Nuclear accumulation of Calcineurin B Homologous Protein 2 (CHP2) results in enhanced proliferation of tumor cells

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The interaction between calcineurin B homologous protein 2 (CHP2) and Na\(^+\)/H\(^+\) exchanger 1 (NHE1), two membrane proteins, is essential for protecting cells from serum deprivation–induced death. Although four putative EF-hands in CHP2 had been predicted for years, Ca\(^{2+}\)-binding activities of these motifs have not been tested yet, their role in this process remain poorly understood. To identify Ca\(^{2+}\)-binding motifs required for the stable formation of CHP2/NHE1 complexes, we developed a mutagenesis-based assay in PS120 cells. We found that \(^{45}\)Ca\(^{2+}\) bond to two EF-hand motifs (EF3 and 4) of CHP2 proteins with high affinity. Complex formation between CHP2 and the CHP2 binding domain of NHE1 resulted in a marked increase in the Ca\(^{2+}\)-binding affinity of CHP2. Co-immunoprecipitation and distribution of GFP-tagged CHP2-EF3m/4m also indicated that Ca\(^{2+}\) affected the membrane location of CHP2 to interact with NHE1. The C-terminal region of CHP2 contains a nuclear export sequence (NES). When the six leucines of NES were mutated to alanines, the resulting CHP2 protein was predominantly localized to the nucleus. Furthermore, mutation of the NES resulted in enhanced proliferation and oncogenic potential of HeLa cells. Together, these results show that calcium and NES control the subcellular distribution of CHP2 and then distinctively regulate cell proliferation.

Introduction

Calcineurin B homologous protein 2 (CHP2), also known as hepatocellular carcinoma antigen 520 (HCA520), belongs to the super family of N-myristoylated, EF-hand Ca\(^{2+}\)-binding protein CHPs (Pang et al. 2001; Wang et al. 2002). Three isoforms of CHPs have been identified to date. CHP1 (also called P22) is expressed ubiquitously in virtually all tissues; CHP 3 (also called tescalcin) is expressed only in a few normal tissues (Zaun et al. 2008). Unlike CHP1 and 3, the expression of CHP2 is restricted to cancer (Pang et al. 2002) and the small intestine (Inoue et al. 2003). Of the three isoforms, CHP1 has been characterized in the most detail. CHP1 has been reported to exhibit multiple functions including vesicular transport, inhibition of calcineurin phosphatase activity (Lin et al. 1999), as well as interaction with NHEs (Barroso et al. 1996), microtubules (Timm et al. 1999), death-associated protein kinase–related apoptosis-inducing protein kinase 2 (DRAK2) (Kuwahara et al. 2003) and kinesin-family1B\(^{2}\) (KIF1B\(^{2}\)) (Nakamura et al. 2002). CHP2 might share the structural and multiple functional similarities to CHP1, for these two proteins show high homology in primary sequence (61% identity).

The intracellular shuttling is an important mechanism to regulate the activities and functions of considerable proteins during cell differentiation and development. On the one hand, we have discovered that CHP2 on plasma membrane increases intracellular pH (pH\(_{i}\)) by activating NHE1 to protect cells from serum deprivation–induced death (Pang et al. 2002). Crystallographic analysis and structure-based
mutagenesis (NHE1 mutants, I534K, I534D and I537K) show the importance of hydrophobic interactions between CHP2 and NHE1 for the activity of NHE1 and plasma location of CHP2 (Ammar et al. 2006). However, little attention has been devoted to identify the key amino acids or motifs of CHP2 determining its affinity for interaction with NHE1, especially the two canonical EF 3&4 Ca\(^{2+}\)-binding motifs. Moreover, intracellular Ca\(^{2+}\) flux plays a crucial role in the proliferation and differentiation of cells (Crabtree & Olson 2002). As for CHP1, tightly bound Ca\(^{2+}\) ions are important structural elements in the ‘pH\(_i\) sensor’ of NHE1 (Pang et al. 2004). Several other groups report the interactions between CHPs and NHE1, but there is still little information available about the role that Ca\(^{2+}\) plays in the interaction of NHE1 with CHP2.

In contrast, putative nuclear export sequence (NES) was also found in CHP2. NES is a short sequence that has been identified as a motif required for the active nuclear export of proteins (Sorokin et al. 2007). Inoue et al. (2003) suggested for the first time that the fluorescence signal of CHP2-GFP in the nucleus was stronger than that of CHP1-GFP (Inoue et al. 2003). But the function of CHP2 located in the nucleus is still obscure.

Recently, a few reports have shown that overexpression of CHP2 accelerates proliferation, growth, invasion and metastasis in ovarian cancer (Jin et al. 2007) and human embryonic kidney cell line 293 (HEK293) (Li et al. 2008). These important roles of CHP2 prompted us to study its functional mechanisms with NHE1, NES and Ca\(^{2+}\) ions in transformed cells. We discovered that the binding of Ca\(^{2+}\) ions to EF-hand 3&4 motifs determined the plasma membrane location of CHP2 via forming a Ca\(^{2+}\)/CHP2/NHE1 complex. More interestingly, we found that accumulation of CHP2 in the nucleus could enhance the proliferation and oncogenic potential of tumor cells. Besides given a profound interpretation of CHP2 located on plasma membrane in a Ca\(^{2+}\)-dependent manner to adjust NHE1 activity, our results have shown that the regulation of subcellular location mediated by NES affects the function of CHP2 in cancer proliferation.

Results

Characterization of Ca\(^{2+}\)-binding motifs in CHP2

Human CHP2 protein has a primary sequence highly homologous to that of CHP1 (61% identity). CHP2 contains four EF-hand Ca\(^{2+}\)-binding motifs, of which two ancestral sites might not bind Ca\(^{2+}\) because of the substitution of critical acidic residues. The canonical EF-hand consists of 29 consecutive residues with two flanking helices and a 12-residue loop (Fig. 1). The chelating loop residues in positions 1 (+x), 3 (+y), 5 (+z), 7 (–y), 9 (–x) and 12 (–z) ligate Ca\(^{2+}\) through seven oxygen atoms arranged three dimensionally on the axes of a pentagonal bipyramid. The –z position, providing the only side-chain oxygen atoms, is crucial for Ca\(^{2+}\) binding (Yap et al. 1999). 45Ca\(^{2+}\) binding to EF-hand motifs of CHP2 was analyzed using recombinant CHP2 and CHP2/NHE1 (aa 515–545) complex. We introduced mutations into

![Figure 1](https://example.com/fig1.png)

**Figure 1** Amino acid sequence alignment of the human calcineurin B homologous protein (CHP) isoform 1 and 2. Amino acid sequences of human CHP 1 and 2 were aligned. The nuclear export sequence of CHP2 (residues 137–148) were boxed, and the loop regions of four Ca\(^{2+}\)-binding EF-hand motifs were indicated by solid underlines, respectively. Two N-terminal ancestral sites (EF 1&2) of the four EF-hand Ca\(^{2+}\)-binding motifs do not have a typical EF-hand sequence and thus might not bind Ca\(^{2+}\).
EF1, EF2, EF3 and EF4 in which acidic residues (aspartic acid or glutamic acid) at the $\alpha$ position were replaced by alanine (Fig. 2A). We co-expressed the wild-type or mutant CHP2s together with the juxtamembrane region of NHE1 (aa 515–545) in *Escherichia coli*. On 12% SDS–PAGE, EF3m and EF4m proteins were found to migrate more slowly than the wild-type, EF1m or EF2m proteins (Fig. 2B), suggesting that a mutation-induced conformational change, which occurs in these mutant proteins, had impaired Ca$^{2+}$ binding.

We measured $^{45}$Ca$^{2+}$ binding to various CHP2 mutant proteins by a membrane filtration procedure. We found that $^{45}$Ca$^{2+}$ bound to the purified CHP2 proteins with an apparent $K_d$ of 88 nM. When CHP2 formed a complex with the NHE1 fragment, the binding affinity for $^{45}$Ca$^{2+}$ increased markedly (42-fold, Fig. 3A). Mutation of either of Ca$^{2+}$-binding motifs EF3 or EF4, but not EF1 and 2, resulted in loss of approximately one half of $^{45}$Ca$^{2+}$ bound to the complex (Fig. 3B). However, $^{45}$Ca$^{2+}$ binding was completely blocked when the experiment was carried out using EF3/m proteins (two sites were simultaneously mutated) even with the NHE1 fragment (Fig. 3B). Together, these results indicate that CHP2 binds two Ca$^{2+}$ ions, one at EF3 and the other at EF4.

To determine how complex formation increases the Ca$^{2+}$-binding affinity, we measured $^{45}$Ca$^{2+}$ release from CHP2 proteins by rapid filtration. As shown in Fig. 4A, most of the $^{45}$Ca$^{2+}$ bound to CHP2 without the NHE1 fragment was released rapidly ($t_{1/2} = \sim 3$ s). In contrast, $^{45}$Ca$^{2+}$ release from CHP2/NHE1 (aa 515–545) complex ($t_{1/2} = \sim 8$ s) was much slower. A slow release of $^{45}$Ca$^{2+}$ also occurred in two mutant CHP2 proteins, EF3m and EF4m, complexed with the NHE1 fragment (Fig. 3B), suggesting that Ca$^{2+}$ binds tightly to each EF-hand and each of the Ca$^{2+}$-binding motifs.

**Figure 2** Amino acid sequences of EF-hand motifs and purified protein of various calcineurin B homologous protein (CHP2) mutants. Panel A shows amino acid sequences of four EF-hand motifs present in CHP2. In five mutant CHP2s (EF1m, EF2m, EF3m, EF4m and EF34m), Asp50, Gly82, Glu135, Glu176 and both Glu135/Glu176 were replaced by alanine. In panel B, recombinant histidine-tagged various CHP2 variant proteins with or without mutations in EF-hand motifs were expressed and purified from an *Escherichia coli* strain and then subjected to SDS–PAGE. The purified of Histagged CHP2 variants were separated by electrophoresis on a 12% SDS–PAGE gel and then visualized by Coomassie Brilliant Blue staining.

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**Figure 3** Equilibrium $^{45}$Ca$^{2+}$ binding to various calcineurin B homologous protein (CHP2) mutant proteins. Recombinant histidine-tagged various CHP2 mutants with its binding region (aa 515–545) in Na$^+$/H$^+$ exchanger 1 (NHE1) was expressed and purified from an *Escherichia coli* strain. In panels A and B, CHP2 or its various mutant proteins and the complex of CHP2 variants with the NHE1 segment (aa 515–545) (0.1–0.2 mg/mL) were incubated for 1 h in solutions containing 50 μM $^{45}$CaCl$_2$ and various concentrations of EGTA, which produce 0.2 nM to 50 μM free Ca$^{2+}$. Symbols corresponding to each protein variant were indicated in figures. The solutions were filtered through Millipore filters, and $^{45}$Ca$^{2+}$ bound to the CHP2 proteins was measured.
EF-hand has a similar Ca\(^{2+}\)-binding property as that of CHP1 (Fig. 4B).

**Plasma membrane location of CHP2 is Ca\(^{2+}\) dependent**

As CHP2 is an EF-hand Ca\(^{2+}\)-binding protein via motifs 3 & 4, we examined the Ca\(^{2+}\)-dependent function of CHP2 due to these two motifs. CHP2-EF3m and EF4m proteins were found to migrate more slowly than the wild-type, EF1m or EF2m proteins on SDS–PAGE (Fig. 2B, lower panel). Disparity of electrophoretic velocity of CHP2 proteins suggested that Ca\(^{2+}\) is indispensable to maintain the natural conformation of CHP2. When Ca\(^{2+}\) is reduced by 10 mM ethylene diamine tetraacetic acid (EDTA), CHP2 did not co-immunoprecipitate with NHE1 (Fig. 5A,B). Consistently, this study shows that Ca\(^{2+}\) is an important factor for regional distribution of CHP2, as weaker green fluorescent protein (GFP) fluorescence was observed on the plasma membrane in CHP2-EF3m\(^{-}\)/EF4m-GFP cells than that of wild-type CHP2-GFP controls (Fig. 5C). The binding of Ca\(^{2+}\) ions to EF-hand 3 & 4 determines CHP2 to locate onto the cell plasma membrane via affecting CHP2/NHE1 complex formation.

**The NES signal mediate the cytoplasmic localization of CHP2**

We tried to explore putative signaling sequences in CHP2 that might control its intracellular distribution. We found a NES-like motif (residues 137–148) in the carboxyl-terminal region of the protein. The NES element of CHP2 is similar to typical NES
sequence, which consists of leucine and other hydrophobic residues (isoleucine, valine, phenylalanine and methionine), with characteristic spacing as CHP1 reported previously (Fig. 1). This putative NES motif of tissue-specific isoform CHP2 is conserved (Inoue et al. 2003), so we suggest that the putative NES sequence of CHP2 is of functional significance.

To determine whether the putative carboxyl-terminal NES was relevant to the nuclear export of CHP2, we examined the localization of CHP2-GFP with point mutants of NES in HeLa cells. For the determination of the functional effect of NES on subcellular location of CHP2, wild-type CHP2-GFP fusion protein and CHP2-GFP fusion protein derivatives with substitutions in NES sequence (L137A/L140A/L142A/V144A/V146A/V148A) were constructed. Then, the stable transfectants with these protein derivatives were selected. These mutant proteins were shown to have the expected molecular size as that calculated for the wild-type CHP2 by SDS–PAGE and immunoblotting (data not shown). Wild-type CHP2-GFP was predominantly located in the cytoplasm of HeLa cells, whereas CHP2-GFP fusion protein derivatives with substitutions in NES sequence (CHP2-NESm-GFP) were found in the nucleus (Fig. 6). This analysis clearly shows that NES functions as a signal for the export of CHP2 from the nucleus and has sufficient activity for the proper nuclear export of CHP2.

Nuclear accumulation of CHP2 accelerates cell proliferation in vitro

Previous studies have documented increased expression of CHP2 in a variety of tumor tissues (Jin et al. 2007; Li et al. 2008). To investigate the potential involvement of NES in tumor cells, we compared the in vitro growth characteristics of three cell lines by means of (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (MTT) and colony-formation assays. Stable transfectants of GFP-tagged CHP2 fusion proteins with NES mutation were obtained for the proliferation observation of HeLa cells in the subsequent studies. Figure 7 showed that transfectants of CHP2 with NES mutant (CHP2-NESm) had an enhanced potential of proliferation, of which the CHP2-NESm protein was accumulated in nucleus. In colony-forming assay, CHP2-NESm cells formed not only more colonies (Fig. 7A,B) but also larger colonies than CHP2 and control cells (Fig. 7A,C). In MTT assays, at each time point examined over the course, the stable transfectant of CHP2-NESm constantly produced more cells than the stable transfectants of CHP2 and normal HeLa cells (Fig. 7D). As further evidence, enhanced cell proliferation was also observed in transiently transfected MCF 7 (breast cancer cell line, Michigan Cancer Foundation-7) and MDA-MB-231 (M.D. Anderson - Metastatic Breast cell line-231) cells in bulk cultures (data was not shown). These data suggested that tumor cells bearing CHP2-NESm had greater growth potential than either wild type or control.

Accumulation of CHP2 in nucleus promotes the oncogenic potential of HeLa cells in vivo

We next examined whether accumulation of CHP2 in nucleus altered the tumorigenic capacity of HeLa cells in vivo. When cells were inoculated subcutaneously, tumors formed in the CHP2-NESm group were consistently larger in size than those in the CHP2 control group (Fig. 8A). At the end, the total mass of the tumors harvested from the CHP2-NESm group was nearly threefold more than that of the CHP2 group (P < 0.01) (Fig. 8B). Furthermore, the CHP2-NESm transfectants appeared to be more aggressive, invading into many abdominal organs, such as spleen, liver and kidney than HeLa cells with CHP2 (data was not shown). Collectively, our present findings strongly suggest that the accumulation of...
CHP2 in nucleus significantly increases the tumorigenic capacity of HeLa cells.

Discussion

Subcellular distribution is an important characteristic of a protein because location is intimately related to its function. Current work shows that subcellular localization of CHP2 is regulated by the following factors: (i) Ca$^{2+}$ ions binding to EF-hand 3&4 motifs of CHP2 determine its location onto cell plasma membrane via complex formation between CHP2 and NHE1; (ii) experiments of point mutations in NES signal implicate that NES signal controls distribution of CHP2 in

Figure 7 Effect of expression of wild-type or nuclear export signal mutant of GFP-tagged calcineurin B homologous protein (CHP2) on cell proliferation in vitro. Stable clones of HeLa cells transfected with GFP-tagged CHP2 or CHP2 of nuclear export sequence mutant (CHP2-NESm) were selected. Colony-forming potential was analyzed by soft agar colony-forming assay. (A) Representative photographs of colony formation for HeLa cells transfected with CHP2, CHP2-NESm or control vector, respectively. Upper panel (40×) showed the number of colonies, and lower panel (100×) showed the size of colonies. (B) Colony numbers for different HeLa cell lines of each sight under microscope were shown. (C) Colony radius under microscope was also shown (D) Cell proliferation potential in liquid culture was determined by (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) method at time point indicated. *$P < 0.05$ by Student’s t-test.
or out of the nucleus. Ca\(^{2+}\) determines the location of CHP2 onto cell plasma membrane to adjust pH\(_i\) via complex formation of CHP2 \(\sim\) NHE1, whereas NES signal regulates CHP2 into nucleus to enhance cell proliferation, but how the two pathways are regulated still needs to be further studied. Our previous study has shown that CHP2 could regulate pH\(_i\) of cells. Here, we focus on how CHP2 localizes on the membrane to interact with NHE1 and how NES inactivation regulates CHP2 into nucleus and then enhances the cell proliferation.

CHP is an EF-hand Ca\(^{2+}\)-binding protein; Ca\(^{2+}\) is crucial for its activity. Here, we first examined the role of EF-hand Ca\(^{2+}\)-binding motifs in CHP2. Our results indicated that a Ca\(^{2+}\) ion bound to each of EF3 and EF4 in CHP2 with an overall apparent \(K_d\) of \(\sim\)88 nM. This Ca\(^{2+}\)-binding affinity was close to that of another family member, CHP1 (apparent \(K_d\) \(\sim\)90 nM). CHP2 potentially has four Ca\(^{2+}\)-binding motifs; the two ancestral sites EF1 and EF2 do not bind Ca\(^{2+}\) as CHP1. The Ca\(^{2+}\) affinity of CHP2 increased markedly upon complex formation with the NHE1 fragment (aa 515–545). Consistently, the release of \(^{45}\)Ca\(^{2+}\) from the complex was much slower than that from CHP2 alone. The interaction between EF-hand and Ca\(^{2+}\) ions in CHP2 is a little stronger than that from CHP2 alone. The interaction between EF-hand and Ca\(^{2+}\) ions in CHP2 is a little stronger than that from CHP1. Increases in the affinity for Ca\(^{2+}\) by interacting with target proteins have also been reported for other Ca\(^{2+}\)-binding proteins. As reported before, the Ca\(^{2+}\)-binding affinity for calmodulin (CaM) was increased 16– to 38-fold upon interaction with myosin light chain kinase (Olwin et al. 1984), 2.6-fold with myristoylated alanine-rich protein kinase C substrate peptide (Johnson et al. 1996), and 75-fold with the CaM-binding peptide in calcineurin A (CNA) (Stemmer & Klee 1994). Figure 5A,B showed that interactions between CHP2-GFP and NHE1 were affected by different concentrations of Ca\(^{2+}\) ions and EDTA. Our results implicate that tightly bound Ca\(^{2+}\) ions are important structural elements for maintaining the normal conformation of CHP family and seriously affect the interaction between CHP2 and NHE1. For the modest differences in Ca\(^{2+}\) bound between these two isoforms, how could we account for the functional differences of CHP1 and 2? As we know, high expression of CHP2 is mainly restricted in tumor cells, whereas CHP1 is widely expressed in various cells. The tissue-specific expression might contribute to their functional difference. Moreover, there is only 61% identity in amino acid sequence, and some other specific motifs of these two proteins might also take part in determining their distinctive functions.

The NES of CHP2 is a kind of low leucine-type NES motif (Nagita et al. 2003). This is different from canonical NES signal that have high leucine contents in some typical nuclear-exported molecules, such as Rev (Fischer et al. 1995) and mitogen-activated protein kinase kinase (MAPKK) (Fukuda et al. 1996). Only one functional NES (aa 137–148) was identified in CHP2, although several studies have reported two putative NES (Nagita et al. 2003). Experiments of point mutations showed that this NES motif is important for the proper intracellular distribution of CHP2. The NES sequence of CHP2 is also conserved in CHP1 and CNB (Nagita et al. 2003). The intracellular distributions of all these proteins might also be controlled by NES signal. The only functional
NES of CHP2 is located in the carboxyl-terminal α-helix of the EF-hands and the flanking region. Phospholipase C-Δ1 also has a typical NES sequence in its EF-hand domain, which is located at a position similar to that of CHP1 and CHP2 (Yamaga et al. 1999). These observations imply that many other EF-hand proteins might also possess a functional NES as reported by Nagita et al. (2003).

We have demonstrated that CHP2 protects cells from serum deprivation–induced death (Pang et al. 2002). Several reports also confirm the result that wild-type CHP2 could enhance the proliferation capacity of tumor cells (Jin et al. 2007; Li et al. 2008), although the effect is less than NES mutation variant (CHP2-DESsm, Fig. 7). No obvious change of NHE1 expression was observed in CHP2-transfected cells, so the effect of CHP2 on proliferation might be from its role on NHE1 activity (Jin et al. 2007). NHE1 is activated only upon cytosolic acidicification in normal cells, whereas in transformed cells NHE1 is hyperactivated even at resting pHi (Kim et al. 2009). CHP2 appears to be exclusively expressed in transformed cells. So the activation of NHE1 by CHP2 might be a key mechanism for the high pHi in these abnormal cells. Both CHP1 and CHP2 could increase the pHi of cells. There is little difference in pHi regulation between them except for under the serum-free culture condition (Pang et al. 2002). At normal conditions, modest difference in proliferation of these two isoforms was also detected (data was not shown).

Previous reports have shown that there are two pathways for CHPs in regulating the function of NHE1 in cells. First, CHP1 plays an essential role in the stabilization of NHE1 for reaching of NHE1 to the plasma membrane (Matsushita et al. 2007). Second, CHPs confer ability to NHE1 to maintain a high exchange activity on the plasma membrane. The former and our present studies show that all of these above biological processes require a direct physical interaction between CHP1/2 and NHE1 via the binding site of CHP1/2 in the juxtamembrane region within the carboxyl-terminal cytoplasmic domain of NHE1, which was shown by the co-localization of GFP-tagged protein (Fig. 5C), co-immunoprecipitation (Fig. 5A,B) and crystallographic analysis. Behaviors of NHE1 regulated by CHPs, such as translocation, stabilization and activity, happen in some limited cell compartments, such as endoplasmic reticulum, plasma membrane and cytoplasm (Pang et al. 2001, 2002, 2004; Ammar et al. 2006). So, it must be another case for CHP2 accumulated into nucleus, irrelevant to NHE1, regulating tumor cell proliferation, because the different subcellular distribution contributes to the loss of direct physical interaction of CHP2-NESm with NHE1. Nuclear CHP2-NESm could induce much greater proliferation activity than wild-type CHP2. It implicates that another NES-dependent pathway for CHP2 regulates the proliferation. Although the molecular pathways have not been elucidated, it should be noticed that CHP2 might regulate proliferation by different mechanisms. CHP2 could enhance oncogenic potential of HEK293 cells via activating the calcineurin/nuclear factor of activated T-cells (NFAT) signaling pathway by increasing nuclear accumulation of NFATc3 (Li et al. 2008). Over-expression of DRAK2, an apoptosis-inducing protein kinase, induces the nuclear accumulation of CHP1 from cytoplasm (Kuwahara et al. 2003). CHP1 exports to the cytoplasm in an NES-dependent manner after entering the nucleus (Nagita et al. 2003). These reports give us clues to further study the mechanism how the nuclear CHP2 regulates the proliferation of cancer cells. CHP2 might transport into nucleus by forming complex with other partners or by forming functional complex with other proteins later in the nucleus. In the extended work, using CHP2 as ‘bait’, melanoma antigen-D1 (MAGE-D1), an apoptosis-related protein, was screened by yeast two-hybrid system. MAGE-D1 has been showed to regulate proliferation, migration and invasion of breast cancer cells (Du et al. 2009), HeLa cells and human hepatocellular carcinoma cell line (BEL7402) (Shen et al. 2007). Co-expression of both CHP2 and MAGE-D1 in COS-7 cells existed around or accumulated in the nucleus. Meanwhile, many EF-hand Ca2+-binding proteins are also known to regulate the functions of their target proteins in response to cytosolic Ca2+ mobilization. CHP1 regulates DRAK2 activity as a result of a conformational change in the DRAK2/CHP complex dependent on the intracellular Ca2+ concentration (Kuwahara et al. 2003). CHP2 enhances the oncogenic potential of HEK293 cells by activating the calcineurin/NFAT signaling pathway, and the dephosphorylation and nuclear translocation of NFAT are sensitive to the increasing intracellular Ca2+ flux (Kim & Usachev 2009). Further studies are presently under way to investigate the role of cytosolic Ca2+ in the proliferation of CHP2 on cancer cells via NES-mediated subcellular location.

Together, these results show that CHP2 is translocated into nucleus and that CHP2-induced cell growth is augmented with NES mutation in HeLa cells. In conclusion, our study provides sufficient evidence that CHP2 interacts with NHE1 in a Ca2+-
dependent manner to regulate NHE1 activity and also enhances cell proliferation via its nuclear accumulation controlled by NES. These results provide new insight into our understanding about the role of CHP2 in tumor cell growth. But detailed understanding of NES-controlled CHP2 in tumorigenesis still needs to be further studied.

Experimental procedures

Materials

$^{45}\text{CaCl}_2$ was purchased from Du Pont-NEN (Boston, MA, USA). The rabbit polyclonal antibodies against CHP2 and NHE1 were made by us as described previously. All other chemicals were of the highest purity available.

Cell cultures and plasmid transfection

The exchanger-deficient cell line PS120 (Pouyssegur et al. 1984), HeLa cell line and corresponding transfectants were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies Inc., Rockville, MD, USA) containing 25 mM NaHCO$_3$ and supplemented with 10% (v/v) fetal calf serum, penicillin (50 units/mL), and streptomycin (50 $\mu$g/mL) at 37 $^\circ$C in an atmosphere of 95% air and 5% CO$_2$. Each plasmid construct (20 $\mu$g) was transfected into PS120 cells or HeLa cells (5 x 10$^5$ cells/100-mm dish) by the calcium phosphate coprecipitation technique and stable clones for NHE1 and its mutant constructs were selected by repetitive H$^+$-killing selection procedures. For the stable over-expression of GFP-tagged CHP2 fusion protein variants, single clones expressing proteins were isolated by monitoring GFP fluorescence under a confocal fluorescence microscope (BioRad) as a marker after selection with G418.

Construction of expression vectors for NHE1 and CHP2 variants

The cDNA for CHP2 was obtained from human blood by reverse transcriptase-PCR based on the reported sequence. Plasmids carrying cDNAs for human NHE1, CHP2 and their mutants containing unique restriction enzyme sites all cloned into the mammalian expression vector pECE. All these constructs were produced by means of the polymerase chain reaction (PCR)-based strategy. PCR fragments were digested and cloned into the appropriate restriction enzyme sites of vector pEGFP-N1 (Clontech, Palo Alto, CA, USA) for GFP-tagged CHP2 fusion protein or its mutant forms in NES motif by means of the PCR-based method. The primary sequences of all constructs were confirmed by sequencing with an automatic DNA sequencer—ABI-PRISM DNA sequencer model 3100 (Applied Biosystems, Foster City, CA, USA).

Protein expression and purification

Recombinant histidine-tagged CHP2 proteins were produced in E. coli (BL21- Star; Invitrogen, San Diego, CA, USA) transformed with pET11 carrying the cDNA encoding CHP2 containing the C-terminal six histidine residues as described previously. The complex of CHP2 (aa 1–196) with its binding region (aa 515–545) in NHE1 was expressed and purified essentially similar to the methods for CHP1 and NHE1 described previously (Pang et al. 2004). Briefly, the cytoplasmic region (aa 515–545) of NHE1 was cloned into the vector pET24 and co-expressed with His-tagged CHP2 in E. coli in the presence of ampicillin and kanamycin. Recombinant proteins were then all recovered in the soluble fraction and partially purified by passage through a Ni$^{2+}$-affinity resin column (ProBond; Invitrogen) according to the manufacturer’s protocol.

Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were carried out essentially as described previously. Briefly, cells were solubilized with 1% Triton X-100 in a solution of 150 mM NaCl, 10 mM hydroxyethyl piperazine ethanesulfonic acid (HEPES)/Tris (pH 7.4) and protease inhibitors. Cell lysates were incubated with respective antibodies and protein A Sepharose. After centrifugation, precipitated materials were separated on 7.5% or 12% polyacrylamide gels and electrophoretically transferred to Immobilon membranes (Millipore). After blocking, incubation with antibodies and washing, protein signals were visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, UK). The signal intensity was measured using a photonic microscope system (ARUGUS-100; Hamamatsu photonics).

Measurement of equilibrium $^{45}\text{Ca}^{2+}$ binding

$^{45}\text{Ca}^{2+}$ binding to the proteins was measured by a filtration method as follows. Purified proteins (0.1–0.2 mg/mL) were incubated for 1 h at 25 $^\circ$C in a solution containing 60 mM KCl, 5 mM MgCl$_2$, 50 mM CaCl$_2$, 0.02 $\mu$Ci/mL $^{45}\text{CaCl}_2$, 10 mM HEPES/Tris (pH 7.2) and different concentrations of ethyleneglycol tetraacetic acid (EGTA) (0–58 mM), giving a free Ca$^{2+}$ concentration of 0.1 mM to 50 $\mu$M. Aliquots (1 mL) of the reaction mixture were transferred onto 0.22-$\mu$m Millipore filters (Millipore, Bedford, MA, USA) and then filtered under vacuum. To measure the background binding of $^{45}\text{Ca}^{2+}$, the same reaction mixtures without proteins were filtered as controls. $^{45}\text{Ca}$ radioactivity was measured by scintillation counting after the filters were dried.

Measurement of $^{45}\text{Ca}^{2+}$ release from proteins

$^{45}\text{Ca}^{2+}$ release from proteins was measured using a rapid filtration apparatus as follows. After preincubation of proteins with a solution containing 50 $\mu$M $^{45}\text{CaCl}_2$ for 1 h, aliquots (1 mL)
of reaction mixtures were filtered through Millipore filters. Filters were then washed at a constant rate (0.2–2 mL/s) for the indicated periods (0.2–30 s) with 0.4–6 mL of 60 mm KCl, 5 mm MgCl₂, 10 mm HEPES/Tris (pH 7.2), and 10 mm EGTA. After the filters were dried, ⁴⁵Ca radioactivity was measured by scintillation counting.

**GFP fluorescence**

Cells were fixed for 10 min at room temperature with 4% paraformaldehyde in phosphate-buffered saline (PBS). For observation of GFP fluorescence, fixed cells stably expressing CHP2-GFP proteins were stained with Hoechest 33342 to allow the identification of the nucleus and then mounted with 50% glycerol/PBS. Images were taken under a fluorescent microscope equipped with a CoolSNAP imaging system (RS Photometrics).

**Soft agar colony formation**

Colony formation in soft agar was assessed as follows. Cells (2.5 × 10³) from CHP2-NESm, CHP2 and mock plasmids were suspended in 0.5 mL top agar medium (DMEM with 10% FBS supplied with 0.4% agar) and layered over 0.75 mL bottom agar medium (DMEM with 10% FBS supplied with 0.8% agar) in 24-well plate. After 3 weeks, the cells were photographed under inverted microscope and the number of colonies was counted. Independent experiments were carried out in triplicates (Shi et al. 2008).

**MTT assay**

The proliferation of cells was assayed by MTT (Sigma) test (Chong et al. 2010). In all, 1 × 10⁴ cells per well were seeded into 96-well plates in 100 µL volume and cultured in DMEM medium (Sigma) supplemented with 10% fetal bovine serum (Sigma) at 37 °C. Cells were cultured for 24 or 48 h. Then, cells were washed once with the corresponding medium and incubated for 4 h in the medium containing 1 mm MTT. The medium was discarded and 100 µL of dimethylsulfoxide (DMSO; Sigma) was added to each well. The absorbance of formazan product was determined by 570 nm absorbance using Versa Max tunable microplate reader (Molecular Devices, Sunnyvale, CA, USA).

**Inoculation of nude mice**

Calcineurin B homologous protein 2 and its variants’ stable 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 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). All mice were kept in aseptic cages and killed 2 months after inoculation by cervical dislocation.

**Statistics analysis**

Equilibrium ⁴⁵Ca²⁺ binding was fitted to the dose–response equation, ⁴⁵Ca²⁺ bound = maximal ⁴⁵Ca²⁺ bound/(1 + ([Ca²⁺])ⁿ) (Kd = apparent dissociation constant for Ca²⁺; n = Hill coefficient). Kinetic parameters were expressed as the best fit values with standard errors, whereas other data were presented as the means ± SD for at least three determinations. Significant differences were defined as P < 0.05, which was determined by the software SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA).

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**References**


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