway (15). In contrast, CHP2 enhanced calcineurin activity and its overexpression induced the activation of NFAT in transfected cells. In concordance with the differential biochemical responses they induce, CHP1 and CHP2 exert opposite effects on cell proliferation. Although proliferation of CHP1 transfectants is shown to be suppressed (15), CHP2 transfectants demonstrated enhanced proliferation. The functional distinction between CHP1 and CHP2 is also observed in their regulation of transmembrane Na\(^+\)/H\(^+\) exchange. Pang et al. (21) reported that activation of NHE1 by CHP1 is serum dependent, whereas NHE1 is constitutively activated in cells overexpressing CHP2. The mechanism underlying the differential effect of CHP1 versus CHP2 remains to be determined. Presumably, studies involving replacement of individual domains and substitution of critical amino acids should allow better understanding of the structural basis for their molecular activity. Besides a regulatory role in calcineurin signaling and Na\(^+\)/H\(^+\) exchange, several other activities have been documented for CHP1. It is found to be able to bind to and suppress the function of DRAK2, an apoptosis-inducing protein kinase (29, 30). Another study shows that CHP1 may interact with microtubules as well as endoplasmic reticulum and modulate the organization and assembly of cytoskeleton and endoplasmic reticulum (18). It will be interesting to determine whether CHP2 has any impact on these processes.

Although absent in the majority of normal tissues, CHP2 is highly expressed in various types of malignant cells (20, 21). However, its specific contribution to tumor development and progression has been poorly understood except for the suggestion that CHP2 may enhance tumor cell survival in an unfavorable environment by maintaining high intracellular pH (21). Our results indicate that CHP2 plays a more direct and fundamental role in the regulation of tumor growth and invasion. Specifically, we showed that stable or transient transfection with CHP2 remarkably increased the proliferation of HEK293 and LO2 cells. Knockdown of endogenous CHP2 expression, on the other hand, inhibited the proliferation of HepG2 cells. When CHP2-transfected HEK293 cells were inoculated into nude mice, tumor formation was accelerated. Moreover, these cells exhibited a newly acquired capacity to invade into multiple adjacent organs. Of note, similar findings have been reported in a recent study with enforced CHP2 expression in an ovarian cell line (31).

As far as the mechanism is concerned, data currently available suggest the implication of a disturbed calcineurin/NFAT signaling pathway. In CHP2-transfected cells, NFAT proteins were detected in the nucleus at a much increased level by immunofluorescence and Western blot analysis, which was coupled with an enhanced transcriptional activity. Of the two isoforms detected in HEK293 cells, CHP2-induced nuclear translocation mainly involved the predominant one, NFATc3. The reason behind the target specificity is unclear. Importantly, both CHP2-induced NFAT activity and CHP2-enhanced proliferation in HEK293 cells could be blocked by the calcineurin inhibitor CsA. Therefore, CHP2-mediated calcineurin activity...
and the NFAT activation induced thereby are at least partly responsible for the increased proliferation of the CHP2 transfectant.

Evidence is accumulating that the deregulation of the calcineurin/NFAT signaling pathway is implicated in tumor development and progression. Aberrant expression or activation of calcineurin and NFAT has been documented in a number of tumor types. Calcineurin, for example, was reported to be highly expressed in human colorectal adenocarcinomas (32) or highly expressed in lymphoma samples (35). Several recent studies provide further evidence for the contribution of the disturbed signaling pathway to tumorigenesis. Neal and Clipstone (36) reported that expression of the constitutively active NFATc1 mutant in several tumor types. Calcineurin, for example, was reported to be involved in the tumor suppressive function of the calcineurin/NFAT signaling pathway. In NFATc3-deficient mice, T cell lymphoma tended to develop faster and more frequently following retroviral infection (38). Targeted deletion of the calcineurin inhibitor DSCR1 (calcipressin) led to hyperactivated calcineurin and suppressed angiogenesis and growth of tumor (39). In contrast, we found that transfection of HEK293 with CHP2, a potential calcineurin activator, increased cell proliferation in vitro and tumor growth in vivo, echoing a most recent study showing that CnB overexpression enhances the oncogenic potential of HEK293 cells (40). More intriguingly, we demonstrated that the observed effect was likely to be mediated by NFATc3. The seemingly conflicting data are reflective of the complexity of the calcineurin/NFAT pathway. Its precise role in tumorigenesis is probably dependent on the specific cellular context.

In conclusion, our studies have demonstrated a novel function of CHP2 as an activator of the calcineurin/NFAT signaling pathway. Malfunction of CHP2 may play a role in tumor development and progression. Understanding of the upstream events and downstream consequences of the disturbed calcineurin/NFAT function in tumor cells may reveal potential targets for clinical intervention.

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