The catalytic activity and intracellular localization of protein kinase C (PKC) are both highly regulated in vivo. This family of kinases contains conserved regulatory motifs, i.e., the C1, C2, and HR1 domains, which target PKC isozymes to specific subcellular compartments and restrict their activity spatially. *Saccharomyces cerevisiae* contains a single PKC isozyme, Pkc1p, which contains all of the regulatory motifs found in mammalian PKCs. Pkc1p localizes to sites of polarized growth, consistent with its main function in maintaining cell integrity. We dissected the molecular basis of Pkc1p localization by expressing each of its domains individually and in combinations as green fluorescent protein fusions. We find that the Rho1p-binding domains, HR1 and C1, are responsible for targeting Pkc1p to the bud tip and cell periphery, respectively. We demonstrate that Pkc1p activity is required for its normal localization to the bud neck, which also depends on the integrity of the septin ring. In addition, we show for the first time that yeast protein kinase C can accumulate in the nucleus, and we identify a nuclear exit signal as well as nuclear localization signals within the Pkc1p sequence. Thus, we propose that Pkc1p shuttles in and out of the nucleus and consequently has access to nuclear substrates. Surprisingly, we find that deletion of the HR1 domain results in Pkc1p localization to the mitotic spindle and that the C2 domain is responsible for this targeting. This novel nuclear and spindle localization of Pkc1p may provide a molecular explanation for previous observations that suggest a role for Pkc1p in regulating microtubule function.

The protein kinase C (PKC) family of serine/threonine protein kinases is involved in a multitude of signal transduction pathways. At least 10 isotypes of mammalian PKCs have been identified, in addition to two PKC-related kinases (31). The catalytic domains of these enzymes are highly related, and they display little substrate specificity in vitro. However, in vivo each PKC isotype seems to serve distinct functions (9). This isotype specificity is largely determined by multiple regulatory domains contained within each PKC and is located upstream of the catalytic domain. These regulatory domains provide specificity, first by recognizing distinct physiological activators, such as phosophatidylserine, Ca$^{2+}$, diacylglycerol (DAG), and lipid mediators, and second by mediating selective translocation of PKC isoforms to distinct cellular compartments in response to different physiological stimuli (53). Translocation of PKC also results in localized activation of the kinase, as engagement of regulatory domains on the membrane causes release of an autoinhibitory motif from the active site of the enzyme, allowing substrate binding and catalysis (37).

The presence and function of regulatory domains varies among different PKC isoforms. For example, the C1 regulatory domain, found in classical PKCs (cPKCs; α, βI, βII, and γ isoforms) and novel PKCs (nPKCs; δ, ε, η, and θ), consists of two cysteine-rich domains arranged in zinc finger motifs that bind to phorbol ester and diacylglycerol (19, 56). However, a single cysteine-rich domain is present on atypical isoforms (aPKC; λ/α and ζ), and a C1 domain is missing in PKC-related kinases (PRKs); consequently, these isoforms are unresponsive to phorbol esters. Similarly, the C2 domain was initially shown to confer Ca$^{2+}$-dependent phospholipid binding to cPKC members (4), but Ca$^{2+}$-independent nPKCs, aPKCs, and PRKs also contain C2 motifs that fail to bind Ca$^{2+}$ (45). Finally, the HR1 domain is present only in PRK isoforms and has been shown to bind Rho-type GTP-binding proteins (30, 52). Thus, the functional and structural heterogeneity of regulatory domains in PKC isoforms confers specific regulation to these otherwise highly homologous kinases.

In addition, the C1, C2, and HR1 regulatory domains mediate the translocation of PKC to specific intracellular compartments in response to different stimuli (31, 33, 53). For example, the C1 domain of PKCδ targets the protein to distinct cellular membranes upon stimulation by different phorbol esters and related ligands (59). C2 domains also provide differential subcellular specificity, as C2 domains in some proteins (i.e., PKCδ) interact preferentially with anionic phospholipids in the plasma membrane, whereas other C2 domains target proteins to the nuclear membrane, which is enriched in phosphatidylcholine (55). Furthermore, some C2 domains form specific protein-protein interactions that are important for subcellular targeting (16, 27, 32). Therefore, the regulatory subdomains restrict the activity of each PKC isozyme by determining its localization to specific subcellular compartments.

In contrast to the differential distribution of the various regulatory modules between different isoforms in mammalian cells, in *Saccharomyces cerevisiae* all of these elements are present together in one enzyme, Pkc1p (26) (see Fig. 1). Like its mammalian counterparts, the HR1 domain of Pkc1p has been shown to interact with a member of the Rho family of
GTPases, Rho1p (50). A C1 domain including two cysteine-rich domains is also present in Pkc1p, and it has also been shown to bind Rho1p (38, 49). The C2 domain present in Pkc1p lacks the conserved Ca\(^{2+}\)-binding motif, and accordingly Pkc1p activity was shown to be Ca\(^{2+}\)-independent (2, 60). Pkc1p also contains, between the C1 and kinase catalytic domains, a sequence that we have designated the interdomain. The interdomain contains a region (residues 672 to 766) whose similarity to C2 domains has been noted (26); however, this homology is too low to identify the region as a true C2 domain (45).

The main function of Pkc1p is to protect cell wall integrity. pkc1 null mutants undergo cell lysis and are inviable under standard growth conditions, but they can be rescued on osmotically stabilized medium (25, 40). Pkc1p is activated by the Rho1p GTPase, which receives upstream signals from the Slg1p and Mid2p transmembrane sensors, upon cell wall stresses such as hypotonic or heat shock (8, 20, 21, 40, 43). However, the mechanisms by which these sensors activate Pkc1p pathway are not well understood. Pkc1p, in turn, activates a mitogen-activated protein (MAP) kinase cascade, causing activation of Rlm1p transcription factor and subsequent expression of cell wall synthesis genes (15, 61). Pkc1p-dependent activation of this MAP kinase pathway also leads to phosphorylation of the SCB (Swi 4,6-dependent cell cycle box) binding factor (SBF), which regulates transcription at the G0/S-phase transition of the cell cycle (3, 29). Other functions of PKC1, however, are less well characterized and are not necessarily mediated through MAP kinase activation. Several observations suggest that PKC1 regulates aspects of microtubule function. PKC1 interacts with genes involved in spindle pole body duplication (22) and with BIM1, which encodes a microtubule-binding protein (17). Also, a specific allele of pck1, stt1-1, causes sensitivity to thiabendazole, a microtubule-depolymerizing drug (17). Other roles for PKC1 include possible regulation of RSC1, an essential chromatin-remodeling complex (6), and relocation of nuclear proteins in response to hypertonic shock (35, 36). Although several of these observations indicate potential roles for Pkc1p in the nucleus, its localization to this organelle has never been reported.

Full-length yeast Pkc1p localizes to sites of polarized growth (1). Early in the cell cycle, Pkc1p-green fluorescent protein (GFP) is found at the prebud site and bud tip, and it relocalizes to the mother-bud neck during cytokinesis. This membrane localization of Pkc1p is consistent with its well-established function in cell wall integrity. The targeting of Pkc1p to different subcellular compartments could be a mechanism by which cells regulate kinase activity both temporally and spatially. Therefore, we decided to investigate the molecular determinants of Pkc1p localization and dissected the role of each regulatory domain in localization of this kinase. We show in this study that, in addition to the previously defined localization to the bud tip and bud neck, Pkc1p can localize to the nucleus. Furthermore, we identify a nuclear exit signal (NES) and a nuclear localization signal (NLS) within the PKC1 sequence. In addition, we report targeting of the C2 domain of Pkc1p to the mitotic spindle, which may reflect a physiological role for the kinase in regulating microtubule function.

**MATERIALS AND METHODS**

**Yeast strains and media.** Yeast strains DL100 (MATa leu2-0 trpl1 ura3-0 his4 CAN1\(^{50}\)) and DL523 (MATa ura3-0 trpl1 his4 CAN1\(^{50}\) pck1::LEU2) were provided by David Levin (Johns Hopkins University). Strain M-YML904 (Mate/α cdc12-6 cdc12-6 cdc12-6 his3-1 leu2-0 ura3-0 his4) was a gift from J. Thorner (University of California). Strain xpo1-1, derived from W303 (ade2-1 ura3-1 his3-11,15 trpl-1 leu2-3,112 can1-100), was provided by Karsten Weis (University of California) (54). SD medium (51) contained twice the recommended levels of supplements, with 3.5 g of ammonium chloride per liter substituted for ammonium sulfate.

**Vector construction.** pVD61 was the backbone of all PKC1-GFP fusions made for this study, and it was constructed by cloning a 635-bp fragment of the
upstream regulatory sequence of PKC1 into the SacI/SmaI sites of pGRU2, provided by B. Daignan-Fornier (Bordeaux, France). Subsequently, PCR fragments containing full-length or fragments of the PKC1 open reading frame were cut at BglII/SalI sites and cloned into the BamHI/SalI sites of pVD61. The primers used for PCR were named after the position of their 5'-end-matching PKC1 sequence and were as follows: A1/H11001 B3453 (to construct pVD67), A1/H11001 B1659 (pVD65), A1/H11001 B1173 (pVD63), A1/H11001 B585 (pVD77), A559/H11001 B3453 (pVD71), A559/H11001 B2457 (pVD182), A559/H11001 B1659 (pVD69), A559/H11001 B1173 (pVD79), A1116/H11001 B3453 (pVD73), A1116/H11001 B1659 (pVD81), A1630/H11001 B2457 (pVD112), and A2376/H11001 B3453 (pVD75).

PKC(K853R)-GFP (pVD124) was constructed by site-directed mutagenesis, replacing an XhoI/XhoI in pVD67 fragment with a similar PCR fragment containing a CGC codon instead of the AAA codon for Lys853, which created a MluI site.

PKC1(L61, 63A)-GFP (p123) was created by a two-step PCR that changed TTA and TTG codons from Leu61 and Leu63 into GCG codons, thereby creating an SmaI site, and subsequently cloning this fragment into sites SacI/HindIII of pVD67 deprived of the corresponding fragment.

PKC1(L54S)-GFP (pVD170) was generated by a two-step PCR strategy followed by replacement of a SacI/HindIII fragment from pVD67, which introduced an AGC codon instead of the TTG codon for Leu54, thereby destroying the SacI site.

To construct the four NLS-3xFP plasmids, we cloned the following pairs of oligomers into pOM4, which was cut at SpeI/HindIII sites: PKC-NLS1A (5'-CTAGTCACAAACCCGAATTCTCCGGAAGAAACAAACTGA-3') and PKC-NLS1B (5'-AGGTCTAGTTGGTTTCTGGTGCATGTGTTTA-3') for NLS1-3xFP; PKC-NLS2A (5'-CTAGTTATAGTGAGGAAAGCAAGCGGA-3') and PKC-NLS2B (5'-CTAGTATTAGTTTAGAGGAAAGCGGAAGA-3') for NLS2-3xFP; PKC-NLS3A (5'-CTAGTAAACGTAATCAAGAGAAAAGAAGGAA-3') and PKC-NLS3B (5'-CTAGTACGAGGAGGTATACAGAGACGAGAAGA-3') for NLS3-3xFP; and PKC-NLS4A (5'-CTAGTCAGACGCTAAACAGTGAGGAAAGAAGAAGAAGGAA-3') and PKC-NLS4B (5'-CTAGTGTTAGTGAGGAAAGCAAGCGGAAGAAGAAGAAGGAA-3') for NLS4-3xFP. PKC1-NES*NLS3*-GFP (pVD155), corresponding to L61,63A/K554,555,556A mutations, was created by a two-step PCR that changed the 5'-AAAAAGAAGA-3' sequence (positions 1660 to 1669 downstream of the start codon) to the 5'-GCGGCCGCTA-3' sequence, thereby creating a NotI site, and the resulting PCR fragment was used to replace the corresponding BamHI/BamHI fragment from pVD123.

PKC1-NES*NLS4*-GFP (pVD159), corresponding to L61,63A/K810,811A/R812A, was generated by a two-step PCR using pVD123 as a PCR template that changed the 5'-AAAAAGAAGA-3' sequence (positions 2428 to 2436 downstream of the start codon) to the 5'-GCGGCCGCTA-3' sequence containing a NotI site. The resulting PCR fragment was subsequently used to replace the corresponding XhoI/XhoI fragment from pVD67.

PKC1-NES*NLS3* NLS4*-GFP (pVD160), corresponding to L61,63A/K554,555,556A/K810,811A/R812A mutations, was constructed as PKC1-NES*NLS4*-GFP, except that pVD155 was used as a PCR template.

CDC3-GFP was a gift from Lucy Robinson (48).

**Microscopic imaging.** Cells were grown at 30°C (except where a different temperature is noted) in SD-leu medium and harvested at log phase (optical density at 600 nm was 0.6 to 1.0). One construct, VD77, was expressed in pkaΔ (DL523) and was grown on SD-leu containing 1.2 M sorbitol at 25°C (see Fig. 2D). Epifluorescent microscopy was performed with a Nikon Eclipse E600 microscope, fitted with a 100× immersion objective and a standard fluorescein isothiocyanate filter set. Images were recorded digitally using a CCD4742-95 camera (Hamamatsu) and QED Imaging, Inc., software. Images were processed digitally using Adobe Photoshop (Adobe Systems, Inc.).

**FIG. 2.** Each domain of Pkc1p is targeted to a specific subcellular localization. Each picture is composed of epifluorescence microscopy (left) and corresponding differential interference contrast microscopy (right). All constructs were expressed in WT (DL100) cells except for pVD77, which was expressed in strain DL523 as indicated (pkaΔ). For correspondence between plasmid names (pVD67 to pVD112) and PKC inserts, refer to Fig. 1. The scale bar on the first picture is applicable to all pictures.
RESULTS

Each subdomain of Pkc1p is targeted to a specific subcellular localization. In order to determine how each conserved regulatory domain of Pkc1p contributes to the in vivo distribution of this protein, we examined the localization of every subdomain as well as that of Pkc1p truncated for one or more of these domains. For this purpose we used the green fluorescent protein gene (GFP), fused to each PKC1 sequence fragment represented in Fig. 1 and carried on a μm vector, under the control of a PKC1 endogenous promoter sequence. The full-length PKC1-GFP construct complemented the heat sensitivity and the high-osmolarity-medium requirement for growth of a pkc1Δ strain, indicating that the construct was functional (data not shown). The localization of each fusion protein was observed both in a wild-type strain and a pkc1Δ strain; localization was indistinguishable in the two strains, except in the case of one construct, pVD77 (see below). As observed previously, full-length Pkc1p localized to the bud tip in small- to medium-sized buds and to the bud neck in large-budded cells (Fig. 2A).

First, we engineered a series of C-terminal deletions of full-length Pkc1p (Fig. 1). Interestingly, C-terminal deletion of the kinase domain and the interdomain resulted in more sustained fluorescence at the surface of the bud, emergence of a surface localization on unbudded cells, and incomplete bud neck localization (Fig. 2B). Indeed, only two small dots appear at the bud neck of large-budded cells performing cytokinesis, instead of the double-ring pattern filling the space in the middle of the neck which was observed for full-length Pkc1p (compare Fig. 2A to B). A further C-terminal deletion of the C1 domain resulted in localization of the HR1-C2-GFP polypeptide exclusively to the bud tip (Fig. 2C). This suggests that either the HR1 or the C2 domain targets the GFP specifically to the bud tip. Next, expression of a GFP fusion containing only HR1 domain (pVD77) in a wild-type strain showed mostly cytosolic fluorescence as well as some localization to the bud tip in a small percentage of cells (data not shown); however, the HR1 domain expressed in a pck1Δ strain typically localized to the bud tip and also faintly at the bud neck of some cells (Fig. 2D). Hence, the HR1 by itself was sufficient to target the GFP to sites of polarized growth, and therefore the HR1 domain is likely to play a major role in the specificity of Pkc1p binding to these sites. The difference between the HR1 domain localization in WT versus pck1Δ cells is possibly due to a competition with endogenous Pkc1p for binding sites at the membrane.

We next deleted the HR1 domain of Pkc1p and made a second series of truncations from the C terminus (Fig. 1). Strikingly, an N-terminal deletion of the HR1 domain from full-length Pkc1p resulted in its localization to the nucleus, and in particular to the mitotic spindle (Fig. 2E). This Pck1pΔHR1-GFP protein specifically decorated nuclear microtubules in cells containing either short or fully elongated spindles. Bud tip and bud neck localization was still observed in some cells, but this fluorescence was fainter than that observed with full-length Pkc1p, suggesting that spindle localization competes with the two other localizations. This novel and surprising localization was also observed for a series of protein fusions, each of which lacked the HR1 in combination with additional and successive C-terminal deletions of the kinase domain, the interdomain, and the C1 domain (Fig. 2D). Comparison of Fig. 2A to D with Fig. 2E to H shows that both the absence of the HR1 domain and the presence of the C2 domain are required to target Pkc1p to the mitotic spindle. In addition, Fig. 2H shows that the C2 domain alone is necessary and sufficient to target GFP to nuclear microtubules.

We next deleted both the HR1 and C2 domains from the N terminus of Pkc1p. This truncated protein localized to sites of polarized growth as well as to the nucleus, suggesting that a sequence in the C1, interdomain, or kinase domain targets the protein to the nucleus (Fig. 2F). Localization of pVD81 showed that the C1 domain alone targeted GFP to the periphery of unbudded and budded cells, with a preference for the bud, whether it was small or large (Fig. 2J). Therefore, the C1 domain might play an important role in tethering Pkc1p to the membrane while not providing the highly specific binding to the bud tip observed with the HR1 alone or with full-length Pkc1p. We next observed the localization of GFP fused to the interdomain region. Strikingly, the interdomain showed a very bright and specific localization to the nucleus (Fig. 2K). Finally, we constructed a fusion of the kinase domain of Pck1p to GFP, but the resulting protein was unstable and produced a signal that was too faint to characterize (data not shown).

In summary, this deletion study showed that the three conserved regulatory subdomains of Pkc1p, i.e., the HR1, C1, and C2 domains, are each targeted to a specific subcellular location. This analysis also indicated that either the interdomain or the kinase domain affected Pkc1p localization to the bud neck, and we investigated this issue in further experiments.

Localization of Pck1p at the bud neck depends upon its own kinase activity and requires the septin ring. The changes in localization observed between full-length Pck1p and a C-terminal deletion of the kinase and the interdomain suggested that at least one of these domains participates in the bud neck localization of Pck1p (Fig. 2B). We tested whether kinase activity was important for normal Pck1p localization by using a catalytically inactive Pck1p(K853R)-GFP fusion protein, which carries a point mutation in the active site (60). We first confirmed that, in contrast to the WT Pck1p-GFP fusion, the Pck1p(K853R)-GFP mutant was unable to complement the growth defects of a pck1Δ strain (data not shown). This catalytically inactive mutant localized to the periphery of unbudded cells as well as buds of all sizes (Fig. 3A). It also localized to the surface of the cell at the bud neck of large-budded cells, appearing on a focal plane as two dots on each side of the neck, but not filling the space within the neck as full-length Pck1p did (compare Fig. 3A to 2A). The difference in the localization patterns of these two fusion proteins is likely due to the loss of kinase activity, because the catalytically inactive version differs from WT Pck1p by only one residue. The localization pattern observed using a C-terminal deletion of the protein (Fig. 2B) was similar to that observed for this catalytically inactive Pck1p, with loss of the typical bud neck pattern and localization of the protein to large buds and unbudded cells. Therefore, kinase activity of protein kinase C is essential for proper localization of the protein.

As a second step toward the analysis of the elements controlling Pck1p localization to the bud neck, we analyzed whether the presence of the septin ring was required for normal Pck1p localization, as shown for other kinases that localize
of Pkc1p to the bud neck is controlled by formation of the septin ring as well as by the kinase activity of Pkc1p itself.

**PKC1 nuclear export is regulated by a leucine-rich NES within the HR1 domain and by the exportin Xpo1p/Crm1p.** The unexpected nuclear localization of both the C2 domain and the interdomain strongly suggested that Pkc1p could localize to the nucleus. Thus, we examined the PKC1 sequence to identify putative regulatory import and export signals. Nuclear export signals are poorly conserved; however, we identified a region (amino acids 54 to 63) that displayed a leucine-rich motif, $L(X)_1LXXL(X)L$, common to at least six NESs, from a variety of proteins involved in signaling pathways, including αPKC and ζPKC (42). Promisingly, this motif was within the HR1 region, and deletion of this domain from PKC1 resulted in localization of the protein to the nucleus and mitotic spindle (Fig. 1E). To disrupt the putative NES, we mutagenized the last two leucines (L61 and L63) of this motif within a Pkc1p-GFP fusion and examined the localization of the resulting protein. This mutant, Pkc1p(L61, L63A)-GFP, displayed nuclear localization in addition to its typical localization to the bud tip and neck (Fig. 4A).

This result suggested that we had defined an NES in the HR1 domain. However, previous analysis of the HR1 domain of Pkc1p and its interaction with Rho1p showed that an L54S substitution in the HR1 domain prevents Rho1p binding to this domain (50). Because this mutation is within the leucine-rich motif we identified, it was possible that the L61, L63A mutation caused a disruption of Rho1p binding rather than inactivation of an NES. To clarify this issue, we introduced the L54S mutation into a Pkc1p-GFP fusion. This mutation had a dramatic effect on Pkc1p localization; we did not observe any fluorescence at the bud tip, bud neck fluorescence was greatly reduced, and cytosolic fluorescence was increased (Fig. 4B). However, in contrast to Pkc1p(L61, L63A)-GFP, Pkc1p(L54S)-GFP did not display nuclear localization. Thus, disruption of the Rho1p-Pkc1p interaction disrupts Pkc1p localization to sites of polarized growth but does not result in nuclear localization. The finding that Pkc1p(L61, L63A)-GFP localized both to the nucleus and to sites of polarized growth supports the conclusion that these mutations disrupt an NES and do not disrupt the interaction of Pkc1p with Rho1p.

Because leucine-rich NES motifs are known to interact with Xpo1p/Crm1p, a member of the importin β family that acts as an exportin (39, 54), we examined Pkc1p localization in an xpo1-1 temperature-sensitive mutant. When shifted to nonpermissive temperature (37°C), Pkc1p-GFP accumulated in the nucleus of xpo1-1 cells, whereas the isogenic WT strain displayed typical Pkc1p localization under these conditions (Fig. 4C). Thus, Xpo1p is responsible for exporting Pkc1p from the nucleus. Altogether, our results concur to show that Pkc1p contains a small, leucine-rich NES motif located within the HR1 domain which is homologous to some previously identified NES motifs from mammalian PKCs.

**PKC1 has two NLSs located within the interdomain region.** To identify potential NLSs within PKC1, we performed an in silico search for motifs homologous to known NLSs and found four putative sequences. Two of these sequences were located in the C2 domain, and two were in the interdomain. Because both domains on their own localize to the nucleus (Fig. 2H and K), all of these sequences were good candidates for NLSs.
Proteins over 50 kDa can enter the nucleus only if they have an NLS. Therefore, we tested each putative NLS from Pkc1p by fusing small DNA oligomers containing each putative NLS sequence to three tandem copies of GFP, which encodes a 75-kDa protein. We observed a diffuse, cytosolic localization for NLS1-3xGFP and NLS2-3xGFP, whereas NLS3-3xGFP and NLS4-3xGFP both localized specifically to the nucleus (Fig. 5A), suggesting that they were functional NLSs. To confirm the role of NLS3 and NLS4 in Pkc1p nuclear import, we performed site-directed mutagenesis on these two sequences in the context of the NES mutant PKC1(L61, L63A)-GFP. As observed in previous experiments, PKC1(L61, L63A)-GFP localized to the nucleus (Fig. 5B). Additional mutation of three key residues in NLS3* (KKK554-556AAA) did not significantly affect nuclear localization of this protein (Fig. 5B). Conversely, mutation NLS4* (KKR810-813AAA) and the double NLS3* NLS4* mutation abolished the nuclear accumulation observed for PKC1(L61, L63A)-GFP (Fig. 5B). Thus, in the absence of a functional NLS4, the presence of NLS3 was not sufficient to target Pkc1p to the nucleus. We concluded that NLS4, identified in the interdomain region, was the main nuclear localization signal responsible for entry of Pkc1p into the nucleus.

**DISCUSSION**

The activities of many proteins are regulated through their subcellular localization; directing enzymes to specific structures or compartments affects their proximity to both upstream regulators and substrates. The highly dynamic localization of mammalian PKCs, which are able to translocate to specific cellular locations in response to different stimuli, is intimately linked to their activation and substrate specificity. The C1 and C2 domains of PKC isozymes are largely responsible for their targeting to membranes (7); however, in each PKC isotype these domains display distinct properties and specificities. Thus, in mammalian cells, characterizing the localization and in vivo activity of each PKC isotype is a challenging task. In contrast, budding yeast, *S. cerevisiae*, contains a single PKC, Pkc1p (26). Like mammalian PKC, Pkc1p is highly dynamic and localizes to several different sites during the cell cycle (1). However, the intramolecular mechanisms that underlie Pkc1p targeting have not been addressed. In the present study, we systematically describe the role of each domain in the localization of Pkc1p. Furthermore, we demonstrate for the first time that this kinase is able to shuttle in and out of the nucleus and that the mitotic spindle is a potential binding site for Pkc1p in the nucleus. Also of note is our failure to observe association of any of the GFP fusions we analyzed with the endoplasmic reticulum. This is of interest because a previous study demonstrated interaction between Pkc1p and luminal portions of three subunits of the endoplasmic reticulum-localized oligosaccharyltransferase enzyme complex (41).

**Pkc1p localization at sites of polarized growth.** Targeting of the HR1 and C1 domains to the membrane reveals their specificity for (HR1) or preference (C1) for the bud tip, and it mirrors the localization of Pkc1p full-length protein to this site. Both domains also localize to the bud neck and partially overlap with Pkc1p distribution to this site, although we show that PKC catalytic activity is required for its complete localization to the bud neck. Thus, the HR1 and C1 domains act together to tether Pkc1p to the bud tip and contribute to Pkc1p bud neck localization. The HR1 and C1 domains likely target Pkc1p by binding Rho1p, the upstream activator of Pkc1p. Rho1p also localizes to sites of polarized growth and is re-
quired for the correct distribution of Pkc1p in vivo (1, 62). In two-hybrid assays, both the HR1 and C1 domains mediate Pkc1p interaction with an activated version of Rho1p (Q68H) (38, 49, 50). This Pkc1p-Rho1p interaction is eliminated only when a point mutation in the HR1A domain (L54S) is introduced into Pkc1p lacking the C1 domain (Pkc1p\textsuperscript{H9004C1}) (50). In contrast, we observed that the mutation in HR1A (L54S) is by itself sufficient to disrupt Pkc1p localization to the bud tip and bud neck. Thus, perturbing HR1-Rho1p association alters Pkc1p localization without affecting the Pkc1p-Rho1p two-hybrid interaction; this may be due to use of activated Rho1p in the two-hybrid assay. In conclusion, association of Rho1p with the HR1 and C1 domains plays an essential role in targeting Pkc1p to sites of polarized growth. Furthermore, our observations confirm that HR1 motifs can serve as localization signals, as shown previously for the HR1b domain from Prk1, which binds to RhoB (30). For targeting of the C1 domain of Pkc1p to the membrane, it remains to be shown whether the interaction between Rho1p and C1 is sufficient for this association or whether binding of additional putative ligands, such as DAG, is also required.

FIG. 5. Identification of Pkc1p nuclear import signals. (A) Expression in yeast of small oligomers (24- to 30-mer) encompassing the four putative NLSs and fused to 3xGFP. See Materials and Methods for the sequence of each oligomer. Each picture is composed of epifluorescence microscopy (left) and corresponding differential interference contrast microscopy (right). NLS3 and NLS4, but not NLS1 and NLS2, were able to target the 3xGFP in the nucleus in WT (DL100) cells. (B) Site-directed mutation analysis of NLS3 and NLS4. Double mutant Pkc1p-NES* NLS3* (pVD155) accumulated in the nucleus as well as Pkc1p-NES* (p123); whereas Pkc1p-NES* NLS4* (pVD159) and triple-mutant Pkc1p-NES* NLS3* NLS4* (pVD160) failed to accumulate in the nucleus in WT (DL100) cells.
Pkc1p localization to the nucleus. The discovery that Pkc1p trafficks in and out of the nucleus raises the intriguing possibility that kinase activity is targeted to the nucleus in response to specific stimuli. We were unable, however, to identify physiological conditions that lead to significant accumulation of Pkc1p in the nucleus. We did observe Pkc1p localization to the nucleus in the spo1/cem1 mutant; this supports the idea that Pkc1p shuttles in and out of the nucleus, with a small subpopulation of Pkc1p molecules being nuclear at any one time. Is Pkc1p nuclear localization physiologically relevant? Several known functions of the enzyme may be performed in the nucleus. Pkc1p activates a MAP kinase cascade, resulting in activation of the Rim1p transcription factor and expression of genes involved in cell wall biogenesis (61); it has yet to be determined in what cellular compartment the phosphorylation of Bck1p, the upstream kinase in the cascade, by Pkc1p takes place. In addition to regulating the MAP kinase cascade, Pkc1p performs other, less well-characterized, functions; as a consequence of these other activities, a pkc1Δ strain displays more severe growth defects than mutants in the MAP kinase pathway. For example, functional interactions between Pkc1p and the chromatin-remodeling complex RSC have previously been described, and these appear to be independent of the MAP kinase pathway (6, 17). Also, in response to hypertonic shock, Pkc1p-dependent relocation of nuclear proteins has been observed, and components of the MAP kinase pathway are not required for this activity (35, 36). Our analysis of Pkc1p localization supports the possibility that Pkc1p serves these and other functions by directly phosphorylating nuclear substrates.

C2 domain localization to the spindle. The novel observation that the Pkc1p-C2 domain localizes to the mitotic spindle suggests further that Pkc1p may directly regulate the microtubule cytoskeleton. In eukaryotic cells, most C2 domains bind lipids in a Ca2+-dependent manner and therefore contribute to PKC targeting to the membrane (7). However, C2 domains that lack the Ca2+-binding pocket, such as those found in Pkc1p, aPKCs, and PRKs, do not bind lipids, and their role is less well characterized. Some C2 domains mediate specific protein-protein interactions. Interestingly, both C2 domains of synaptotagmin I and synaptotagmin IX bind tubulin, and these protein-protein interactions. Interestingly, both C2 domains of synaptotagmin I and synaptotagmin IX bind tubulin, and these protein-protein interactions are involved in microtubule-mediated locomotion (57, 58). In addition, Pkc1p localization to sites of polarized growth. J. Cell Sci. 2000. Dynamic, Rho1p-dependent localization of Pkc1p to sites of polarized growth. J. Cell Sci. 113:2685–2693. 2. Antonsson, B., S. Montessuit, L. Friedli, M. A. Payton, and G. Paravicini. 1994. Protein kinase C in yeast. Characteristics of the Saccharomyces cerevisiae PKC1 gene product. J. Biol. Chem. 269:16821–16828. 3. Baetz, K., J. Moffat, J. Haynes, M. Chang, and B. Andrews. 2001. Transcriptional coregulation by the cell integrity mitogen-activated protein kinase Slt2 and the cell cycle regulator Swi4. Mol. Cell. Biol. 21:5155–5162. 4. Bazzi, M. D., and G. L. Nelsestuen. 2000. Dynamic, Rho1p-dependent localization of Pkc1p to sites of polarized growth. J. Cell Sci. 113:2685–2693. 5. Bouquin, N., Y. Barral, R. Courbejeotte, M. Blondel, M. Snyder, and C.
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