The Pleckstrin Homology Domain of CK2 Interacting Protein-1 Is Required for Interactions and Recruitment of Protein Kinase CK2 to the Plasma Membrane*  

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CKIP-1 is a recently identified interaction partner of protein kinase CK2 with a number of protein-protein interaction motifs, including an N-terminal pleckstrin homology domain. To test the hypothesis that CKIP-1 has a role in targeting CK2 to specific locations, we examined the effects of CKIP-1 on the localization of CK2. These studies demonstrated that CKIP-1 can recruit CK2 to the plasma membrane. Furthermore, the pleckstrin homology domain of CKIP-1 was found to be required for interactions with CK2 and for the recruitment of CK2 to the plasma membrane. In this regard, point mutations in this domain abolish membrane localization and compromise interactions with CK2. In addition, replacement of the pleckstrin homology domain with a myristoylation signal was insufficient to elicit any interaction with CK2. An investigation of the lipid binding of CKIP-1 reveals that it has broad specificity. A comparison with other pleckstrin homology domains revealed that the pleckstrin homology domain of CKIP-1 is distinct from other classes of pleckstrin homology domains. Finally, examination of CK2α for a region that mediates interactions with CKIP-1 revealed a putative HIKE domain, a complex motif found exclusively in proteins that bind pleckstrin homology domains. However, mutations within this motif were not able to abolish CKIP-1-CK2 interactions suggesting that this motif by itself may not be sufficient to mediate interactions. Overall, these results provide novel insights into how CK2, a predominantly nuclear enzyme, is targeted to the plasma membrane, and perhaps more importantly how it may be regulated.

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1 The abbreviations used are as follows: CK2, protein kinase CK2 or casein kinase II; CKIP-1, CK2 interacting protein-1; PH, pleckstrin homology; LZ, leucine zipper; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescent protein; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; HA, the VPDY epitope of influenza virus hemagglutinin; AKAP, A-kinase anchoring protein; JIP, JNK-interacting protein; JNK, c-Jun N-terminal kinase; MSA, multiple sequence alignment; GST, glutathione S-transferase; CMV, cytomegalovirus; PKD, protein kinase D.

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but not α in yeast two-hybrid assays. Interactions between CK2 were subsequently confirmed using GST pull-down assays and co-immunoprecipitation from transfected and non-transfected cells indicating that CKIP-1 and CK2 interact under physiological conditions (37).

CKIP-1 cDNA is broadly expressed and encodes a protein with a predicted molecular mass of 46 kDa. Although its overall biological functions remain poorly understood, CKIP-1 has recently been implicated in phosphatidylinositol 3-kinase-regulated muscle cell differentiation (38). Examination of the amino acid sequence of CKIP-1 revealed the presence of an N-terminal pleckstrin homology (PH) domain, which could mediate interactions between CKIP-1 and cellular membranes or proteins, and a putative C-terminal leucine zipper, which is thought to be involved in protein-protein interactions (37). In addition, this protein contains five PX/PH motifs, two of which match the consensus sequence for phosphorylation by cyclin-dependent kinases and mitogen-activated kinases.

Based on the fact that CKIP-1 contains a number of protein-protein interaction motifs, and its discrete cellular localization, we hypothesized that CKIP-1 is a non-enzymatic regulator of CK2. To test this hypothesis we examined the interactions between CK2 and CKIP-1 in cells and in vitro. Our data demonstrate that CKIP-1 is capable of redistributing CK2 to the plasma membrane and that the PH domain of CKIP-1 is required for this activity. This demonstration provides novel insight into a possible mechanism for how CK2, a predominantly nuclear enzyme, is capable of phosphorylating proteins present at the cellular membrane.

EXPERIMENTAL PROCEDURES

Materials

Human osteosarcoma Saos-2 cells and U2-OS cells were obtained from ATCC (Manassas, VA) and were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (CanSera International, Inc.). Anti-CKIP-1 antibodies were raised in rabbits by BabCO (Richmond, CA) as previously described (37), and anti-GST monochlonal antibodies, anti-FLAG M2 monochlonal antibodies, and rhodamine-conjugated goat anti-rabbit antibodies were obtained from Sigma; horseradish peroxidase-conjugated goat antimouse antibodies were obtained from Bio-Rad. PIP-Strips were obtained from Echelon Research Laboratories Inc., and reagents for enhanced chemiluminescence (ECL) were obtained from Pierce. Plasmids encoding enhanced yellow fluorescent protein (EYPF-C1) and enhanced cyan fluorescent protein (ECFP-C1) were obtained from Clontech, encoding enhanced yellow fluorescent protein (EYFP-C1) and enhanced green fluorescent protein (EGFP-C1) were obtained from Clontech, encoding C-terminal GFP (GFP-C1) were obtained from Clontech, encoding Myc-C2β (Myc-C2β) were obtained from Clontech, encoding glutathione-S-transferase (GST) were obtained from Echelon Research Laboratories Inc., and reagents for expansion of the GST gene were obtained from New England Biolabs, Inc. pEGFP-C3 with EcoRI/SalI and inserted into EcoRI/XhoI-digested pACT2.

Plasmid Constructs

**pEYFP Construct**—Full-length CK2α was amplified out of pGBT9-CK2α using the following forward 5′-TGATGCTAATGTTCCCGGAATTCGGCTCGACCATCACGACTG-3′ and reverse 5′-CTTAGTGACTGCCGAGAGAGGAGAGTAGTG-3′ primers. The amplified product was gel-purified and ligated into the pCR-Blunt vector (Invitrogen) then digested with EcoRI and Sall. The resulting EcoRI/Sall fragment was subcloned into the EcoRI/Sall sites of pEYFP-C1.

**pECPF Construct**—Full-length CK1δ was amplified out of pGBT9-CK1δ using the following forward 5′-ACATAGTTCCCGGAATTCGGCTCGACCATCACGACTG-3′ and reverse 5′-CTTAGTGACTGCCGAGAGAGGAGAGTAGTG-3′ primers. The amplified product was gel-purified, ligated into the pCR-Blunt vector, digested with EcoRI and Sall, and subcloned into EcoRISall-digested pECPF-C1 (Clontech). A monomeric form of ECPF-C1 was constructed by incorporating an A206K mutation as described previously (39) using a sequential PCR strategy.

**pECF Construct**—Full-length CK1α encoding CKIP-1 was subcloned into the EcoRI and SalI sites of the pECPF-C3 vector. To examine the residues important for interactions with CK2, key residues within the PH domain (Lys42, Arg44, and Trp123) of CKIP-1 were mutated. Full-length cDNA encoding CKIP-1, as described previously (37), incorporated into a pEPEF vector (pRS99), was utilized as the template in a PCR reaction to amplify the DNA and incorporate specific point mutations. To begin with, W123A was incorporated into the full-length cDNA using sequential PCR. The first two PCR products for the W123A mutation were generated using the CKIP-1 forward primer 5′-TGA TCG GAA TTC CCA TGA AGA AGA ACA ATG CCG CCC AAT CCT-3′ and the W123A reverse primer with 5′-AAT GAT CCA GGA TCT CTC TCT CTC-3′, and the W123A forward primer 5′-GAA GAG AAG GAA TCG CGC ATG CCT CTC-3′ with the CKIP-1 reverse primer 5′-GAA TTC GTC GAC CCC ATG CTG CTC-3′. These products were gel-purified and added to a second PCR reaction mixture along with the CKIP-1 forward and reverse primers. The PCR product was gel-purified and ligated into pCR-Blunt. The clone was then digested with EcoRI/Sall, and the resulting fragment was subcloned into the EcoRI/Sall-digested pEFP-C3 vector to create a construct with an N-terminal GFP tag. The sequence of this clone, pMO7, was confirmed by sequencing using the pEFP-C5 primer. The K42C mutant, R44C, and K42C/R44C double mutant were made in a similar fashion, using K42C forward 5′-TTC AGG GAG ATT TGG TGT TGT ACG CAG TGG TGT-3′ and K42C reverse 5′-GCG CGG ATC AAT CCT CTC CCT-3′ primers. The resulting clones, pMO8, pMO55, and pCZ12, respectively, were confirmed by sequencing using the pEFP-C5 primer. pMO7 was used as the template to construct the double mutants containing K42C and W123A, and R44C and W123A, and the triple mutant containing K42C, R44C, and W123A. PCR was used to create point mutations utilizing either K42C or R44C primers for the double mutants, and the K42C/R44C primers for the triple mutant. The resulting clones, pMO12, pMO54, and pMO13, respectively, were verified by sequencing using the pEFP-C5 primer.

**pACT2 Constructs**—Full-length cDNA encoding CKIP-1 was amplified using the CKIP-1 forward primer and the CKIP-1 reverse primer. Primers were designed to create the number of CKIP-1 deletion mutants. CKIP-PH was constructed using the CKIP-1 forward primer and PH reverse primer (5′-GAA TTC GTC GAC TCA GGT GAT GGC AGA GAG ATT TGG TGT AAC TGC TAT GTG-3′). These products were gel-purified and ligated into pCR-Blunt. The clone was then digested with EcoRI/Sall and inserted into EcoRI/Sall-digested pACT2. The Ras membrane-targeting signal is a 19 amino acid peptide ending in CAAAX, where C = cysteine, A = aliphatic amino acid, and X = any amino acid (40). The resulting construct was designated Myc-C2β-CAAX.

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porate a HindIII site and a FLAG tag on its N terminus and a reverse primer (5′-GGC CAG AAT TAG CCT GTC TGC AGG TGC GGC CGC GTC TCC CCT CAT-3′) that added a NotI site for subcloning. The amplified PCR product was gel-purified, ligated into pCR-Blunt, then digested using HindIII/NotI and subcloned into HindIII/NotI-digested pR/CMV. To clone into FLAG-pR/CMV, CKIP-1 PH domain mutants were amplified out of the pEGFP-C3 vector using FLAG-pR/CMV forward, 5′-AGC GTA AAT TTC TTT GAA AAG AAG CAA TCT CAC-3′ and FLAG-pR/CMV reverse, 5′-CCG GGT ACC GTG CCG CCG CTC TCG CCA TCA CAT C-3′, primers and ligated into pCR-Blunt, before subcloning into EcoRI/NotI-digested FLAG-pR/CMV vector. The sequence of each clone, pMO41 (R42), pMO58 (R44C), pMO40 (W123A), pMO60 (KR), pMO42 (KW), pMO39 (RW), and pMO43 (triple mutant), was confirmed by sequencing.

**Myristoylation Signal Constructs**—A myristoylation sequence from v-Src (41), MSGSSKRPFQPSQR, was used to replace the PH domain at the N-terminal of CKIP-1 using the following myristoylation primer, 5′-AAG GAA GTA TTC ATG GTG AGC AGA ACG ACG CAC GAT GCC ACC CAG CGA GCC AAC AAG CGT ATC ATG GAG GTC AC-3′. To subclone myristoylated CKIP-1 into FLAG-pR/CMV, EGFP-CKIP-1 was used as a template for PCR using the myristoylation primer and FLAG-pR/CMV reverse primer. The PCR product was gel-purified, ligated into pCR-Blunt, digested with EcoRI/NotI, ligated into EcoRI/NotI-digested FLAG-pR/CMV vector and sequence verified, resulting in clone pMO47. Similarly, a construct lacking the FLAG tag was generated by amplifying a pR/CMV Blunt clone with HindIII/Apal, and then subcloning into HindIII/Apal-digested pR/CMV. The resulting clone, pMO48, was verified by sequencing.

**pGBT9 Constructs**—Full-length cDNAs encoding CK2α or CK2α were previously subcloned into the BamHI site of the pGBT9 vector, and orientation was sequence-verified (37). To prepare constructs incorporating mutations in a putative HIKE domain, full-length CK2α and CK2α were amplified from the pGBT9 vector by PCR using the pGBT9 forward primer, 5′-CTA GAA ACA GTG CCA CAG CTA TTT-3′, with the CK2α (5′-CTT TCT GTG ATG ATC ATA CAT GAC-3′) or CK2α (5′-CTT TCT GTG ATG ATC ATA CAT GAC-3′) HIKE domain primers, and using pGBT9 reverse primer, 5′-GTC ATG ACG ACA GAG TCA TTA-3′ or CK2α (5′-GTC ATG ACG ACA GAG TCA TTA-3′) or CK2α (5′-GTC ATG ACG ACA GAG TCA TTA-3′) and were subjected to SDS-polyacrylamide gel electrophoresis. Proteins that were bound to GST or GST-CK2α, generated using pTNT S. E. coli and purifying on glutathione-agarose as described previously (31), were verified by sequencing.

**Transfection of Saso-2 and U2-OS Cells**

U2-OS and Saso-2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen). Cells were grown to 80% confluence, harvested and resuspended in complete medium lacking Trp and Leu (Trp-Leu-), and on synthetic complete medium lacking Trp and Leu (Trp-Leu-). Precipitates were left on the cells for 16 h, washed once with PBS, and then incubated for 1 h with rhodamine-conjugated goat anti-rabbit antibodies (1/1000 dilution). Finally, cells were washed twice with PBS, and then mounted in AirVol. For non-confocal images, EYFP and rhodamine fluorescence were visualized using a Zeiss LSM 410 or Zeiss LSM 510 Meta inverted confocal microscope. The images were pseudocolored using Adobe Photoshop. For confocal images, EGFP, EYFP, and rhodamine fluorescence were visualized with a Zeiss LSM 410 or Zeiss LSM 510 Meta inverted confocal microscope. The images were pseudocolored using built-in Zeiss LSM software, and transferred into Adobe Photoshop. In all cases, controls were used to ensure that the settings used for microscopy eliminated bleed-through between channels.

**Transformation and Maintenance of Yeast**

pGBT9-C2K and pACT2-CKIP-1 constructs (including mutants of CK2 and CKIP-1) were transformed into the yeast strain BY47–4 (MATa, his3 –112, thr1 –1, IIP25–1, leu2 –3, ura3 –52, gal1–1, gal2–1, ade2–1, ade1–1, his3–1, lys2–2, his4–0, trp1–1) using the method of Schiestl and Gietz (43). Transformations were plated on synthetic complete medium lacking Trp and Leu (Trp-Leu-) and on synthetic complete medium lacking His, Ade, Trp, and Leu (His-Ade-Leu-Trp-). Pure transformants were selected at 30°C and were grown on Trp-Leu- used as a measure of transformation efficiency, whereas growth on His-Ade-Leu-Trp- was an indication of positive interactions between the pGBT9 and pACT2 constructs used in the transformation.

**GST Pull-downs and in vitro Binding Assays**

Glutathione S-transferase (GST) fusion proteins encoding full-length CKIP-1, CKIP-1 deletion mutants (LZ, ΔPH, etc.), CK2α or GST itself were expressed in BL21(DE3) bacteria and purified using glutathione-agarose as described previously (31) and left on the beads for use in GST pull-down assays or in vitro binding studies. GST pull-down assays were performed by incubating the purified proteins with whole cell lysates from CK2α-HA-transfected U2-OS cells for 1 h at 4°C. Alternatively, in vitro binding assays were performed by incubating 11 μl of GST or GST-CK2α beads in 10 μl of Nonidet P-40 buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40) with 5 μl of labeled FLAG-CKIP-1, and FLAG-CKIP-1 PH domain mutants produced using a TNT kit (Promega) with T7 polymerase according to the manufacturer's recommendations and incubating for 1 h at 4°C. Following incubation, glutathione-agarose was collected by centrifugation, supernatant was removed, and the beads were washed four times with interaction buffer or Nonidet P-40 buffer, respectively. Proteins remaining on the glutathione-agarose were eluted by the addition of Laemmli sample buffer and were subjected to SDS-polyacrylamide gel electrophoresis. Proteins from GST pull-down assays were transferred to a polyvinylidene difluoride membrane, and the presence of CK2α-HA was confirmed by Western blot with anti-HA antibodies. In the case of the in vitro binding assays, radiolabeled proteins from three independent experiments were visualized using a phosphorimager (Amersham Biosciences), and quantitated using ImageQuaNT software (Amersham Biosciences) to determine the percentage of total radiolabeled product that was bound to the GST fusion proteins. The average percentage of total radiolabeled protein that was bound to GST or GST-CK2α ≥ 5.0 is displayed in a graphical format.

**Phospholipid Binding Assay**

GST fusion proteins encoding full-length CKIP-1, as well as each of the CKIP-1 PH domain mutants, and GST alone were expressed in BL21(DE3) bacteria and purified using glutathione-agarose as described previously (31). Purified GST fusion proteins were used in aoverlay assay with a nitrocellulose membrane spotted with various phospholipids (44, 45) (PIP-Strip, Echelon Research Laboratories Inc.) with the protein at a final concentration of 0.5 μg/ml as directed by the manufacturer. Anti-GST monoclonal antibodies and horseradish peroxidase-conjugated goat anti-mouse antibodies were used along with ECL.
to determine the spectrum of phospholipid binding. Tris-buffered saline with 3% fatty acid-free bovine serum albumin (Sigma) was used to block the PIP-Strip and was also used in the antibody mixtures.

RESULTS

CKIP-1 Recruits CK2 to the Plasma Membrane—Based on previous results (37, 46), we hypothesized that CKIP-1 could be a non-enzymatic regulator of CK2 involved in recruitment of CK2 to specific cellular locations. To test this hypothesis, we examined the ability of CKIP-1 to alter the distribution of CK2 within cells using CK2α as an EYFP fusion and CKIP-1 as an ECFP fusion. Constructs encoding each of these fusions were transfected into human osteosarcoma Saos-2 cells and analyzed by fluorescence microscopy (Fig. 1). As expected, EYFP-CK2α displays mainly nuclear localization with some cytoplasmic staining (Fig. 1, top left panel). By comparison, ECFP-CKIP-1 is localized to the plasma membrane and/or membrane ruffles with additional cytoplasmic staining (Fig. 1, top right panel). When EYFP-CK2α was expressed in cells together with ECFP-CKIP-1, a notable alteration in the distribution of EYFP-CK2α is observed with an apparent increase in the amount of EYFP-CK2α in the cytoplasm and some of the EYFP-CK2α localized on the membrane (Fig. 1, bottom left panel). Examination of the ECFP-CKIP-1 in the same cells (Fig. 1, bottom right panel) revealed that the re-localized CK2α present on the plasma membrane was co-localized with ECFP-CKIP-1. A similar redistribution of EYFP-CK2α in cells expressing ECFP-CKIP-1 was observed in human osteosarcoma U2-OS cells examined by confocal microscopy (data not shown). Furthermore, the redistribution of EYFP-CK2α to the plasma membrane is unaffected when a dimerization-defective mutant of ECFP (39) is used for the expression of ECFP-CKIP-1 indicating that this redistribution does not result from dimerization of EYFP and ECFP fusion. Overall, the differences observed in CK2α localization in the presence and absence of CKIP-1 indicates that CKIP-1 possesses the ability to re-localize CK2α in vivo.

Comparison of the Re-localization of CK2 to the Membrane by CKIP-1 and CK2β-CAAX—Results illustrated in Fig. 1 demonstrate re-localization of CK2α to the plasma membrane by CKIP-1. However, it is evident that a major proportion of CK2α was still localized within the nucleus. Accordingly, to examine the full extent to which CK2α can be re-localized to the plasma membrane, we devised a strategy to examine the ability of a membrane-targeted version of CK2β, an integral component of tetrameric CK2 complexes, to recruit CK2α to the membrane. In this respect, we constructed a mutant of CK2β designated Myc-CK2β-CAAX with a C-terminal CAAX motif that is isoprenylated in cells to direct membrane localization and an N-terminal Myc-tag to permit detection (40). Initial immunoprecipitation studies demonstrated that Myc-CK2β-CAAX is not impaired in its ability to interact with the catalytic CK2 subunits (data not shown). Indirect immunofluorescence indicates that Myc-CK2β-CAAX is localized primarily to the plasma membrane (Fig. 2A) with some cytoplasmic staining. Occasionally, this mutant exhibited nuclear staining in some cells (not shown). To compare the localization of CKIP-1 to that of Myc-CK2β-CAAX using indirect immunofluorescence, we constructed a variant of CKIP-1 with an N-terminal FLAG epitope. Indirect immunofluorescence (Fig. 2A) demonstrates that FLAG-CKIP-1 localizes to the plasma membrane and/or membrane ruffles with some cytoplasmic staining (Fig. 2A) in a manner identical to that seen with ECFP-CKIP-1. Staining with either anti-Myc or anti-FLAG was absent in non-transfected cells (Fig. 2A, top panels). To compare the abilities of Myc-CK2β-CAAX and CKIP-1 to re-localize CK2 to the plasma membrane, U2-OS cells were transfected with EYFP-CK2α in the presence or absence of either Myc-CK2β-CAAX or FLAG-CKIP-1 and EYFP fluorescence visualized by confocal microscopy. When EYFP-CK2α was co-transfected with Myc-CK2β-CAAX or with FLAG-CKIP-1, a proportion of the EYFP-CK2α could be visualized at the plasma membrane (Fig. 2B, middle and right panels, respectively), which was not evident when EYFP-CK2α was expressed by itself (Fig. 2B, left panel). It is also evident with Myc-CK2β-CAAX (Fig. 2B, middle panel), as is the case with FLAG-CKIP-1 (Fig. 2B, right panel) and with ECFP-CKIP-1 (Fig. 1), that a major proportion of CK2 is retained within the nucleus. The latter observation indicates that, even with an integral component of CK2 complexes, it is only possible to re-localize a fraction of the total cellular CK2, an observation that likely reflects the complex regulation of CK2 and its interactions with a large number of cellular proteins. In relation to Myc-CK2β-CAAX, it is therefore apparent that CKIP-1 is nearly as effective at eliciting the re-localization of CK2α to the plasma membrane.

To confirm that the fluorescent proteins were located at the membrane, cells were treated with digitonin to permeabilize the cells and release their soluble cytoplasmic contents without releasing membrane-associated proteins. U2-OS cells were transfected with EYFP-CK2α in the presence or absence of either Myc-CK2β-CAAX or FLAG-CKIP-1 and incubated with, or without digitonin, prior to visualization of EYFP-CK2α by fluorescence microscopy (Fig. 2C). Digitonin treatment (Fig. 2C, bottom left panel) results in the complete loss of EYFP-CK2α indicating that EYFP-CK2α by itself is not membrane-associated. By comparison, a significant proportion of the
EYFP-CK2α was retained at the cell membrane following digitonin treatment of cells that were co-expressing EYFP-CK2α with either Myc-CK2β-CAAX or FLAG-CKIP (Fig. 2C, bottom middle and bottom right panels). These results validate the membrane localization observed without digitonin treatment and reinforce the conclusion that CKIP-1 re-localizes CK2.

Elucidation of Domains on CKIP-1 Responsible for Interactions with CK2—To understand the mechanistic basis for interactions between CK2 and CKIP-1 we performed yeast two-hybrid assays to identify the domain(s) on CKIP-1 responsible for interactions with CK2. Constructs encoding full-length CKIP-1 and different CKIP-1 deletion mutants fused to the GAL4 activation domain in the pACT2 plasmid (Fig. 3A) were each co-transformed into yeast with GAL4 binding domain fusions of either CK2 or CK2α in the pGBT9 plasmid. Each of the co-transformations into the PJ69-4a yeast strain was plated on both Trp-Leu-, to monitor transformation efficiency, and His-Ade-Leu-Trp-, to examine interactions. Positive inter-
CKIP-1 is indicated as is the putative leucine zipper (LZ) from residues 347-373. Plasmids encoding CKIP-1 or CK2LZ/H9251 are important for interactions with CK2. The possibility that the membrane localization itself may be involved in membrane localization, we decided to investigate because PH domains are necessary but, on its own, is not sufficient for interactions with CK2. To confirm the results of the yeast two-hybrid studies, indicating that the PH domain of CKIP-1 is protected when CKIP-1 was co-transformed with pACT2-CKIP-1, CK2 interacts with GST-CKIP-PH. Overall, the results concur with yeast two-hybrid studies, suggesting that the PH domain of CKIP-1 is required for interactions with CK2.

**Fig. 3. The PH domain of CKIP-1 is required for interactions with CK2.** A, to examine the interactions between CK2 and CKIP-1, and CKIP-1 with itself, cDNA encoding full-length CKIP-1 was expressed in yeast as a fusion with the DNA activation domain (AD) of GAL4, using the plasmid pACT2 as described under “Experimental Procedures.” Constructs encoding GAL4 DNA activation domain fusions encoding deletion mutants of CKIP-1 (LZ, PH, ΔLZ, ΔPH, and ΔPH ΔLZ, as indicated) were also prepared. The pleckstrin homology (PH) from residues 21-132 of CKIP-1 is indicated as is the putative leucine zipper (LZ) from residues 347-373. Plasmids encoding CKIP-1 or CK2a as GAL4 DNA binding domain fusions were co-transformed into yeast along with pACT2 constructs encoding wild type CKIP-1 or its various deletion mutants. Positive interactions were indicated by the ability of transformants to grow on synthetic complete media deficient in His, Ade, Leu, and Trp (designated -HALT). B, GST pull-down assays were performed on whole cell lysates from CK2a-HA-transfected U2-OS cells. GST, GST-CKIP-1, GST-CKIP-LZ, GST-CKIP-PH, GST-CKIP-ΔLZ, GST-CKIP-ΔPHΔLZ, and GST-CKIP-ΔPH, were used in pull-down assays on the whole cell lysates. Proteins bound to GST-fusion proteins were eluted from beads using Laemmli sample buffer. CK2a-HA was detected on immunoblots using anti-HA antibodies and enhanced chemiluminescence.

The PH Domain of CKIP-1, Not Its Membrane Localization, Is Required for CK2 Re-localization—Because PH domains are involved in membrane localization, we decided to investigate the possibility that the membrane localization itself may be important for interactions with CK2α in vivo, rather than the PH domain. Therefore, the PH domain of CKIP-1 was replaced with a myristoylation signal (MGSSKSKPKDPSQR) to target CKIP-1 to the cell membrane in the absence of its PH domain (41). Confocal microscopy (Fig. 4A) demonstrates that FLAG-CKIP-1 and Myr-CKIP exhibit identical distribution when expressed in Saos-2 displaying primary localization on the cell membrane/ruffles together with some cytoplasmic staining.

The ability of FLAG-CKIP-1 or FLAG-Myr-CKIP to interact directly with CK2α was then tested in vitro employing pull-down assays using GST or GST-CKIP as affinity matrices with radiolabeled FLAG-CKIP-1 (50 kDa) or FLAG-Myr-CKIP (42 kDa) that were obtained by in vitro transcription and translation (Fig. 4B). Approximately 50% of FLAG-CKIP-1 was retained on GST-CK2α with negligible amounts (<0.5%) retained on GST alone (Fig. 4B, left panel). Negligible amounts (i.e. <0.5%) of FLAG-Myr-CKIP were retained on either GST-CK2α or GST beads (Fig. 4B). These results indicate that the PH domain of CKIP-1 is required for its interactions with CK2. To extend these findings, we examined the ability of CKIP-1 to interact with CK2α in vivo (Fig. 4C). Saos-2 cells were cotransfected with EYFP-CK2α and either ECFP-CKIP-1 or Myr-CKIP, and the subcellular localization of EYFP-CK2α was examined by confocal microscopy. As with the experiments shown elsewhere (Figs. 1 and 2) expression of CKIP-1 resulted in re-localization of some CK2α to the cell membrane (indicated by arrows, Fig. 4C). However, when EYFP-CK2α was expressed with Myr-CKIP, CK2α was found solely in the nucleus.
of the cells. Overall, these results demonstrate that, although FLAG-CKIP-1 and Myr-CKIP exhibit the same subcellular distribution, the presence of the PH domain itself is required for interactions with CK2.

Evaluation of the Role of a Potential HIKE Domain in CK2—As reported previously, the unique C-terminal of CK2 is not responsible for interactions between CK2 and CKIP-1 but, rather, a portion of the N-terminal that is highly related to CK2/H9251 (37). It is conceivable that the interaction between the two lies in a region of the N-terminal of CK2 exhibiting non-identity to CK2/H9251. Closer examination of amino acid sequences of the N termini of the CK2 catalytic subunits revealed a potential HIKE domain present in CK2/H9251 (Fig. 5A). HIKE is a highly conserved sequence motif, as shown in Fig. 5A, that selectively occurs in proteins that bind PH domains (47). This motif was originally identified by sequence homology analysis of the proteins considered the strongest PH binding candidates, such as PKC and Akt (48). A conserved β strand-loop-β strand structure is exhibited by HIKE, despite the fact that the proteins in which they are found have widely different three-dimensional structures (48). There is a highly conserved acidic residue 8 amino acids downstream from the most C-terminal conserved Lysine. It is this conserved acidic residue, present in CK2α but not CK2α' (Fig. 5A), at which mutagenesis was directed to evaluate the domain. The resulting constructs were co-transformed along with pACT2-CKIP-1 into the PJ69-4a yeast strain and subjected to yeast two-hybrid analysis. It was found that the E167Q mutation in the HIKE domain of CK2/H9251 was not sufficient to abolish interactions between CKIP-1 and CK2/H9251, nor was the Q168E mutation in CK2/H9251/H11032 sufficient to promote interactions with CKIP-1 (Fig. 5B). These results suggest that residues other than the mutated residue contribute to the interaction between protein kinase CK2 and CKIP-1, reflecting the complexity of the HIKE domain.

Multiple Sequence Alignment of the PH Domain of CKIP-1 with Other PH Domains—PH domains are found in over 70 proteins involved in signal transduction and cytoskeletal structures (49) and are thought to have a role in either membrane localization and/or protein-protein interactions (37, 50, 51). Multiple sequence alignments of PH domains indicate that the domain itself is a series of rather poorly conserved peptides, ~120 amino acids in length, containing a nearly invariant Trp
residue in the C-terminal 15 amino acids (51), and a fairly conserved "+X+" motif (52). A multiple sequence alignment (MSA) was performed on the PH domain of CKIP-1, along with representative PH domains from established PH domain classes. The alignment illustrated in Fig. 6A shows that the domain itself is not highly conserved. A phylogenetic tree was then drawn from a MSA containing nine different PH domains. The branch lengths serve as a basis of the evolutionary conservation between items on the tree. As shown in Fig. 6B, there is not a high degree of conservation between PH domains, and CKIP-1 does not show high conservation to any PH domain in the given alignment. Overall, the results demonstrate the poor sequence identity and large amount of diversity exhibited by PH domains and make it evident that the PH domain of CKIP-1 is distinct from the known classes of PH domains.

**Fig. 5.** Evaluation of a potential HIKE domain in CK2α. A, alignment of a portion of the amino acid sequence of CK2α and CK2α' showing residues conserved between the two catalytic subunits. The consensus sequence for the HIKE domain, which is found in a number of proteins known to bind PH domains (47) is also shown. HIKE residues in CK2α and CK2α' are underlined and indicated in bold. B, mutations were made in the potential HIKE domain of CK2α and/or CK2α' as described under "Experimental Procedures." A yeast two-hybrid assay was performed using constructs in which the potential HIKE domain was mutated and expressed in yeast as a fusion with the DNA binding domain (BD) of GAL4, using the plasmid pGBT9 as described under "Experimental Procedures." CKIP-1 was co-transformed as a fusion with the GAL4 DNA activation domain. Positive interactions were indicated by the ability of transformants to grow on synthetic complete media lacking His, Ade, Leu, and Trp (-HALT).
**Fig. 6.** Multiple sequence alignment of the PH domain of CKIP-1 with other well known PH domains. A. a multiple sequence alignment was done using ClustalW (version 1.82, available at www.ebi.ac.uk/clustalw/). The PH domain of CKIP-1 and representative PH domains from the known classes of PH domains were aligned using default parameters. The representative proteins from each grouping of PH domains were as follows: Bruton’s tyrosine kinase (group 1), the N-terminal PH domain of Pleckstrin (group 2), Akt-2 (group 3), and Dynamin-1 (group 4). Acidic residues are shown in blue, basic residues in magenta, small hydrophobic residues in red, and residues that contain hydroxyls and amines and are basic are shown in green. The consensus symbols are as follows: ‘*’ indicates that the residue is identical in all sequences in the alignment, ‘:’ indicates that conserved residues have been observed according to color designations, and ‘.’ indicates that semi-conserved substitutions are observed. Overall, the alignment of the PH domains shows a very poorly conserved domain. B. a phylogenetic tree was drawn for...
To determine whether membrane localization exhibited any correlation with phospholipid binding, we examined the effects of these CKIP-1 PH domain mutations on binding to various phosphoinositols using overlay assays performed with PIP strips (44, 45). Accordingly, GST fusion proteins of CKIP-1 and each of the CKIP-1 PH domain mutants were expressed in bacteria. Each of the GST fusion proteins was expressed to comparable degrees (Fig. 7B). As illustrated in Fig. 7C with representative data from the PIP strip assays, it is evident that GST-CKIP-1 exhibits broad spectrum of binding. The GST-CKIP-K42C mutant shared the same broad spectrum of binding; however, the K42C mutant bound the phosphoinositols to a lesser degree (Fig. 7C) than was observed for GST-CKIP-1. The R44C and K42C/R44C double mutant showed similar binding to that observed with the K42C mutant (data not shown), again to a weaker extent than GST-CKIP-1. All mutants with a variety of proteins containing PH domains, including the proteins shown in the MSA shown in panel A, using NJ Plot (www.phil.univ-lyoulu.fr/njplot.html). Also included in the phylogenetic tree are β-ARK-1 (group 2), the C-terminal PH domain of pleckstrin (unclassed), and the N-terminal (group 1) and C-terminal PH domains (group 4) of TIAM1. Phylograms are branching tree diagrams assumed to be an estimate of phylogeny, with branch lengths being proportional to the amount of inferred evolutionary change. The phylogram shown here demonstrates the uniqueness of the PH domain CKIP-1 from other known PH domains.
the W123A substitution, including the K42C/R44C/W123A mutant (Fig. 7C) and W123A, K42C/W123A, and R44C/W123A mutants (data not shown) exhibited a complete loss of phospholipid binding (Fig. 8C). These observations concur with the cellular localization observed by confocal microscopy. Overall, these results suggest that the highly conserved tryptophan residue is important for the integrity of the entire domain. Mutation of this residue to alanine causes the protein to no longer be associated with the plasma membrane, with a corresponding loss of phospholipid binding.

Examination of Critical Residues in CKIP-1 Important for Interactions with CK2—Based on the differences in subcellular localization and phospholipid binding of the PH domain mutants of CKIP-1 we were interested in examining their effect on the ability of CKIP-1 to interact with CK2α. As a means of achieving this objective, representative FLAG-tagged CKIP-1 mutants (K42C, W123A, and/or R44C/W123A) were co-transfected into U2-OS cells with EYFP-CK2α. As illustrated in Fig. 8A (middle left panel), FLAG-CKIP-K42C caused re-localization of a proportion of CK2α to a degree similar to that observed with wild-type FLAG-CKIP-1 (upper right panel). By comparison, CK2α was not observed at
the plasma membrane when co-transfected with the W123A, K42C/W123, or K42C/R44C/W123A mutants, an observation consistent with the failure of these mutants to localize to the plasma membrane.

To complement the localization studies, we examined the ability of the PH domain mutants of CKIP-1 to interact directly with CK2α using GST pull-down assays with radiolabeled translation products of each of the PH domain mutations prepared by *in vitro* transcription and translation. Representative autoradiograms of these pull-down assays are shown in Fig. 8B with graphical representations illustrated in Fig. 8C. The K42C and K44C mutants both retained the ability to interact directly with CK2α *in vitro* (Fig. 8, B and C) to the same extent as wild-type CKIP-1. By comparison, all CKIP-1 mutants harboring a W123A substitution, including W123A, K42C/W123A, R44C/W123A, and K42C/R44C/W123A displayed a marked reduction of at least 70% in binding to CK2α as compared with wild-type CKIP-1.

Taking into account the results demonstrated in Figs. 7 and 8 (localization studies, phospholipid binding, and *in vitro* and *in vivo* interactions with CK2α), it is apparent that the CKIP-1 PH domain mutations can be grouped into two classes. First, there are the mutations (K42C and/or R44C) that are still localized to the plasma membrane, retain phospholipid binding, and retain interactions with CK2α both *in vitro* and *in vivo* to the same extent as is seen with wild type CKIP-1. Second, there are the mutations (W123A by itself or in combination with K42C and/or R44C) that are no longer localized to the plasma membrane, have lost phospholipid binding, and have diminished interactions with CK2α both *in vitro* and *in vivo*. Collectively, these results demonstrate the importance of the integrity of the PH domain of CKIP-1 not only for the plasma membrane localization of CKIP-1, but also for interactions with CK2α and recruitment of CK2 to the plasma membrane.

**DISCUSSION**

In this study, we report the investigation of the interaction between protein kinase CK2 and CKIP-1 together with a functional analysis of CKIP-1. As mentioned previously, CKIP-1 has a number of protein-protein interaction motifs. Based on the presence of these motifs and the discrete cellular localization of CKIP-1, we hypothesized that CKIP-1 is a non-enzymatic regulator of CK2. In this respect, one can envisage the possibility that CKIP-1 functions in a manner analogous to the AKAP family of proteins. Protein kinase A anchoring proteins (AKAPs) bind the regulatory subunit of cAMP-dependent protein kinase (protein kinase A) to direct the kinase to discrete intracellular locations (57–60). By binding to additional signaling molecules, AKAPs function to coordinate multiple components of signal transduction pathways. CK2, like protein kinase A, has multiple substrates in a variety of cellular locations, and to date, it is unclear how subpopulations of CK2 are targeted to specific cellular locations (1, 61). CKIP-1 has domains such as a PH domain and putative LZ that make it ideal as a potential targeting molecule. The possibility also exists that CKIP-1 could act as a scaffold/adaptor protein, similar to the JIPs (JNK-interacting proteins), that coordinates signal transduction events involving CK2α and distinct signaling pathways. JIPs were previously identified as scaffolding proteins in the JNK (c-Jun NH2-terminal kinase) signaling pathway (62, 63) but were recently found to bind directly to kinesin (64). JIPs act as a linker between kinesin and its cargo, allowing kinesin to transport many different cargoes and to concentrate and respond to signaling pathways at certain sites within the cell (64–66).

The recruitment of CK2α to the plasma membrane by CKIP-1 may bring the CK2 tetramer or CK2α itself into complexes requiring kinase activity, thereby leading to specific phosphorylation events. In this respect, CK2 phosphorylates a broad range of cellular proteins (61), which includes substrates located at the plasma membrane such as spectrin (24), the insulin receptor (67), caveolin (28), IGF-II receptor (68), dynamin (25), and IRS-1 (69). Although it remains to be determined how the phosphorylation of each of these specific proteins by CK2 is regulated, this present study with CKIP-1 may provide a novel paradigm for understanding how CK2 can phosphorylate targets that are localized at specific sites within cells.

Our results from co-localization studies on CKIP-1 and CK2α indicate that CKIP-1 is capable of re-localizing a proportion of the cellular CK2 to the plasma membrane in both U2-OS and Saos-2 cells (Figs. 1 and 2). Although it was apparent that CKIP-1 could recruit CK2 to the plasma membrane, the majority of the CK2 was retained in the nucleus. Consequently, we performed studies to examine the amount of CK2α that can be re-localized to the membrane by CK2β, an integral CK2 subunit to which a membrane localization signal had been fused. These studies demonstrated that, as was the case with CKIP-1, Myc-CK2β-CAAX is capable of re-localizing only a small fraction of the CK2 to the plasma membrane. In fact, in relation to Myc-CK2β-CAAX, it is apparent that CKIP-1 is nearly as effective at eliciting the re-localization of CK2α to the plasma membrane. This inability of even an integral component of the CK2 complex to re-localize the entire population of CK2α may be due in part to the complex regulation of CK2 as well as the diverse and dynamic nature of its interactions within cells (70, 71).

It is currently unknown whether the interaction between CKIP-1 involves only the catalytic CK2α subunit, or the entire CK2 tetramer. In addition, it becomes clear that CKIP-1 interacts with CK2α through a region of the N-terminal 330 amino acids, and not the C-terminal, which is unique from CK2α’. It should be noted that protein phosphatase 2A also interacts with CK2α but not CK2α’. Similarly, in this case the residues critical for interactions between protein phosphatase 2A and CK2α also lie within the N-terminal of CK2α (21), where there is a region of non-similarity between the two catalytic subunits.

In an effort to characterize interactions between CK2 and CKIP-1, we focused our attention on a putative HIKE domain in CK2α. This potential HIKE domain was identified in the C-terminal of CK2α in an area where there is non-identity between α and α’ (Fig. 5A). HIKE is a highly conserved sequence motif that selectively occurs in proteins that bind pleckstrin homology domains (47, 48). HIKE was identified in strong PH-binding candidates such as Gp, protein kinase C, and Akt. HIKE also encompasses a consensus sequence for phosphoinositide binding, specifically charged residues and their spacing appear highly conserved between HIKE and PI binding sequences (48). The HIKE motif in different proteins shares a β strand-loop-β strand structure despite the widely different three-dimensional structures displayed by these proteins, with the angle between the two β strands varying considerably in amplitude, suggesting that this may contribute to PH-domain binding specificity (47, 48). In keeping with these features, the potential HIKE domain identified in CK2α is in a region of a β strand-loop-β strand structure. Initial studies on the HIKE domain of CK2α were performed by swapping a residue that fits the HIKE motif with the residue found in CK2α’, and vice versa. These constructs were utilized in yeast two-hybrid studies (Fig. 5B), however this residue alone was found not to be responsible for the differences seen between CK2α and α’ and their interaction with CKIP-1. These results suggest that this
particular residue of the putative HIKE domain is not solely responsible for the interaction between protein kinase CK2 and CKIP-1 and that residues other than this one are likely to be involved in the interaction between CK2 and CKIP-1. Overall, the observed results reflect the complexity of the HIKE domain.

Studies on the PH domain of CKIP-1 have revealed that the domain itself is unique from other PH domains classified on the basis of phosphoinositide binding. From the experiments shown in Fig. 7, we conclude that CKIP-1 exhibits a very broad spectrum of binding to phosphoinositols (Fig. 7C). CKIP-1 also shows uniqueness from other PH domain in the MSA and phylogenetic tree (Fig. 6). By comparison, a recent report, implicating CKIP-1 in muscle cell differentiation (38), showed a more specific phospholipid binding spectrum for CKIP-1 and suggested that its membrane localization was dependent on cell stimulation. We do not have a precise explanation for the apparent discrepancies. However, it is noteworthy that we find that CKIP-1 is consistently localized to the plasma membrane even in the absence of stimulation when introduced into cells. This latter observation is consistent with its broad spectrum phosphoinositide binding (Fig. 7). Polyphosphoinositide binding is a property shared by most, if not all PH domains, however, in only very few cases is phosphoinositide binding by PH domains of high affinity and specificity (72–74). By far, the majority of PH domain/phosphoinositide interactions reported thus far are of low affinity and display little or no stereospecificity (72). Although “high affinity” PH domains can function independently as signal-related membrane-targeting modules, the functions of “low affinity” PH domains is less clear, although evidence exists supporting the importance of these PH domains and a role for their weak interactions (72). It has also been suggested by Lemmon et al. (72) that the weak phosphoinositide binding by a PH domain may cooperate with interactions mediated by an entirely separate domain within the same protein, as is seen with Tiam-1 (75). The domains that cooperate in membrane association may be in different proteins, and the regulation of oligomer formation could control the membrane targeting, as suggested for dynamin (72, 76).

In conclusion, we have demonstrated that CKIP-1 is capable of redistributing a proportion of CK2α to the plasma membrane. As noted earlier, the PH domain of CKIP-1 was found to be responsible for the interaction between CK2α and CKIP-1, but on its own is not sufficient. Replacement of the PH domain with a myristoylation signal results in the inability of CKIP-1 to recruit CK2α to the plasma membrane and causes a loss of interaction between the two in vitro. Based on these findings we speculate that CKIP-1 is acting as a non-enzymatic regulator of CK2, with the ability of sequestering a fraction of the CK2 to the plasma membrane. CKIP-1 may also function as an adaptor protein that integrates signals from CK2 to other signaling molecules or possibly to mediate interactions between CK2α and other proteins that could be targets of CK2. Overall, this study has provided a means of how the predominantly nuclear CK2 can have targets at the plasma membrane and provided novel insights into the complex regulation of CK2.

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The Pleckstrin Homology Domain of CK2 Interacting Protein-1 Is Required for Interactions and Recruitment of Protein Kinase CK2 to the Plasma Membrane
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