Identification of a Novel Domain in Two Mammalian Inositol-polyphosphate 5-Phosphatases That Mediates Membrane Ruffle Localization

THE INOSITOL 5-PHOSPHATASE SKIP LOCALIZES TO THE ENDOPLASMIC RETICULUM AND TRANSLOCATES TO MEMBRANE RUFFLES FOLLOWING EPIDERMAL GROWTH FACTOR STIMULATION®

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SKIP (skeletal muscle and kidney enriched inositol phosphatase) is a recently identified phosphatidylinositol 3,4,5-trisphosphate- and phosphatidylinositol 4,5-bisphosphate-specific 5-phosphatase. In this study, we investigated the intracellular localization of SKIP. Indirect immunofluorescence and subcellular fractionation showed that, in serum-starved cells, both endogenous and recombinant SKIP colocalized with markers of the endoplasmic reticulum (ER). Following epidermal growth factor (EGF) stimulation, SKIP transiently translocated to plasma membrane ruffles and colocalized with submembranous actin. Data base searching demonstrated a novel 128-amino acid domain in the C terminus of SKIP, designated SKICH for SKIP carboxyl homology, which is also found in the 107-kDa 5-phosphatase PIPP and in members of the TRAF6-binding protein family. Recombinant SKIP lacking the SKICH domain localized to the ER, but did not translocate to membrane ruffles following EGF stimulation. The SKIP SKICH domain showed perinuclear localization and mediated EGF-stimulated plasma membrane ruffle localization. The SKICH domain of the 5-phosphatase PIPP also mediated plasma membrane ruffle localization. Mutational analysis identified the core sequence within the SKICH domain that mediated constitutive membrane association and C-terminal sequences unique to SKIP that contributed to ER localization. Collectively, these studies demonstrate a novel membrane-targeting domain that serves to recruit SKIP and PIPP to membrane ruffles.

Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) serves as a precursor to two major signaling pathways. This central phosphoinositide is phosphorylated by phosphoinositide 3-kinase, forming PtdIns(3,4,5)P3, which transiently accumulates at the plasma membrane of growth-factor-activated cells and is subsequently rapidly metabolized by PtdIns(3,4,5)P3 5- or 3-phosphate-specific lipid phosphatases. PtdIns(3,4,5)P3 recruits various effector proteins that contain PH domains, such as the serine/threonine kinases Akt and PDK1 (1), and Rho, Rac, and ADP-ribosylation factor guanine nucleotide exchange factors, including P-Rex1 (2) and SWAP-70 (3), which in turn regulate many signaling pathways, including inhibition of cell death, cell cycle progression, actin polymerization, membrane ruffling, cell migration, and secretion (4–7). In addition, PtdIns(4,5)P2 is hydrolyzed by phospholipase C to produce inositol 1,4,5-trisphosphate (Ins(1,4,5)P3) and diacylglycerol, which mobilize intracellular calcium and activate protein kinase C, respectively. PtdIns(4,5)P2 itself regulates the activity of actin-binding proteins by suppressing the function of vinculin, coflin, gelsolin, and profilin and by activating the actin-gelatinizing activity of α-actinin (8).

The inositol-polyphosphate 5-phosphatases (referred to as 5-phosphatases) are a large family of signal-modifying enzymes that hydrolyze the 5-phosphate from the inositol ring of both inositol phosphates, such as Ins(1,4,5)P3 and Ins(1,3,4,5)P4, and/or PtdIns-derived messenger molecules, including PtdIns(4,5)P2, PtdIns(3,4,5)P3, and PtdIns(3,5)P2 (9). Ten mammalian and four yeast homologs have been identified and characterized. Two recent studies using a bioinformatics approach (10) and x-ray crystallography (11) have demonstrated that the 5-phosphatases belong to the family of apurinic/apyrimidinic endonucleases and share the same catalytic mechanism of action. However, clarification of the substrate specificity of the various 5-phosphatases, in particular their in vivo substrates, awaits further delineation.

Many recent studies have demonstrated that the mammalian 5-phosphatases play critical roles in regulating phosphoinositide 3-kinase and PtdIns(4,5)P2 signals. Gene-targeted deletion of SHIP-1 results in a phenotype of myeloid cell expansion, splenomegaly, and lung infiltration (12, 13), whereas SHIP-2 knockout mice demonstrate insulin hypersensitivity and die from hypoglycemia (14). Synaptotagmin-1-deficient mice show accumulation of clathrin-coated vesicles in post-synaptic neurons and early death (15). Humans deficient in OCRL 5-phosphatase (16) demonstrate growth and mental retardation, renal tubular acidosis, and cataracts, although mice that lack this enzyme surprisingly show no phenotype (17). Homozygous male 5-phosphatase II-deficient mice are infertile due to disrupted sperm function (18).

Two novel mammalian 5-phosphatases designated SKIP for skeletal muscle and kidney enriched inositol phosphatase (19) and PIPP for proline-rich inositol-polyphosphate 5-phosphatase.

Lost in translation
tase (20) have recently been cloned, but not extensively char-
acterized. The 5-phosphatase SKIP is a 51-kDa enzyme that
contains a central catalytic 5-phosphatase domain with no
other reported motifs. Kinetic analysis using recombinant
5-phosphatase indicates that the enzyme’s major substrates in
vitro are PtdIns(3,4,5)P3 and PtdIns(4,5)P2. Ectopic overex-
pression of SKIP in COS-7 cells results in loss of actin stress
fibers and disruption of intracellular membranes. Immunocy-
chemistry using antibodies specific for SKIP in transfected
K562 cells revealed a localization of SKIP in membrane ra-
folds (21). PIPP is a 107-kDa 5-phosphatase that hydrolyzes
PtdIns(4,5)P2, Ins(1,4,5)P3, and Ins(1,3,4,5)P4 and localizes to
membrane ruffles (20).

Here we investigate the intracellular localization of SKIP
and show that, in the unstimulated cell, this 5-phosphatase
localizes to the ER. Following growth factor stimulation, SKIP
translocated to plasma membrane ruffles, mediated by the
novel C-terminal domain SKICH, for SKIP carboxyl homology.

Bioinformatics analysis demonstrates that this C-terminal do-
main is also present in PIPP and several other signaling pro-
teins. We propose that the SKICH domain directs plasma mem-
brane ruffle localization in these 5-phosphatases.

EXPERIMENTAL PROCEDURES

MATERIALS—Restriction and DNA-modifying enzymes were obtained
from New England Biolabs Inc. or MBI Fermentas. The human testsis
Marathon-Ready cDNA kit was from Clontech. Synthetic oligonucleo-
tides were from Generows (Adelaide, Australia) or the Department of
Microbiology, Monash University (Melbourne, Australia). Antibodies
were from the American Type Culture Collection. The pCGN vector and anti-
EEA1 (early endosomal autoantigen-1) antibody were gifts from Drs. T.
Tiganis and Ban-Hock Toh (Monash University), respectively. Mono-
clonal antibodies were from the following sources: anti-hemagglutinin
(HA; Babco), anti-green fluorescent protein (GFP; Roche Molecular
Biochemicals), anti-calnexin (Affinity Bioreagents Inc.), and anti-
LAMP2 (Hybridoma Bank, University of Iowa). Alexa fluor/Texas Red-
conjugated anti-mouse IgG, or with Texas Red-conjugated concanavalin A for 5 min at
4 °C. Coverslips were mounted using SlowFade (Molecular Probes, Inc.)
and visualized by confocal microscopy (Leica).

**Transient Expression of GFP- or HA-tagged SKIP/SKICH and SKIP Domain Mutants**—The open reading frame of SKIP was PCR-
amplified and cloned in frame into the pEGFP-C2 (Clontech) or pCGN
vector, adding an N-terminal GFP or HA tag, respectively, to SKIP. The
 SKIP truncation mutants SKIPΔSKICH (amino acids (aa) 1–324) and the
SKIP-SKICH (aa 321–448) and the SKICH domain mutants SKIP-
ΔSKICH (aa 321–431), SKIP-(321–431), and SKIP-(321–448) were also generated by PCR amplification and cloned in frame into the pEGFP or pGFP vector. Site-directed mutagenesis of the SKICH domain (aa 321–448) was performed by dideoxy se-
quencing of both strands. COS-7 cells were transiently transfected by
electroporation with 1–2 μg of full-length SKIP or its truncation con-
structs. Approximately 3 × 10⁵ cells were electroporated in DMEM in a
total volume of 200 μl in a 0.4-cm sterile cuvette at 200 V and 975
microfarads using the Bio-Rad Gene Pulser II. After 48 h, the cells were
fixed and permeabilized as described above. The HA-tagged proteins
were visualized with anti-HA antibody and diaminobenidine (DAB) or
Alexa 488-

conjugated anti-mouse IgG. The expression of the GFP- or HA-tagged recombinant protein was confirmed by immunoblot analysis of the
transfected cells by Western blotting with anti-GFP or anti-HA antibody.

**Intracellular Localization of Recombinant 107-kDa PIPP**—The full-
length cDNA encoding mouse PIPP or the SKICH domain (aa 767–836)
of PIPP was PCR-amplified and cloned in frame into the pCGN vector,
adding an N-terminal HA tag. The constructs were transiently trans-
fected into COS-7 cells as described for the SKIP constructs. The HA-
tagged recombinant proteins were stained with anti-HA antibody, and
fluorescence was analyzed by confocal microscopy as described above.

**Redistribution of Endogenous or Recombinant SKIP upon EGF or Insulin Stimulation**—COS-7 and U87 cells were grown on coverslips in
DMEM supplemented with 10% FCS, and C2C12 cells were grown in DMEM supplemented with 20% FCS. The cells were serum-starved by
placement in DMEM containing 0.1% FCS for 24 h. COS-7 or C2C12 cells transfected with various SKIP constructs were grown in normal
medium for 24 h before serum starvation. Following overnight serum
starvation, the cells were stimulated with EGF (100 ng/ml), insulin (100 nm), fixed, permeabilized, and stained with affinity-purified anti-
SKIP antibodies or anti-HA antibody as described above; and where
indicated, the cells were also stained with phallloidin. The localization of
the wild-type and truncation fusion proteins at the plasma membrane was quantified by scoring >200 transfected cells over three independ-
ent experiments. For all constructs, cells demonstrating plasma mem-
brane staining were expressed as a percentage of total transfected cells.

**Phosphoinositide 5-Phosphatase Assays**—The open reading frame
of SKIP was PCR-amplified and cloned in frame into the pTrcHisB vector
(Invitrogen), adding an N-terminal hexahistidine tag. The recombinant

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² Available at www.ncbi.nlm.nih.gov.
pressed in COS-7 cells. Immunoblot analysis of SKIP-transfected cells using antibodies specific to each tag indicated that HA- or GFP-tagged recombinant 51-kDa SKIP was expressed intact with minimal proteolysis (Fig. 1C).

The localization of endogenous 51-kDa 5-phosphatase was further investigated by comparing the SKIP staining pattern in U87 cells with that of antigens commonly used as markers of specific intracellular compartments (Fig. 2A). Specific antibodies were used in separate samples to stain the lysosomes (LAMP2), clathrin-coated vesicles (γ-adaptin), ER (antibodies to the ER-specific protein calnexin or concanavalin A), or poly-merized actin (phalloidin). Little colocalization was observed with the lysosomes (Fig. 2A). Some areas of colocalization were detected between SKIP and γ-adaptin, as shown in the merged images; however, 5-phosphatase expression also appeared in areas distinct from γ-adaptin staining. The majority of SKIP staining coincided with the ER, as shown by colocalization with calnexin and concanavalin A. No colocalization ofSKIP with phallolidin staining or with the endosomal marker EEA1 was demonstrated (data not shown). Little expression of SKIP was detected at the plasma membrane. Analysis of the expression of HA-tagged recombinant 51-kDa SKIP demonstrated colocalization with the ER-specific marker concanavalin A when the recombinant protein was expressed in COS-7 cells, but no colocalization with early endosomes (EEA1) and only partial colocalization with the Golgi marker β-COP (Fig. 2B).

We also determined the SKIP localization in subcellular fractions of serum-starved and EGF-stimulated U87 cells, isolated using a simplified differential centrifugation that enables enrichment of fractions with markers for the plasma membrane/ER, endosomes and clathrin-coated vesicles (high density microsomes), and intracellular membranes comprising clathrin-coated vesicles and other membranes in the high speed pellet (24). The supernatant designated the cytosol contained all elements that did not sediment at 177,000 × g (Fig. 2C, CYT). In whole cell lysates of U87 cells, two isoforms of SKIP migrating at ~51 and 36 kDa, respectively, were detected, consistent with the two predicted isoforms identified by cloning studies. Following subcellular fractionation, the 51-kDa SKIP isoform was found in the plasma membrane/ER fraction, colocalizing with markers specific for the plasma membrane (EGF receptor) and the ER (calnexin). Markers for the early endosomes (EEA1) also co-sedimented with this fraction. Neither SKIP isoform was detected in the cytosolic fraction. However, the smaller SKIP isoform was consistently found in the high speed pellet, which co-sedimented with the marker for trans-Golgi-derived clathrin-coated vesicles, γ-adaptin, consistent with the colocalization data shown in Fig. 2A. The smaller isoform of SKIP may have a discrete membrane localization; however, this isoform is unlikely to express 5-phosphatase activity, as it lacks the 57 amino acids of the N-terminal 5-phosphatase domain (19) and therefore lacks critical conserved residues that have recently been shown to be essential for 5-phosphatase catalytic activity (10).

**RESULTS**

**Characterization of the Intracellular Localization of SKIP**—To investigate the subcellular distribution of SKIP isoforms, we developed anti-peptide antibodies raised against a fusion peptide representing the N- and C-terminal seven amino acids of human 51-kDa SKIP (19). Immunoblot analysis using this affinity-purified antibody detected a 51-kDa polypeptide in both undifferentiated Sol8 myoblasts and differentiated myotubes. A weakly staining polypeptide of 36 kDa was also detected (Fig. 1A), which may represent the smaller spliced isoform of SKIP predicted by cloning studies (19). In U87 cells, a 51-kDa peptide and a faint 36-kDa peptide were detected in the Triton-soluble fraction.

The intracellular localization of endogenous SKIP was investigated by indirect immunofluorescence microscopy of several cell lines, including COS-7 and U87 cells. In all cell lines, the predominant staining pattern was punctate, localizing to the perinuclear region (Fig. 1B). Preimmune antiserum was non-reactive. Similar results were obtained when the recombinant 51-kDa isoform of SKIP fused to either HA or GFP was expressed in COS-7 cells. Immunoblot analysis of SKIP-transfected cells using antibodies specific to each tag indicated that HA- or GFP-tagged recombinant 51-kDa SKIP was expressed intact with minimal proteolysis (Fig. 1C).
and enzyme assays were performed using \(^{32}\)P-labeled PtdIns(3,5)P\(_2\) as the substrate. The identity of PtdIns(3,5)P\(_2\) was verified as described previously (25). No hydrolysis of PtdIns(3,5)P\(_2\) was detected using purified recombinant enzyme. However, SKIP actively hydrolyzed PtdIns(3,4,5)P\(_3\), forming PtdIns(3,4)P\(_2\) (Fig. 3), indicating that the enzyme is active, but unlikely to regulate PtdIns(3,5)P\(_2\).

**SKIP Translocates to the Plasma Membrane of EGF- or Insulin-stimulated Cells**—A significant in vitro substrate for SKIP is PtdIns(3,4,5)P\(_3\) (19). Because it is not clear if PtdIns(3,4,5)P\(_3\) is present on the ER, we investigated the subcellular distribution of SKIP in COS-7 cells following EGF stimulation, conditions under which PtdIns(3,4,5)P\(_3\) is transiently synthesized on the inner surface of the plasma membrane (28, 29). COS-7 cells were serum-starved for 24 h and then stimulated with 100 ng/ml recombinant EGF for up to 20 min. The localization of endogenous SKIP was detected by indirect immunofluorescence (Fig. 4). In >90% of unstimulated cells, SKIP showed a predominantly perinuclear distribution. However, within 1 min of EGF stimulation, SKIP staining was detected at the plasma membrane initially at membrane ruffles, areas at the leading edge of the cell (lamellipodia) where the plasma membrane detaches from the support and rolls up (Fig. 4, arrows). By 5 min of stimulation, SKIP localized diffusely at the plasma membrane. Despite the increase in plasma membrane staining, there was no obvious decrease in perinuclear staining. The localization of SKIP at

![Fig. 3. SKIP phosphoinositide 5-phosphatase activity.](image)

**Fig. 3. SKIP phosphoinositide 5-phosphatase activity.** Purified His-tagged recombinant SKIP in duplicate samples (lanes 2 and 3) or the His tag alone (lane 1) was used in 5-phosphatase enzyme assays and analyzed by thin-layer chromatography using the \(^{32}\)P-labeled phosphoinositide substrates PtdIns\(^{32}\)P(3,5)P\(_2\), (a) and PtdIns\(^{32}\)P(3,4,5)P\(_3\), (b). The migration positions of phosphoinositides are shown on the right.

![Fig. 2. Colocalization of SKIP with ER-specific markers.](image)

**Fig. 2. Colocalization of SKIP with ER-specific markers.** A, U87 cells were labeled with affinity-purified anti-peptide antibodies to SKIP and antibodies to organelle-specific markers as indicated: LAMP2 (lysosomes), \(\gamma\)-adaptin (Golgi), calnexin (ER), or concanavalin A (ER). Anti-SKIP antibodies were visualized with Alexa 488-conjugated anti-rabbit IgG, and organelle-specific markers were detected with Alexa 594-conjugated anti-mouse IgG or Texas Red-labeled concanavalin A. Fluorescence was analyzed by confocal microscopy. Regions of colocalization appear yellow in overlay images. B, COS-7 cells were transiently transfected with construct encoding HA-tagged SKIP and processed for indirect (anti-HA, Alexa 488-conjugated anti-mouse IgG) immunofluorescence. The transfected cells were also counterstained with antibody to the Golgi marker \(\beta\)-COP, anti-EEA1 antibody, or Texas Red-labeled concanavalin A as indicated. Areas of colocalization appear yellow in overlay images. C, serum-starved (0 min) and EGF (100 ng/ml)-stimulated (5 min) U87 cells were fractionated using sequential differential centrifugation. Fractions including the whole cell lysate (WCL), high density microsomes (HDM), cytosol (CYT), plasma membrane/ER (PM/ER), and high speed pellet (HSP) were analyzed by immunoblotting with anti-peptide antibodies to SKIP. The fractions were also immunoblotted with organelle-specific markers: calnexin (ER), \(\gamma\)-adaptin (Golgi), EGF receptor (EGFR; plasma membrane), and EEA1 (early endosomes). The migration positions of molecular mass markers are shown on the left.
the plasma membrane was only transient; and within 20 min, the 5-phosphatase relocalized to a perinuclear distribution, with little plasma membrane staining detected. Co-staining of COS-7 cells with phalloidin, which stains polymerized actin, demonstrated no colocalization of SKIP with actin in unstimulated cells. However, following EGF stimulation, conditions under which membrane ruffles and actin are actively formed and remodeled (30), SKIP colocalized with submembranous actin (as shown by co-staining with phalloidin) at the leading edge of the cell, specifically at membrane ruffles (Fig. 4A, arrow). As previous studies have demonstrated that SKIP is most highly expressed in skeletal muscle, we repeated these studies by staining endogenous SKIP (Fig. 4C) or by expressing HA-tagged recombinant SKIP in the skeletal muscle cell line C2C12 (Fig. 4D). In unstimulated cells, SKIP localized in a perinuclear distribution, with minimal colocalization with submembranous actin detected by phalloidin staining. Following insulin stimulation, conditions that promote membrane ruffling (31), both endogenous (Fig. 4C) and recombinant (Fig. 4D) SKIP staining was detected at areas of increased submembranous actin staining, consistent with localization at membrane ruffles (arrows in merged images). Membrane staining was not detected in cells expressing empty vector (data not shown).

Identification of a Novel C-terminal Domain Designated SKICH—To identify the possible domains mediating membrane ruffle localization, we undertook a bioinformatics analysis of the SKIP amino acid sequence. We have identified a novel domain in human SKIP, a 128-amino acid region C-terminal to the 5-phosphatase domain. Data base searching revealed that this region is highly conserved in mouse SKIP (68% identity, EXPECT score of $2 \times 10^{-49}$) and is also present C-terminal to the 5-phosphatase domain in 107-kDa PIPP (38% sequence identity, EXPECT score of $2 \times 10^{-36}$). Our searches revealed that the C-terminal region of human SKIP shares significant sequence similarity (20–23% identity, EXPECT scores ranging from $5 \times 10^{-36}$ to $2 \times 10^{-48}$) with an ~130-amino acid region in the N terminus of the recently described protein T6BP (TRAF6-binding protein), which has also been characterized as Tax1 (human T-cell leukemia virus type I)-binding protein-1, TXBP151 (32, 33). We have also noted this domain in a chicken (Gallus gallus) protein homologous to T6BP (GenBank™ accession number BAA94854). We also identified this domain in a cytoplasmic protein of unknown function, NDP52 (34). All EXPECT scores are well below the significance threshold of $1 \times 10^{-6}$ as described by Park et al. (35). A sequence alignment of all domains revealed that this novel domain is characterized by the highly conserved motif $DWXGX_{3}VGY_{3}W$ and a second motif, $GX_{3}PF$ (Fig. 5A). We suggest that this region in the C terminus of SKIP thus represents a novel domain, which we have designated SKICH for SKIP carboxyl homology. The original cloning studies reported that the SKIP amino acid sequence contains a central 5-phosphatase domain and no other signaling or targeting motifs; however, our analysis demonstrates that this domain is present in the C terminus of 2 of the
10 mammalian 5-phosphatases and in the N terminus of a distinct family of proteins, the TRAF6/Tax1-binding protein family (Fig. 5B). Hydrophobicity analysis demonstrated no obvious transmembrane region or C-terminal anchoring tail within the SKICH domain (data not shown).

Role of the SKICH Domain in Plasma Membrane Localization—To investigate the role of the SKICH domain in regulating intracellular localization, the wild-type 5-phosphatase (HA-SKIP), mutant recombinant SKIP lacking the SKICH domain (aa 1–324) (HA-SKIPΔSKICH), and the SKICH domain alone (aa 321–448) (HA-SKIP-SKICH) were expressed as fusion proteins with an HA tag in COS-7 cells (Fig. 6A). Immunoblot analysis using antibodies to the HA tag of transfected cell lysates demonstrated that these mutant recombinant proteins were expressed intact and migrated at their predicted molecular mass (Fig. 6B). To quantitate the localization of the wild-type and mutant fusion proteins at the plasma membrane, >200 transfected cells were scored (over three independent transfections for each construct), and the percentage of cells demonstrating SKIP plasma membrane expression was determined in both serum-starved and EGF-stimulated cells (Fig. 6C). In resting cells, wild-type recombinant SKIP localized in a perinuclear distribution, as shown for the endogenous protein, consistent with an ER localization; and 25% of cells demonstrated plasma membrane staining (Fig. 6, A and C). We noted that expression of recombinant SKIP resulted in a greater percentage of cells with plasma membrane staining in serum-starved cells than observed upon staining the endogenous protein using anti-peptide antibodies, when <5% of cells demonstrated plasma membrane staining (data not shown). In resting cells, the recombinant 5-phosphatase that lacked the SKICH domain (HA-SKIPΔSKICH) localized in a perinuclear distribution; however, in some cells, a more diffuse perinuclear localization was demonstrated compared with wild-type SKIP localization. The SKICH domain alone (HA-SKIP-SKICH) localized in serum-starved cells in a perinuclear distribution, with <25% of cells showing plasma membrane staining (Fig. 6A); however, in some cells, a more diffuse perinuclear localization was demonstrated compared with wild-type SKIP localization. The SKICH domain alone (HA-SKIP-SKICH) localized in serum-starved cells in a perinuclear distribution, with <25% of cells showing plasma membrane staining (Fig. 6A); however, in some cells, a more diffuse perinuclear localization was demonstrated compared with wild-type SKIP localization.
COS-7 cells were transiently transfected with constructs (100 ng/ml) for 10 min, and prepared for indirect immunofluorescence.

A

B

C

<25% of cells) following growth factor stimulation (Fig. 6, A and C). The SKICH domain alone (HA-SKIP-SKICH) behaved like the wild-type 5-phosphatase, with 80% of stimulated cells demonstrating significant plasma membrane staining.

We further characterized the SKIP SKICH domain, in particular, the role the regions surrounding the conserved motifs DWGXV, VXW, and GXPF play in mediating plasma membrane and/or ER localization. The SKICH domain truncation mutants included SKIP(321–431), which lacks the SKIP C-terminal region beyond the highly conserved motifs; SKIP-(360–448), which lacks the N-terminal region of the SKICH domain prior to the conserved motifs; and SKIP(360–431), which comprises only the conserved regions within the SKICH domain common to both SKIP and PIPP (Figs. 5 and 7A). For clarity, we have also shown the localization of the intact SKIP SKICH domain. We expressed these various recombinant proteins and determined the percentage of cells demonstrating plasma membrane expression in resting and growth factor-stimulated cells. These recombinant proteins were expressed at the predicted size with minimal proteolysis. In serum-starved cells, expression of SKIP(321–431), which lacks the C-terminal region of the SKICH domain, and expression of SKIP-(360–448), which lacks the N-terminal region of the SKICH domain, demonstrated that 33% of cells showed plasma membrane staining, an increased percentage compared with the intact SKICH domain (Fig. 7B versus Fig. 6C). Following growth factor stimulation, these mutant recombinant proteins behaved comparable to the intact SKICH domain, with 75–80% of cells showing plasma membrane staining (Fig. 7B).

The SKICH domain mutational analysis demonstrated that SKIP sequences C-terminal to residue 431 might play a role in preventing constitutive SKICH plasma membrane localization. We therefore investigated the localization of the C-terminal 18 amino acids of SKIP. This sequence is not found in the 5-phosphatase PIPP (Fig. 5), which did not localize to the ER, but rather localized constitutively to the plasma membrane (see Fig. 8). The SKIP C-terminal 18 amino acids (SKIP(431–448)) were fused to GFP and expressed in serum-starved and EGF-stimulated COS-7 cells (Fig. 7C). This fusion protein demonstrated a perinuclear expression under both resting and stimulated conditions, with little plasma membrane localization under either serum-starved or stimulated conditions. We also noted intense nuclear expression of this fusion protein, consistent with the passive diffusion of the recombinant 28-kDa fusion protein into the nucleus. Collectively, this analysis identified a “core sequence” within the SKICH domain that mediates plasma membrane localization in both serum-starved and growth factor-stimulated cells.

We noted, within the core sequence of the SKICH domain, a number of highly conserved aromatic amino acids, which, in other proteins such as the annexins, play a significant role in regulating membrane association (36). To investigate the role of conserved residues within the SKICH domain, we generated, by site-directed mutagenesis, mutations of three of the highly conserved aromatic residues within the intact SKICH domain (SKIP aa 321–448), Y349A or Y349F, W362A, and Y376A or Y376F, and one other highly conserved residue, D361A. Mutation of the conserved tyrosines (Y349A or Y349F and Y376A or Y376F) had no effect on SKICH EGF-stimulated membrane ruffle localization (Fig. 7D). However, the D361A mutant and mutation of the adjacent conserved aromatic residue (W362A) resulted in a significant decrease in SKICH membrane association following EGF stimulation. These data support the contention that the SKICH domain interacts in a specific manner with a specific partner (either protein or lipid) within the plasma membrane. However, we have not yet identified the precise nature of the partner and are currently investigating both possibilities.

The SKICH Domain Mediates Membrane Ruffle Localization of the 107-kDa 5-Phosphatase PIPP—Our bioinformatics analysis predicts that the recently identified 5-phosphatase PIPP also contains a SKICH domain. Studies by Mochizuki and Takenawa (20) have shown that PIPP localizes constitutively to membrane ruffles. PIPP contains extensive N- and C-terminal proline-rich domains. Overexpression of Myc-tagged recombinant PIPP and various deletion mutants in COS-7 cells by these investigators demonstrated that deletion of the N-terminal proline-rich domain does not result in loss of 5-phosphatase plasma membrane expression. However, deletion of the C-terminal 277 amino acids (aa 725–1001 of rat PIPP), which includes the SKICH domain followed by the C-terminal proline-rich domain, results in loss of localization of the 5-phosphatase to membrane ruffles (20). The sequences mediating membrane localization were not further delineated. Our studies predict

Fig. 6. Role of the SKICH domain in plasma membrane translocation. COS-7 cells were transiently transfected with constructs encoding HA-SKIP, HA-SKIPSKICH, or HA-SKIP-SKICH as shown. A, transfected cells were serum-starved for 24 h, stimulated with EGF (100 ng/ml) for 10 min, and prepared for indirect immunofluorescence (anti-HA, Alexa 488-conjugated anti-mouse IgG). B, 100-μg lysates of cells transfected with HA-SKIP-SKICH (lane 1) or HA-SKIP-SKICH (lane 2) were analyzed by immunoblotting with anti-HA antibody. C, transfected cells were scored for staining of recombinant fusion proteins at the plasma membrane in serum-starved (white bars) versus EGF-stimulated (gray bars) cells, and the results are expressed as the mean percentage ± S.D. of all cells examined. A minimum of 200 cells were counted for three independent transfections.
that the PIPP C-terminal region contains a SKICH domain located within aa 725–837, which encodes the analogous sequence in the SKIP-(360–431) mutant and may constitutively associate with the plasma membrane (Fig. 7A). We investigated whether this region in PIPP mediated localization to membrane ruffles. HA-tagged wild-type PIPP and the HA-
tagged PIPP SKICH domain alone (aa 767–360) (PIPP-SKICH) were expressed in resting and EGF-stimulated COS-7 cells. In >90% of cells, wild-type PIPP localized in both the resting and EGF-stimulated cells to membrane ruffles and also demonstrated perinuclear staining (Fig. 8, A and B). The isolated PIPP SKICH domain localized to the plasma membrane ruffles in both resting (70%) and EGF-stimulated (86%), comparable to the analogous region in the SKIP domain (SKICH; 360–431).

**DISCUSSION**

This study reports novel findings for two recently identified signal-terminating 5-phosphatases, SKIP and PIPP. First, SKIP, a PtdIns(4,5)P$_2$ and PtdIns(3,4,5)P$_3$ 5-phosphatase, localizes to the ER in the unstimulated cell. Second, SKIP translocates to membrane ruffles meditated by a novel resting and EGF-stimulated cells we have designated SKICH, which is found in another 5-phosphatase (PIPP) and members of the TRAF6-binding protein family. Third, we have delineated the core sequences within the SKICH domain that mediate constitutive membrane association and the surrounding sequences specific to SKIP that contribute to ER localization. Fourth, the 107-kDa 5-phosphatase PIPP also contains a SKICH domain that constitutively localizes to membrane ruffles in both resting and EGF-stimulated cells. The SKICH domain may thereby provide a mechanism for localizing these 5-phosphatases to their specific substrates on the inner wall of the plasma membrane.

**Intracellular Localization of SKIP at the ER**—In the unstimulated cell, SKIP is predominantly localized to the ER. PtdIns(3,4,5)P$_3$ has not been localized to the ER; however, it is of interest that another PtdIns(3,4,5)P$_3$ phosphatase, a PTEN homolog designated TPIP (TPEP (transmembrane phosphatase with tensin homology)) and PTEN homologous inositol lipid phosphatase a) that hydrolyzes the 3-phosphate from PtdIns(4,5)P$_2$, PtdIns(3,5)P$_2$, PtdIns(3,4)P$_2$, and PtdIns(3)P, also localizes to the ER (37). Although PtdIns(3,4,5)P$_3$ has not been identified in the ER, recent studies using the PH domain of phospholipase C$_6$, have identified, by electron microscopy, that PtdIns(4,5)P$_2$, in addition to its prominent localization on the plasma membrane, is also detected on the ER, Golgi stack, endosomes, and nucleus (38). SKIP also hydrolyzes PtdIns(4,5)P$_2$, forming PtdIns(4)P. Several recent studies have implied a potential role for PtdIns(4)P and PtdIns(4,5)P$_2$ in ER vesicular function. In yeast, there exists a substantial pool of the precursor of PtdIns(4,5)P$_2$, PtdIns(4)P, which is present in the ER (39). The mammalian PtdIns 4-kinase, the yeast homolog Stt4p (40, 41), and a novel PtdIns(5)P 4-kinase localize to the ER (42). PtdIns(4)P promotes Golgi exocytic trafficking and inhibits ER-to-Golgi transport. Yeast sac1 null mutants demonstrate accumulation of PtdIns(4)P, and trafficking from the ER to the Golgi is slowed (39). In a manner analogous to SKIP, the yeast PtdIns(4,5)P$_2$ 5-phosphatase Inp54p localizes to the cytosolic surface of the ER, anchored to this site via its C terminus (43). Furthermore, inp54 null mutants demonstrate enhanced secretion of reporter proteins from the ER, consistent with the contention that PtdIns(4,5)P$_2$ may play a positive role in the regulation of secretory transport from this compartment (43). The localization of SKIP to the ER may play a role analogous to Inp54p in regulating PtdIns(4,5)P$_2$ levels and thereby vesicular trafficking from this compartment.

**The SKICH Domain**—We have identified the SKICH domain as a novel plasma membrane-targeting domain. In addition to SKIP and PIPP, the SKICH domain occurs in several other proteins, including NDP52 and TXBP151 (T6BP). TXBP151 was originally identified as a Tax1-binding protein that binds the zinc finger protein A20, which acts as an inhibitor of cell death. Overexpression of TXBP151 inhibits tumor necrosis factor-induced apoptosis by an unknown mechanism (32). More recently, TXBP151, also called T6BP, has been shown to associate with TRAF6 in response to interleukin-1 stimulation. However, the interaction between TRAF6 and T6BP does not appear to regulate TRAF6-induced NF-κB or JNK (c-Jun N-terminal kinase) activation (33). In addition to the N-terminal SKICH domain, the T6BP family contains three coiled-coil regions that are proposed to be involved in TRAF6 binding (Fig. 5B). Other T6BP family members that contain an N-terminal SKICH domain include a 62-kDa muscle-derived protein from chicken, which promotes neurite outgrowth (44). Our bioinformatics analysis predicts that NDP52, whose function is currently unknown, and T6BP are structurally related (Fig. 5B). NDP52 is a protein that was originally identified as a component of nuclear domain 10 (34), but was subsequently shown to have a cytosolic localization (45). The intracellular localization of T6BP has not been reported; however, the results of our studies suggest that this protein and NDP52 may localize to the plasma membrane in resting or growth factor-activated cells, mediated by the SKICH domain.

The lipid microenvironment in which a signaling molecule is located can direct the activation of downstream signaling cascades and interaction with other signaling components. The SKICH domain may mediate either protein/protein or protein/lipid interactions. It may function as a lipid-binding domain, as
has been shown for PH domains, PX domains, and C2 domains; and this is the subject of ongoing laboratory investigations. However, we have noted that, although the PIPP SKICH domain constitutively associates with the plasma membrane, the SKIP SKICH domain mediates plasma membrane localization predominantly in response to growth factor stimulation. Deletion mutant analysis identified the highly conserved core sequence within the SKICH domain that mediates constitutive association with the plasma membrane in both SKIP and PIPP. These data suggest that the proteins or lipids to which the SKICH domain binds are constitutively present in the plasma membrane. Because PtdIns(3,4,5)P3 is not detected in serum-starved cells, this implies that this region of the SKICH domain does not act like the PH domain and bind to PtdIns(3,4,5)P3 or to other signaling proteins that are localized to the plasma membrane only in stimulated cells.

**Localization of SKIP and PIPP at Membrane Ruffles**—The translocation of SKIP to membrane ruffles in response to EGF stimulation was not reported by Ijuin et al. (19). However, this may relate to the transient nature of the 5-phosphatase association with membrane ruffles, as the enzyme rapidly relocates to the ER within 20 min of stimulation. Recent studies have demonstrated that the spatial and temporal accumulation of PtdIns(3,4,5)P3 at membrane ruffles correlates with actin repolymerization at this site (46). In contrast to SHIP-2, which also contains a SKICH domain and this is the subject of ongoing laboratory investigations. However, we have noted that, although the PIPP SKICH domain binds are constitutively present in the plasma membrane. Because PtdIns(3,4,5)P3 is not detected in serum-starved cells, this implies that this region of the SKICH domain does not act like the PH domain and bind to PtdIns(3,4,5)P3 or to other signaling proteins that are localized to the plasma membrane only in stimulated cells.

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