Activation of the Akt-related cytokine-independent survival kinase requires interaction of its phox domain with endosomal phosphatidylinositol 3-phosphate

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Protein kinases of the Akt and related serum- and glucocorticoid-regulated kinase (SGK) families are major downstream mediators of phosphatidylinositol (PI) 3-kinase signaling to many cellular processes including metabolic flux, membrane trafficking, and apoptosis. Activation of these kinases is thought to occur at the plasma membrane through their serine and threonine phosphorylation by the phosphoinositide-dependent kinase 1 (PDK1) protein kinase, which interacts with membrane 3′-polyphosphoinositides through its pleckstrin homology (PH) domain. Here, we demonstrate that the SGK family member cytokine-independent survival kinase (CISK) binds strongly and selectively to the monophosphoinositide PI(3)P through its phox homology (PX) domain. Comparing native green fluorescent protein-CISK (EGFP-CISK) to a mutant EGFP-CISK (Y51A) that displays attenuated binding to PI(3)P reveals that this interaction is both necessary and sufficient for its localization to early endosome antigen (EEA1)-positive endosomes. Furthermore, early endosome association of expressed epitope-tagged CISK in COS cells directed by binding of its PX domain to PI(3)P is required for activation of the CISK protein kinase by both insulin-like growth factor-1 and epidermal growth factor. Taken together, these results reveal a critical role of endosomal PI(3)P in the signal transmission mechanism whereby this survival kinase is activated in response to PI3-kinase stimulation by growth factors.

Phosphorylated derivatives of phosphatidylinositol (PI) in the cytoplasmic leaflet of cellular membranes serve both as substrates for lipid hydrolases that produce signaling molecules as well as ligands for a wide variety of proteins involved in membrane-associated cellular functions (reviewed in refs. 1 and 2). A large body of recent work has identified a number of these target proteins and elucidated their roles in the various phosphoinositide-regulated processes (3, 4). Many of these proteins possess pleckstrin homology (PH) domains, some of which confer high affinity binding to 3′-polyphosphoinositides produced by hormone- and growth factor-regulated PI3-kinases (5, 6). One set of downstream targets for the polyphosphoinositides PI(3,4)P_2 and PI(3,4,5)P_3 are PH domain-containing protein kinases of the Akt family (7, 8), which are in turn activated through phosphorylation by the phosphoinositide-dependent kinase 1 (PDK1; ref. 9). PDK1 also contains a PH domain through which it is brought into contact with Akt at the plasma membrane of stimulated cells. The Akt protein kinases appear to mediate a remarkable array of biological responses to extracellular stimuli, including membrane trafficking of glucose transporters (10, 11), transcription of metabolic enzymes and other proteins (12), and cell survival (13, 14). Thus, Akt activation in response to generation of the 3′polyphosphoinositides PI(3,4)P_2 and PI(3,4,5)P_3 has emerged as one of the major control mechanisms for regulating key functions of many cell types.

Recently, two types of protein modules that bind to the monophosphorylated PI(3)P have been identified. One is the FYVE domain, a zinc-finger motif originally identified in the Fab1,YOTB, Vac1 and early endosome antigen 1 (EEA1) proteins (15). The FYVE domain is essential for the localization and function of EEA1 in early endosomes that display PI(3)P (16, 17). We and others have discovered that a second protein motif, the phox (PX) domain, can also function as a phosphoinositide-binding protein module that, in some proteins, is selective for PI(3)P. Originally described in two different subunits of the neutrophil NADPH oxidase complex, the PX domains of the yeast protein Vam7p (18, 19), human p40-phox (20, 21), and sorting nexin 3 (22) bind to PI(3)P, whereas that of the mouse Cpk PI3-kinase shows preferential binding to PI(4,5)P_2 (19). The p40-phox PX domain was shown to localize the protein to endosomes through its binding of PI(3)P (20, 21).

In searching for PX domain-containing proteins that may regulate membrane trafficking, we noted the report of the cytokine-independent survival kinase (CISK) isolated in a screen for IL-3-dependent survival factors in 32D cells (23). CISK is a member of the Akt and serum- and glucocorticoid-regulated kinase (SGK) family and is the mouse homolog of human SGK-3 (also known as SGK-L). Its kinase domain is highly similar to that of Akt as well as the two other SGK family members, but CISK contains a PX domain in place of the Akt PH domain. CISK contains the two regulatory phosphorylation sites also present in Akt and SGK proteins, which are known to be the substrates for the upstream PI3-kinase-dependent PDK1. Furthermore, CISK shares at least several substrates with Akt. It phosphorylates histone H2B and caspase *in vitro* and regulates the forkhead family member FKHRL1 *in vivo* (23). Thus, it seems likely that CISK shares some elements in its activation pathway as well as downstream targets with Akt and SGK. We demonstrate here that, unlike Akt and other SGK family members, CISK localizes specifically to endosomes in COS cells via interaction of its PX domain with PI(3)P. This interaction is required for growth factor activation of CISK, revealing a critical role of PI(3)P in signal transduction through this protein kinase.

Materials and Methods

CISK Expression Vector Construction. The coding sequence of CISK from amino acid 55–496 was obtained from a mouse expressed sequence tag (EST) clone (IMAGE EST 2631682). The remaining sequence was generated by reverse transcription (RT)-PCR of mouse spleen RNA by using primers matching the published CISK cDNA sequence (23). The cloned RT-PCR product was then fused to the EST to generate the complete CISK sequence. A PX

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Abbreviations: SGK, serum- and glucocorticoid-regulated kinase; PI, phosphatidylinositol; PDK, phosphoinositide-dependent kinase; PH, pleckstrin homology; CISK, cytokine-independent survival kinase; HA, hemagglutinin; EGFP, enhanced green fluorescent protein; IGF-1, insulin-like growth factor-1; EGF, epidermal growth factor; EEA, early endosome antigen; PX, phox homology; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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domain-encoding fragment (amino acids 3–157) with *Xho*I and *Bam*HI ends was generated by PCR and ligated to the same sites in pEGFP-C1 (CLONTECH) to generate enhanced green fluorescent protein (EGFP)-CISK PX. Full length EGFP-CISK was generated by using the same N-terminal *Xho*I site and a *Pst*I site in the CISK 3′-non-coding sequence for ligation into EGFP-C1. Triple hemagglutinin (HA)-tagged vectors were generated by ligation of the *Xho*I-*Pst*I insert of the EGFP-C1 vectors into the plasmid pCMV5–3xHA. The mutations Y51A and K191A were generated by PCR-mediated mutagenesis and ligation of the altered DNA fragment into the relevant expression vector.

Preparation of His-Tag Proteins. The PX domain (amino acids 23–124) and the PX domain with Y51A mutation were expressed as the His-tagged protein by cloning the PCR fragments corresponding to the PX domain from vector pCMV5–3xHA into the pXL1a vector (modified from pET15b). Fusion proteins were expressed in *Escherichia coli* strain BL21(DE3) at room temperature overnight, after induction by isopropyl β-D-thiogalactoside (IPTG) in the range from 50 μM to 0.5 mM. The His-tagged PX domains were purified from lysates with Ni-NTA beads (Qiagen, Chatsworth, CA).

Liposome Binding Assays. Phosphatidylinositol, phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine were purchased from Avanti Polar Lipids. PI(4)P, PI(5)P and PI(3,5)P_2 were purchased from Echelon (Salt Lake City). PI(3)P, PI(3,4)P_2, PI(4,5)P_2, and PI(3,4,5)P_3 were purchased from Matreya (Pleasant Gap, PA). A published liposome binding assay protocol was used for the binding experiments in this report (24), summarized as follows: The phospholipid mixture (100 μg per reaction) was dried in a SpeedVac (Savant). The dried mixture was then resuspended in 100 μl of liposome buffer (50 mM Hepes/100 mM NaCl, pH7.2), sonicated in a bath sonicator for 15 min, and spun for 10 min at 14,000 rpm, 4°C. The liposomes were resuspended in binding buffer (50 mM Hepes/100 mM NaCl/1 mM $MgCl_2$, pH 7.2) at the concentration of 1 mg/ml. The liposomes were then incubated with 8 μg of purified protein for 15 min at room temperature, and centrifuged as before. The supernatant was saved, and the pellet was resuspended in 100 μl of binding buffer. Then, 25 μl of both fractions was used for analysis by SDS/PAGE gel and Coomassie blue staining. The intensity of the stained bands was quantitatively determined with an LKB Ultroscan laser densitometer.

Protein-Lipid Overlay Assay. Nitrocellulose membranes spotted with phospholipids (PIP-Strips) were purchased from Echelon. Protein-lipid overlay was performed as described (25, 26). The membrane was blocked in 3% BSA (Sigma) in TBST buffer (50 mM Tris·HCl, pH 7.5/150 mM NaCl/0.1% Tween 20) for 1 h at room temperature. The membrane was then incubated overnight at 4°C in the same solution with 0.2 μg/ml His-tagged CISK protein. After three washes for 10 min with TBST buffer, the membrane was incubated for 1 h at room temperature with 1:1,000 dilution of the anti-6xHis-tag monoclonal antibody (Novagen). The membrane was washed and then incubated with anti-mouse alkaline phosphatase conjugate. Finally, the membrane was washed in TBST buffer, and the His-tagged protein binding to the membrane was detected by enhanced chemiluminescence (Bio-Rad, Immun-Star).

Expression, Immunoprecipitation and CISK Kinase Assays. 3xHA-tagged CISK in pCMV5 or HA-Akt in pCMV6 (a gift of Thomas Franke, Columbia University) were transfected into COS cells by using Lipofectamine Plus reagent (GIBCO). After 24 h, cells were serum-starved overnight in DMEM + 0.5% BSA. Cells were treated with wortmannin (200 nM, Sigma) for 30 min before treatment with insulin-like growth factor-1 (IGF-1, 10 nM, Calbiochem) or epidermal growth factor (EGF, 25 ng/ml, Calbiochem) for 5 min. Cells were washed in PBS and then scraped in lysis buffer (20 mM Tris·HCl, pH 7.4/137 mM NaCl/1% Nonidet P-40/10% glycerol/1 mM Na_3VO_4/20 mM NaF/1 mM PMSF/5 μg/ml leupeptin/5 μg/ml aprotinin); and lysates were cleared by centrifugation. After incubation of lysates for 2 h with a rabbit anti-HA peptide antiserum, immunoprecipitates were collected by incubation with protein A-Sepharose (Sigma) for 1 h. Immunoprecipitates were washed and assayed for kinase activity as described (27). Briefly, beads were washed three times in lysis buffer, once in water, and resuspended in 500 μl of kinase buffer (20 mM Hepes-NaOH, pH 7.4/10 mM $MgCl_2$/10 mM $MnCl_2$). Of this, 300 μl was pelleted and the beads were resuspended in 20 μl of assay buffer. Substrate (1 μg of histone H2B, Roche) and ATP (5 μM containing 10 μCi [γ-^{32}P]ATP, 6,000 Ci/mmol, New England Nuclear) were added and incubated 30 min. The reaction was stopped by adding SDS/PAGE sample buffer. The entire sample was fractionated on a 15% polyacrylamide gel, and phosphorylated histone was detected by autoradiography of the dried gels. H2B phosphorylation activity was quantitated by densitometric scanning. The remaining beads were boiled in SDS/PAGE sample buffer, fractionated on 10% gels, and transferred to nitrocellulose; HA-tagged protein was detected with 12C5A mouse monoclonal anti-HA antibody. Aliquots of the cell lysates were also immunoblotted with anti-phospho-Akt (Ser-473) antibody (New England Biolabs).

Immunofluorescence Microscopy. EGFP-CISK or EGFP-CISK PX expression vectors were transfected into COS cells via calcium phosphate coprecipitation. For transferrin labeling, cells were changed to DMEM + 0.5% BSA for 2–3 h before labeling and then incubated with 5 μg/ml rhodamine-transferrin (Molecular Probes) for 15 min. Cells were then fixed in 4% paraformaldehyde and mounted. For EEA1, staining cells were fixed in 4% paraformaldehyde and permeabilized in methanol for 2 min at room temp. Cells were then incubated with mouse anti-EEA1 (Transduction Laboratories, Lexington, KY) diluted 1:200 in PBS with 5% BSA and 1% FBS for 2 h. After washing, cells were incubated for 30 min with rhodamine-conjugated goat anti-mouse IgG (BioSource International, Camarillo, CA), washed, postfixed in 4% paraformaldehyde, and mounted. Images were collected on a Bio-Rad MRC1024 confocal microscope with LASERSHARP software. Pseudocoloring and merging of images was performed by using Adobe Photoshop.

Results

The CISK PX Domain Binds Specifically to PI(3)P. We recently determined that PX domains from different proteins display distinct specificities for binding to phosphoinositides. Among the three that we tested, only the PX domain of Vam7p displayed selectivity for PI(3)P (19). However, the reported localization of the PX-domain containing protein kinase CISK to vesicular structures in 32D cells (23) suggested this PX domain as another candidate for binding to PI(3)P. We therefore determined the phosphoinositide binding specificity of the isolated CISK PX domain (amino acids 23–124) expressed in bacteria as a 6xHis-fusion protein (Fig. 1). As previously determined with other PX domains, the expressed CISK PX domain failed to bind significantly to liposomes consisting of only phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and remained in the supernatant when the liposomes were recovered by centrifugation (Fig. 1*A*). However, when such liposomes included 5% PI(3)P, a substantial proportion of the CISK-PX fusion protein remained associated with the liposomes and was recovered in the liposome pellet (Fig. 1*A Inset*). In contrast, neither of the other two monophosphorylated phosphatidylinositols, PI(4)P and PI(5)P, promoted binding of CISK-PX to the liposomes. Furthermore, CISK-PX failed to bind liposomes containing any other 3′-phosphorylated isomers or PI(4,5)P_2. We have also tested a CISK-PX construct including the complete N-terminal region (3–157) and found that it also binds strongly and selectively to PI(3)P (data not shown).

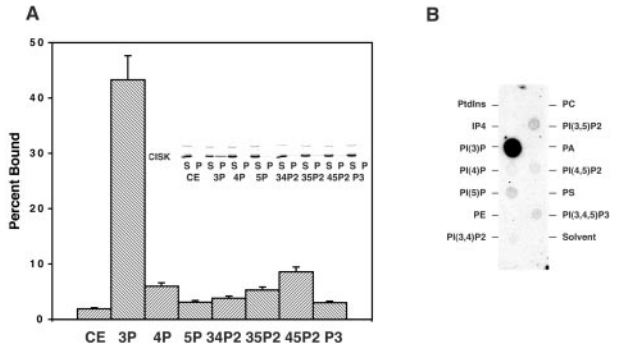

Fig. 1. The CISK PX domain selectively binds PI(3)P. (*A*) Binding percentage of the 6xHis-tagged PX domain of CISK to liposomes containing 5% phosphatidylinositol phosphates. Liposomes were composed of 50 μg of phosphatidylcholine (PC) and 50 μg of phosphatidylethanolamine (PE) or of 47.5 μg of PC, 47.5 μg of PE, and 5 μg of the tested phospholipids. Purified 6xHis-CISK PX protein was added to the liposomes and incubated for 15 min. After centrifugation, liposome pellets (P) and supernatants (S) were analyzed by SDS/PAGE and Coomassie blue staining. 3P, PI(3)P; 4P, PI(4)P; 5P, PI(5)P; 34P2, PI(3,4)P_2; 45P2, PI(4,5)P_2; 35P2, PI(3,5)P_2; P3, PI(3,4,5)P_3; CE, 50% PC and 50% PE. The results are the average of at least three experiments. (*B*) The ability of wild-type CISK protein to bind a variety of phospholipids was assessed by a protein-lipid overlay assay. Nitrocellulose membrane spotted with different phospholipids (100 pmol each) was incubated with the purified His-tagged PX domain of CISK. The fusion protein bound to the membrane by interacting with lipid was detected by using an anti-his·tag antibody. A representative of three experiments is shown here.

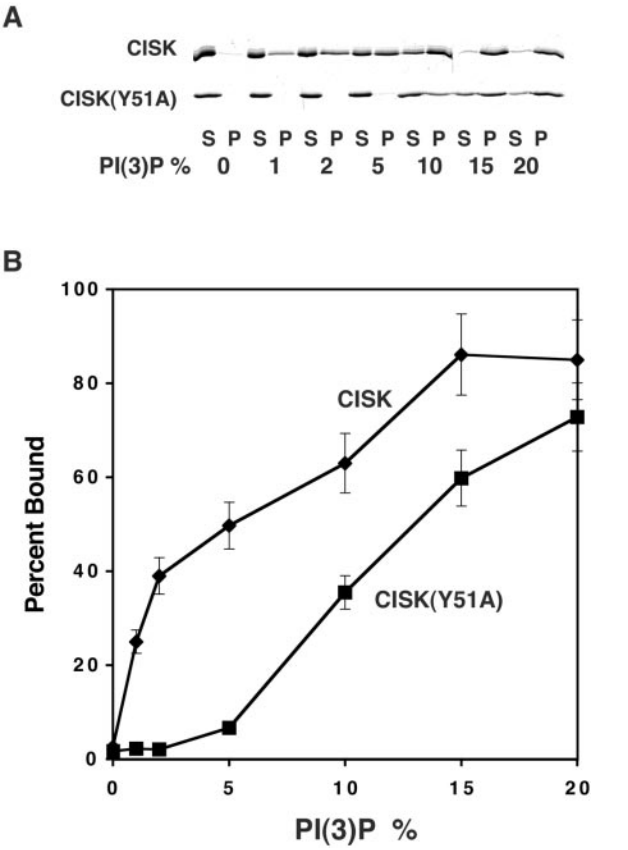

Fig. 2. High affinity, saturable binding of the CISK PX domain is diminished by the Y51A point mutation. (*A*) Liposomes were composed of PC/PE, plus the indicated percentages of PI(3)P. Purified 6xHis-CISK PX protein or the Y51A mutant was added, and the mixture was incubated 15 min at room temperature. (*B*) The graph shows the percentage binding of the PX domains to liposomes containing different amounts of PI(3)P. The results are the average of three independent experiments.

We confirmed the specificity of CISK-PX binding to PI(3)P with a filter-spotted lipid binding assay (Fig. 1*B*). A filter containing PI and each of its phosphorylated forms, as well as PC, phosphatidylserine (PS), PE, and inositol-1,3,4,5-tetrakis-phosphate (IP4), was incubated with His-tagged CISK-PX, and bound protein was detected with anti-His-tag antibody. Consistent with the results of the liposome binding assay, CISK-PX bound strongly only to PI(3)P, with only minimal binding to several other ligands.

One of two loss-of-function mutations in the yeast t-snare protein Vam7p is in a highly conserved tyrosine residue likely to be in close contact with PI(3)P, based on NMR studies of the Vam7p PX domain bound to PI(3)P-containing liposomes (18). We therefore generated the analogous mutant version of CISK-PX and compared its binding to PI(3)P with that of the wild type. Purified His-tagged CISK-PX or CISK-PX Y51A was incubated with PE/PC liposomes or liposomes containing 1–20% PI(3)P, and the protein remaining with the supernatant or associated with the liposome pellet was measured (Fig. 2*A*). As shown in Fig. 2*B*, CISK-PX bound strongly to liposomes containing only a small fraction of PI(3)P, reaching 50% of maximal binding at less than 2% PI(3)P. In contrast, although CISK-PX Y51A could associate with liposomes with high levels of PI(3)P, half-maximal binding required the inclusion of at least 10% PI(3)P. Thus, the Y51A mutation in CISK-PX substantially reduces its ability to bind PI(3)P *in vitro*.

CISK Localizes to Endosomal Membranes via PI(3)P Interaction. Studies by using a dimerized PI(3)P-binding FYVE domain have established that PI(3)P is localized almost exclusively to early endosomes as well as multivesicular endosomes derived from early endosome fusion (28). To determine CISK localization, we fused the CISK coding sequence to the C terminus of EGFP and expressed the construct in COS cells. Analysis by confocal microscopy shows that EGFP-CISK is localized to numerous small vesicular structures distributed throughout the cytoplasm but concentrated in the perinuclear region (Fig. 3*A*). This distribution was similar to the pattern of endosomes labeled by incubation of the cells with rhodamine-labeled transferrin for 15 min (Fig. 3*B*). Examination of the merged confocal images (Fig. 3*C*) revealed a high degree of coincidence between EGFP-CISK and rhodamine-transferrin distribution. Thus, full-length CISK is able to localize to the endosomal compartment of COS cells consistent with its ability to bind to PI(3)P through its PX domain. Expression of the protein kinase-deficient mutant EGFP-CISK K191A resulted in a punctate, vesicular pattern of fluorescence (Fig. 3*E*) and overlap with transferrin (not shown), indistinguishable from that of the wild type. Thus, protein kinase activity is not required for endosomal localization of CISK.

To further verify that localization of CISK to the endosomal compartment depends on a PI3-kinase product, cells expressing EGFP-CISK were treated with the PI3-kinase inhibitor wortmannin (Fig. 3*D*). Wortmannin treatment resulted in a diffuse cytoplasmic distribution of EGFP-CISK, with some accumulation in nuclei. Likewise, EGFP-CISK carrying the Y51A mutation that substantially reduces binding to PI(3)P *in vitro* displayed a diffuse pattern when expressed in COS cells (Fig. 3*F*). These results indicate that high affinity binding of the CISK PX domain to PI(3)P is required for its endosomal localization in mammalian cells.

To test whether the CISK PX domain is sufficient for endosomal targeting, we fused the isolated PX domain of CISK to EGFP and expressed the construct in COS cells. As shown in Fig. 4, EGFP-CISK-PX is localized to cytoplasmic vesicles (Fig. 4*A*) in a pattern similar to that of the full-length protein (Fig. 3). This vesicular pattern depends on PI3-kinase products because wortmannin treatment of the transfected cells results in diffuse cytoplasmic fluorescence (data not shown). Immunostaining of the same cells with antibody against the early endosome marker EEA1 showed a very

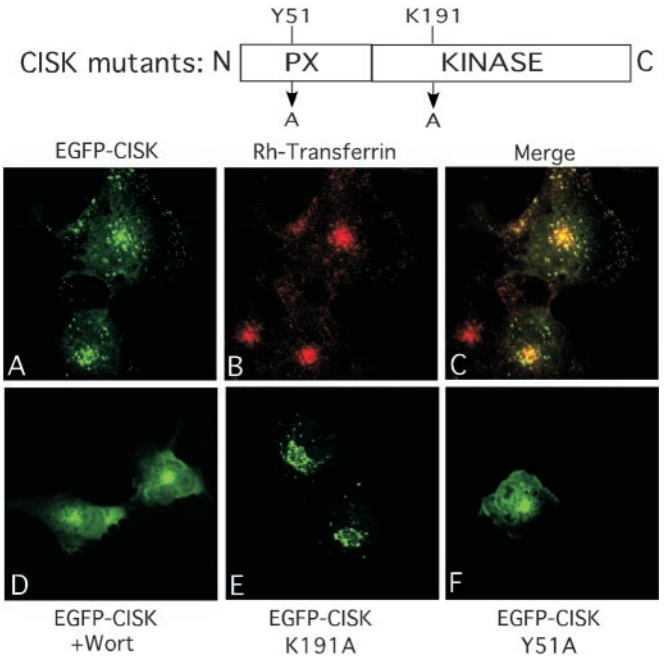

Fig. 3. CISK localizes to the endosomal compartment via interaction with PI(3)P. COS cells were transfected with EGFP-CISK (*A–D*), kinase domain mutant K191A (*E*), or PX-domain mutant Y51A. (*A–C*) Confocal images of cells labeled for 15 min with rhodamine-transferrin show coincidence of EGFP-CISK (*A*) with transferrin-containing cytoplasmic vesicles (*B* and *C*). (*D*) Vesicular localization of CISK is disrupted by treatment of cells with wortmannin (100 nM, 15 min). (*E* and *F*) Localization of CISK mutants. Vesicular localization of CISK is not affected by a kinase domain mutation (K191A; *E*) but is disrupted by a point mutation in the PX domain (Y51A; *F*).

similar pattern (Fig. 4*B*). The merged image (Fig. 4*C*) reveals a high degree of coincidence, consistent with the binding of CISK-PX as well as EEA1 to PI(3)P-containing early endosomes. However, numerous vesicles, particularly in the periphery of the cell shown, label strongly with EGFP-CISK PX but not with anti-EEA1. We assume that this result reflects the persistence of PI(3)P in fused endosomes because Gillooly *et al.* (28) have demonstrated the presence of PI(3)P in structures identified as multivesicular endosomes, which lack EEA1. Their FYVE-domain dimer, like CISK-PX, was shown to bind strongly and selectively to PI(3)P.

Interestingly, cells expressing high levels of EGFP-CISK-PX (Fig. 4*D*) display a disruption in the perinuclear collection of transferrin-labeled endosomes (Fig. 4*E*). Endosomes in these cells expressing EGFP-CISK-PX appeared enlarged as well as more dispersed, compared with untransfected cells. Similar effects have been reported on treatment of cells with wortmannin and in cells overexpressing a FYVE-domain dimer, which binds PI(3)P (28).

As anticipated, the CISK PX domain Y51A mutation that eliminates PI(3)P binding *in vitro* also prevented endosomal localization *in vivo* (Fig. 4*G*). Instead, diffuse cytoplasmic and nuclear staining was observed. Overexpression of EGFP-CISK-PX Y51A had little or no effect on the transferrin-containing endosomal compartment (Fig. 4*H*), supporting the hypothesis that competition for PI(3)P binding is responsible for the disruptive effect of the functional PX domain.

CISK Activation by Growth Factors Depends On Its Binding to PI(3)P. Next, we investigated whether regulation of CISK depends on endosomal localization through binding of its PX domain to PI(3)P. CISK was reported to be activated in 32D cells by IL-3 in a PI3-kinase-dependent pathway (23), similar to the regulation of Akt and SGK protein kinases downstream of receptor tyrosine kinase-stimulated PI3-kinase (7). In the present experiments, triple HA-tagged CISK was expressed in COS cells treated with or without IGF-1 or EGF, lysates prepared, and protein kinase activity by using histone H2B as substrate measured in anti-HA immunoprecipitates (Figs. 5 and 6). Immunoprecipitates from cells transfected with the 3xHA vector alone contained only trace amounts of H2B kinase activity, whether or not cells were treated with IGF-1 (Fig. 5 *Top*, lanes 1 and 2) even though endogenous Akt was activated by IGF-1 treatment (Fig. 5 *Bottom*). On transfection of HA-tagged CISK, protein kinase activity was observed in anti-HA immunoprecipitates, and this activity was increased severalfold on treatment of cells with IGF-1 (Fig. 5, lanes 3 and 4) or EGF (Fig. 5, lane 13). Growth factor stimulation of H2B kinase activity was eliminated by pretreatment of the cells with the PI3-kinase inhibitor wortmannin (Fig. 5, lanes 6 and 14), which also prevented activation of endogenous Akt (Fig. 5 *Bottom*). Recovery of expressed proteins in the immunoprecipitates was verified by immunoblotting with anti-HA antibody (Fig. 5 *Middle*). No significant protein kinase activity was recovered in immunoprecipitates from cells expressing CISK K191A, indicating that the measured kinase activity of the wild type is due to the transfected protein and not components associated with the immunoprecipitates. Transfected HA-tagged Akt could be stimulated under these assay conditions, as expected, by IGF-1 (Fig. 5, lanes 11 and 12). The magnitude of activation by IGF-1 is probably somewhat underestimated because of the elevated basal activity of both CISK and Akt when overexpressed (Fig. 5, lanes 2 and 11). Thus, the results reveal that IGF-1 and EGF activate both CISK and Akt in a PI3-kinase-dependent pathway.

We then tested whether endosomal location of CISK is required for growth factor activation by analyzing immunoprecipitates from cells expressing mutant CISK Y51A. Basal protein kinase activity in anti-HA immunoprecipitates was comparable to that from unstimulated cells expressing wild-type CISK (compare Fig. 5, lanes 3 and 9). Unlike wild-type CISK, however, CISK Y51A activity was not increased by IGF-1 (lane 10) or EGF (data not shown). These results for IGF-1 stimulation in three independent experiments are quantified in Fig. 6. Thus, an intact PX domain capable of binding PI(3)P and targeting CISK to endosomes is required for its activation by growth factors.

Discussion

The data presented here demonstrate that the protein kinase CISK is a member of an emerging group of proteins that bind to PI(3)P via a PX domain (Figs. 1 and 2). In the case of CISK, we provide strong evidence supporting the conclusion that its PX domain is both necessary and sufficient for binding PI(3)P and localizing this protein kinase to endosomes. A point mutation within the PX domain that attenuates PI(3)P binding in biochemical assays (Fig. 2) also disrupts endosome localization of CISK in intact cells (Fig. 3). Further, the CISK PX domain itself localizes to endosomes when expressed in COS cells (Fig. 4), in contrast to the PX domain of Vam7p, which also binds PI(3)P (18, 19, and data not shown). This result suggests that the CISK PX domain has a particularly high affinity for PI(3)P or perhaps forms oligomers with enhanced affinity for PI(3)P. Our results do not exclude other interactions, perhaps protein–protein, that might also stabilize localization to endosomes. Many PX domains include a proline-rich domain, a target of SH3 domain binding. In $p47^{phox}$, this PXXP motif was shown to be on an accessible surface of the domain and binds the SH3 domain of the same protein (29). CISK PX is one of the few PX domains that lack a PXXP motif, but it could conceivably contain other unrecognized protein binding targets.

A key finding of these studies is that targeting of CISK to endosomal membranes via its highly specific binding to PI(3)P is required for its activation by IGF-1 or EGF (Figs. 5 and 6). Both of these growth factors stimulate Class I PI3-kinase activity in COS cells and by this means also activate Akt (30). The sequence similarity between CISK and Akt, particularly in the putative

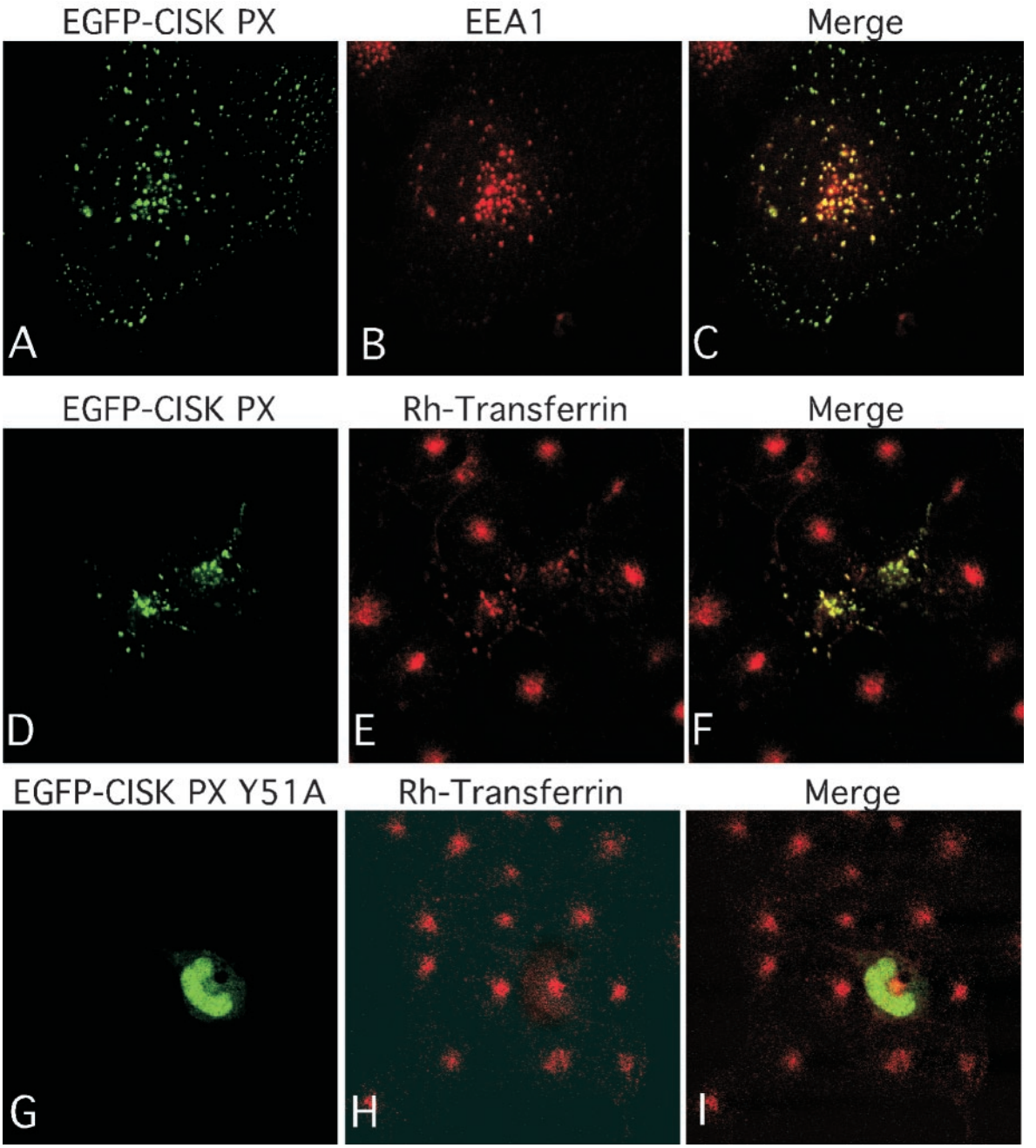

Fig. 4. The PX domain of CISK is sufficient for endosomal localization. COS cells were transfected with EGFP-CISK PX (*A–F*) and then stained with anti-EEA1 antibody (*A–C*) or loaded with rhodamine-transferrin for 15 min (*D–I*). Wild-type EGFP-CISK-PX (*A*) overlaps with the early endosome marker EEA1 (*B* and *C*). Expression of high levels of EGFP-CISK-PX (*D*) enlarges and disrupts perinuclear of rhodamine-labeled endosomes (*E* and *F*) whereas expression of the Y51A mutant with attenuated PI(3)P binding (*G*) has no effect on the transferrin compartment (*H* and *I*).

regulatory phosphorylation sites analogous to serine-473 and threonine-308 of Akt, strongly suggest that CISK is also a regulated target of the PI3-kinase-dependent protein kinase PDK1. However, the prevailing model for Akt activation invokes the ability of both PDK1 and its substrate Akt to localize to the plasma membrane by virtue of interaction of their respective PH domains with Class I PI3-kinase products PI(3,4)P_2 and PI(3,4,5)P_3 (31, 32). It is not clear how a similar mechanism can be invoked to explain CISK activation. Several studies by using selective PH domains as probes have detected PI(3,4)P_2 and PI(3,4,5)P_3 exclusively at the plasma membrane (33–36). By contrast, by using a dimerized FYVE domain as probe for PI(3)P, Gillooly *et al.* (28) have detected PI(3)P only on the limiting membranes of early endosomes and on intralumenal vesicles of multivesicular endosomes. It may be that, under some circumstances, perhaps during the earliest stages of ligand-stimulated receptor internalization, PI(3)P and PI(3,4,5)P_3 or PI(3,4)P_2 exist in proximity whereupon activated PDK1 can contact and phosphorylate PI(3)P-bound CISK. A recent report that a Class II PI3-kinase, denoted Cpk or PI3-kinase C2α, localizes to clathrin-coated endocytic vesicles (37) raises the intriguing possibility that PI(3)P, the favored product of Cpk *in vitro* (38, 39), could be generated early in endocytosis, as well as via the Rab5-dependent activation of the vps34 PI3-kinase in early endosomes (40). Alternatively, an as yet undiscovered PDK1-like kinase lo-

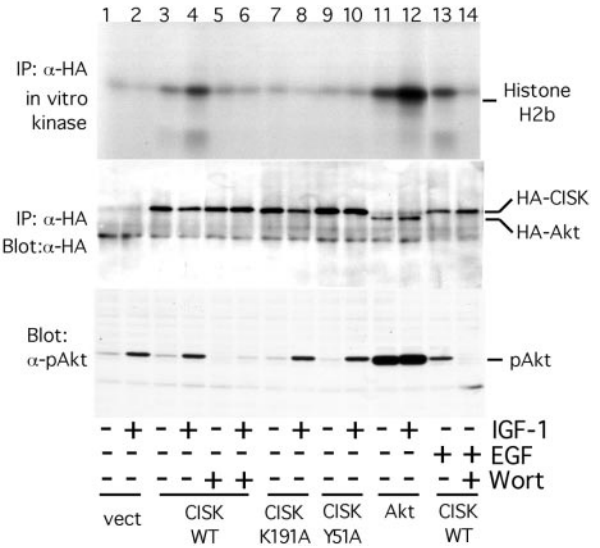

Fig. 5. CISK activation by growth factors depends on PI 3-kinase activity and binding of its PX domain to PI(3)P. (*A*) Cos cells were transfected with empty vector (lanes 1 and 2) or the HA-tagged CISK or Akt constructs as indicated (lanes 3–14). After treatment of cells with IGF-1, EGF, and/or wortmannin as indicated, cell lysates were prepared, and HA-tagged proteins were immunoprecipitated with anti-HA antiserum. Immunoprecipitates were split, and one portion was assayed for kinase activity toward histone H2B substrate. (*Top*) The autoradiogram showing ^{32}P-labeling of histone. Another portion of the anti-HA immunoprecipitate was analyzed by immunoblotting using anti-HA antibody. (*Middle*) Chemiluminescence detection of HA-tagged CISK or AKT in the immunoprecipitates. Cell lysates from the same experiment were subjected to immunoblotting with anti-phospho-Akt (Ser-473) antibody. Detection of phospho-Akt (*Bottom*) shows growth factor activation of Akt dependent on PI3-kinase activity.

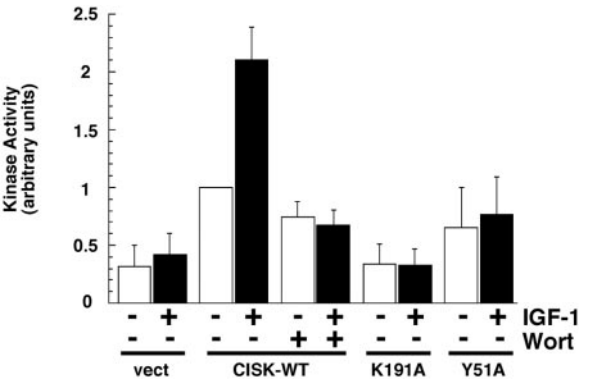

Fig. 6. CISK activation by IGF-1 requires a functional, PI(3)P-binding PX domain. The bars indicate histone phosphorylation activity in immunoprecipitates as shown in Fig. 5. Activity shown is relative to that measured in CISK-wild type immunoprecipitates, which is assigned 1 unit to compare multiple experiments. Data shown are the averages ± SD for three independent assays.

cated and activated in endosomal membranes could be responsible for the activation of CISK.

Regardless of the precise means of activation, the localization of CISK to the endosomal compartment suggests a distinct set of substrates and therefore distinct functional consequences of CISK activation. Because it was identified as a factor in IL-3-mediated survival of lymphocytes and shares activation pathways as well as substrates of Akt (23), functional overlap of CISK with Akt seems probable. Akt has been implicated in numerous cellular functions besides survival, including membrane trafficking phenomena such as the insulin-stimulated translocation of glucose transporters to the cell surface in fat and muscle cells (10, 11). We speculate that CISK may similarly be a key element in connecting PI3-kinase-dependent signaling pathways to trafficking events involving the endosomal membrane system. In addition, growth factor-regulated protein phosphorylation by CISK adds to the variety of functions of PI(3)P binding proteins with FYVE or PX domains (reviewed in refs. 41 and 42). These functions, as well as the importance of a recently described PI(3)P phosphatase in myogenesis (43), point to roles of PI(3)P in many other aspects of cell growth, differentiation, and regulation.

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