Syntrophins are scaffold proteins of the dystrophin glycoprotein complex (DGC), which target ion channels, receptors, and signaling proteins to specialized subcellular domains. We used a yeast two-hybrid screen of a human brain cDNA library with the PSD-95, Discs-large, ZO-1 (PDZ) domain of γ1-syntrophin yielded overlapping clones encoding the C terminus of TAPP1, a pleckstrin homology (PH) domain-containing adapter protein that interacts specifically with phosphatidylinositol 3,4-bisphosphate (PI(3,4)P₂). In biochemical assays, the C terminus of TAPP1 bound specifically to the PDZ domains of γ1, α1, and β2-syntrophin and was required for syntrophin binding and for the correct subcellular localization of TAPP1. TAPP1 is recruited to the plasma membrane of cells stimulated with platelet-derived growth factor (PDGF), a motogen that produces PI(3,4)P₂. Cell migration in response to PDGF stimulation is characterized by a rapid reorganization of the actin cytoskeleton, which gives rise to plasma membrane specializations including peripheral and dorsal circular ruffles. Both TAPP1 and syntrophins were localized to PDGF-induced circular membrane ruffles in NIH-3T3 cells. Ectopic expression of TAPP1 potently blocked PDGF-induced formation of dorsal circular ruffles, but did not affect peripheral ruffling. Interestingly, coexpression of α1- or γ1-syntrophin with TAPP1 prevented the blockade of circular ruffling. In addition to syntrophins, several other proteins of the DGC were enriched in circular ruffles. Collectively, our results suggest syntrophins regulate the localization of TAPP1, which may be important for remodeling the actin cytoskeleton in response to growth factor stimulation.

Many cellular signaling pathways involve dynamic changes in lipid composition at discrete sites in the plasma membrane (1). These changes are thought to create lipid microdomains that provide a platform for the formation of signaling complexes. Scaffold proteins, which are characterized by multiple protein-protein and protein-lipid interaction domains, form multiprotein aggregates that direct signaling components to specialized membrane domains. In addition to facilitating the proper intracellular localization of their partner proteins, scaffold proteins also provide spatial and temporal regulation of signaling events (2).

The syntrophin family of scaffold proteins consists of five isoforms (α1, β1, β2, γ1, and γ2) with the same modular domain organization. Each has two tandem pleckstrin homology (PH) domains, an intervening PSD-95, discs-large, ZO-1 (PDZ) domain, and a C-terminal syntrophin-unique region (SU) (3–5). The PDZ domain of syntrophins serves as an adaptor for recruiting membrane channels, receptors, kinases, and other signaling proteins (6). The latter half of syntrophin, which includes the PH2 and SU domains, mediates the interaction with members of the dystrophin family of cytoskeletal proteins, including dystrophin, utrophin, and dystrobrevin (6). Dystrophin associates with a multimeric transmembrane complex (the dystrophin glycoprotein complex or DGC) that serves as a link between extracellular matrix proteins such as laminin, and cortical actin (7). Thus, syntrophins provide a link between signaling proteins and the actin cytoskeleton via dystrophin.

The exact function of the DGC has not been precisely determined, but it is thought to provide structural stability to the plasma membrane of skeletal muscle cells during cycles of contraction and relaxation (8). In accordance with this idea, mutations in genes encoding proteins of the DGC are the cause of various muscular dystrophies (9). The DGC may have additional roles, including modulating the actin cytoskeleton through its ability to recruit signaling proteins that regulate actin cytoskeletal organization (10). In this report, we identified an interaction between syntrophins and “tandem PH domain-containing protein 1” (TAPP1).

TAPP1, TAPP2, and Bam32/DAPP1 are a closely related group of PH domain-containing adapter proteins that are recruited to the plasma membrane of cells in response to phosphoinositol 3-kinase (PI3K) activation (11–15). PI3K is a key component of multiple signaling pathways, including those that regulate cell survival, growth, and motility (16). PI3K

The abbreviations used are: PH, pleckstrin homology; BAS-GRIP, PTP-BAS GLGF-repeat interaction protein; DG, dystroglycan; DGC, dystrophin glycoprotein complex; DGK-ζ, diacylglycerol kinase-ζ; Db, dystrobrevin; Dys, dystrophin; ECM, extracellular matrix; GST, glutathione S-transferase; HA, hemagglutinin; PDGF, platelet-derived growth factor; PDZ, postsynaptic density protein-95/discs-large/gonadotropin-releasing hormone-receptor-associated protein; PI, phosphoinositide; PH, pleckstrin homology; mAb, monoclonal antibody; PI3K, PI 3-kinase.
catalyzes the transient production of phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3), which triggers the activation of downstream signaling pathways by recruiting PH domain-containing signaling proteins to the plasma membrane. At the membrane, these signaling proteins are activated and initiate various local responses including reorganization of the actin cytoskeleton.

One potential fate of PI(3,4,5)P3 is dephosphorylation at the 5 position of the inositol ring by Sip homology 2 (SH2)-containing phosphatases (SHIP1 and SHIP2), which produces PI(3,4)P2 (17). Accumulating evidence suggests PI(3,4)P2 functions as a signaling lipid. First, it is present at low levels in unstimulated cells, and its cellular concentration markedly increases following growth factor stimulation (18). Second, agents such as hydrogen peroxide and platelet-derived growth factor (PDGF) that produce PI(3,4)P2 induce the rapid translocation of TAPP1, TAPP2, and Pam32/DAPP1 to the plasma membrane of stimulated cells (13–15,19,20). Finally, several proteins possess PH domains with dual specificity for PI(3,4,5)P3 and PI(3,4)P2 and the C-terminal PH domain of proteins is highly selective for PI(3,4)P2 and not PI(3,4,5)P3. These findings also suggest TAPP1 participates in PI(3,4)P2-mediated signaling in response to growth factor stimulation and PI3K activation.

PI3K-driven signaling pathways downstream of PDGF stimulation induce a rapid reorganization of the actin cytoskeleton, which manifests as plasma membrane specializations including lamellipodia, filopodia, and membrane ruffles (21). In serum-starved fibroblasts, PDGF induces at least two types of membrane ruffles that can occur independently of each other and that can be distinguished on the basis of their morphology and longevity: 1) peripheral (edge) ruffles are persistent components of an extending lamellipodium and 2) dorsal circular ruffles, which appear within minutes after stimulation, then contract and disappear, lasting only 5–20 min. Here, we investigated the role of syntrophins and TAPP1 in the formation of dorsal circular ruffles. We show that expression of TAPP1 blocks circular ruffle formation induced by PDGF stimulation, but does not affect PDGF-induced peripheral ruffling. This effect was not evident when TAPP1 was coexpressed with syntrophins. We show that endogenous TAPP1, syntrophins and proteins of the DGC are prominent components of dorsal circular ruffles. Our results suggest syntrophins and TAPP1 regulate actin-mediated membrane ruffling downstream of growth factor activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—FuGENE™ 6 transfection reagent was purchased from Roche Diagnostics. Dulbecco’s modified Eagle’s medium was purchased from Invitrogen Life Technologies, Inc. All other cell culture reagents were from Sigma. PDGF-bb was obtained from Sigma and was dissolved in 0.004 M HCl and 0.1% bovine serum albumin at a concentration of 50 µg/ml. Aliquots were stored at −20 °C. Aliquots were thawed and diluted to 500 ng/ml in the same buffer. This 100× stock solution was stored at 4 °C for up to 2 weeks.

A polyclonal antibody to BAS-GRIP (also known as TAPP1; see below) was made by immunizing rabbits with a glutathione S-transferase (GST) fusion protein corresponding to the C-terminal 115 amino acids. Antibodies directed against the GST portion were removed by absorbing the antisera against GST immobilized on glutathione-Sepharose (Amersham Biosciences). A polyclonal antibody against TAPP1 was a gift from Dario Alessi and has been described previously (13). Monoclonal and polyclonal antibodies against syntrophins and monoclonal antibody 13H1 against dystrobrevin have been characterized previously (22, 23). AlexaFluor 488- and 594-conjugated goat anti-mouse and anti-rabbit secondary antibodies and AlexaFluor 488-conjugated phalloidin were purchased from Molecular Probes/Invitrogen. Rabbit polyclonal anti-HA was from Zymed Laboratories (San Francisco, CA). Synthetic peptides corresponding to the C-terminal 10 amino acids of human TAPP1 (DDasLPVSDV) and human DGK-α (IGHDELETAV) were synthesized by the University of Waterloo Peptide Synthesis Facility (Waterloo, ON). All peptides contained an additional 4 amino acid linker (SOSG) at the N terminus and an N-terminal biotin.

**Yeast Two-hybrid Assay**—Yeast two-hybrid screens were performed using the EGY48 yeast strain harboring the reporter genes β-galactosidase (β-gal) on the plasmid EGY48 and Leu2 on the plasmid pSH18-34. These reporter genes are under the control of 6 and 8, respectively, upstream LexA operators (Invitrogen). The PDZ domain bait consisted of amino acids 51–149 of human γ1-syntrophin fused in-frame with the LexA DNA binding domain in vector pHybLex/Zeo (Invitrogen). A human brain cDNA library constructed in the activation domain vector pB42AD (Clontech, Palo Alto, CA) was screened with the PDZ domain bait.

Several small scale yeast transformations were performed, where 2.5 × 10⁶ cells containing the bait plasmid were transferred to 50 ml of YPD media and subsequently incubated until the density reached 2 × 10⁷ cells/ml, after which the liquid yeast culture was transformed with 5 µg of cDNA library and 0.5 mg of salmon sperm carrier DNA using the lithium acetate method with 50% polyethylene glycol (w/v). After transformation, the yeast were grown 1–2 h in selective ura amino acids to allow expression of the Leu2 reporter gene, before adding Zeco (Invitrogen) at 200 µg/ml. Cultures were grown overnight then plated on ura amino acids containing plates containing 25 µg/ml Zeocin, 2% galactose, and 1% raffinose.

DNA was isolated from yeast colonies and transformed into the KC8 bacterial strain by electroporation. Positives were isolated from the bacteria and transformed into yeast with either the bait or a negative control (pHybLex/Zeo + Lamin) and plated on selective 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) plates. Positive colonies were sequenced at the University of Ottawa Biotechnology Research Institute.

**Cloning**—Clone 4 (encoding amino acids 348–404 of TAPP1) was excised from pB42AD with EcoRI and XhoI restriction enzymes and subcloned into pGEX-4T-1 cut with the same enzymes. A construct encoding amino acids 348–399 of TAPP1 (ΔC-term) was made by amplifying clone 4 with the primer ATATCTCGAGCTAAAGGCTCG-TCATCGTCCAAG and a sense primer from pB42AD. The PCR product was digested with EcoRI and XhoI and then ligated into pGEX-4T-1 cut with the same enzymes. Myc-tagged α1-syntrophin has been described previously (10). To create a Myc-tagged γ1-syntrophin, α1-syntrophin was excised from a modified pQB125-IC1 (Quantum Biotechnologies Inc.) containing two N-terminal Myc epitope tags (in place of sequences encoding green fluorescent protein) by digesting with Apal and HindIII restriction enzymes. A full-length T7-tagged γ1-syntrophin construct described previously (24) was digested with the same enzymes and ligated with the cut vector fragment.

In separate studies, a cDNA clone was isolated in a yeast two-hybrid screen of a human kidney library with the second PDZ domain of PTP-BAS (amino acids 1206–1496) as bait (25). Of the 1 × 10⁵ colonies screened, 4 positives were isolated. Sequencing of the inserts revealed they were identical and encoded 34 amino acids fused in-frame to the GAL4 activation domain. One of the clones (G2Y13) was radiolabeled with [α-32P]dCTP and used to probe a human kidney library in αgt10. The hybridization was carried out at 60 °C in a solution composed of 5× SSPE, 5× Denhardt’s solution, 0.5% SDS, and 20 µg/ml salmon sperm DNA. The filters were washed at 50 °C in 0.2× SSC and 0.1% SDS. The resul tant positive clones were sequenced using a T7 polynucleotide sequencing kit (Amersham Biosciences) and 5′-labeled fluorescent primers. One of the clones, s4–1, had a 1.9 kb insert with an open reading frame, which encoded a novel protein (at the time of submission) of 404 amino acids (GenBank™ accession number E16311). The protein was named PTP-BAS GLGF repeat-interacting protein (BAS-GRIP). This sequence is the same as subsequent data bank entries for human TAPP1. Hereafter, we refer to BAS-GRIP as TAPP1.

An N-terminal HA epitope tag was cloned in-frame with the N terminus of TAPP1 in pGEM-1. The insert was excised with EcoRI and ligated into pcDNA3.1(−) (−) that had been cut with EcoRI and treated with calf intestine alkaline phosphatase. TAPP1ΔCT, a full-length construct encoding a C-terminally truncated TAPP1 (missing the last 5 amino acids) was amplified terminal in pUCD3.1(−) using the T7 promoter primer and 5′-ATATGAAATGCCTAAAGGCTCG-TCATCGTCCAAG-3′. The 1.3-kb product was digested with EcoRI and ligated into pcDNA3.1(−) as described above. All constructs were verified by DNA sequencing.

**Preparation and Purification of Bacterial Fusion Proteins**—Fusion proteins were purified as described previously (26) with the following modifications: BL21(pDE3)Plys cells were transformed and grown in
domains and a C-terminal PDZ binding motif (PVSDV), which conforms to the type I consensus sequence.

2 ml of LB medium supplemented with 200 μg/ml ampicillin and 34 μg/ml chloramphenicol (LB-Amp-Chlor) overnight at 37 °C in a shaking incubator. Bacteria were pelleted by centrifugation and resuspended in 1 ml of fresh LB-Amp-Chlor. This was used to inoculate 1 liter of LB-Amp-Chlor, which was grown until the optical density at 600 nm reached between 0.6 and 1.0. Protein expression was then induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (BioShop, Canada, Inc., Burlington, ON), and the culture was incubated for 3 h at 37 °C or overnight at 28 °C. All subsequent steps were as described.

Overlay Assays—Overlay assays were carried out as described previously (26, 27) with the following modifications: signals generated by enhanced chemiluminescence were captured with a Digital Image Station (Kodak). The amount of fusion protein loaded in each lane was compared by digitally capturing an image of the Ponceau S stained blot prior to blotting.

Cell Culture—NIH-3T3 cells were maintained in Dulbecco’s modified Eagle’s medium high glucose supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, and 100 units/ml penicillin-streptomycin and were cultured on 10-cm diameter plastic dishes at 37 °C in a humidified atmosphere of 5% CO2. For immunolocalization experiments, cells were infected 6 h using FuGENETM 6 transfection reagent following the manufacturer’s protocol. The cells were serum-starved (0.5% fetal bovine serum) overnight, treated for 5 min with 5 ng/ml PDGF or carrier alone, then fixed in 0.5% paraformaldehyde in phosphate-buffered saline, pH 7.2.

Immunolabeling—Coverslips were removed from the 24-well plates and transferred to a covered dish lined with Parafilm “M” laboratory film. Cells were permeabilized for 5–10 min with 0.1% Triton X-100 in phosphate-buffered saline, pH 7.2. Nonspecific binding sites were blocked for 1 h at room temperature with a filtered solution of 1% bovine serum albumin in phosphate-buffered saline, pH 7.2.

Overlay Assays—Overlay filter binding assays were performed. A GBP-1-syntrophin PDZ domain of each PDZ domain of 1- or 2-syntrophin (Fig. 1). The amino acid sequence of the PDZ domain of 1-syntrophin and from PSD-95 and Chapsyn-110 were analyzed by SDS-PAGE and transferred to a nitrocellulose membrane. The number at the left of each black line indicates the identity of the clone. TAPP1 contains two tandem PH domains and a C-terminal PDZ binding motif (PVSDV), which conforms to the type I consensus sequence.

Identification of TAPP1 as a γ1-Syntrophin-interacting Protein—A yeast two-hybrid screen of a human brain cDNA library using the PDZ domain of γ1-syntrophin as bait yielded 2 independent overlapping cDNA clones encoding TAPP1. The structure of TAPP1 is shown schematically in Fig. 1. Controls for the specificity of the yeast two-hybrid interaction included retesting positive clones by back-transformation into yeast and lack of interaction with a nuclear lamin prey. In addition, γ1-syntrophin PDZ did not self-activate when transformed into yeast with an empty prey vector. The cDNAs isolated by the yeast two-hybrid screen overlapped in the C-terminal region of TAPP1, suggesting this region mediates the binding to the PDZ domain of γ1-syntrophin (Fig. 1). The amino acid sequence (PVSDV) at the C terminus of TAPP1, which is conserved in human and mouse TAPP1, is consistent with the class I PDZ domain ligand consensus ([ST]X[V]) (28) but differs from the consensus sequence identified for strong binding to α- and β-syntrophin PDZ domains ([Q/R/K][E/S/T][X/V/L]I) (26, 29, 30).

Direct Interaction between Syntrophin PDZ Domains and the C Terminus of TAPP1—To show direct biochemical association between the PDZ domain of γ1-syntrophin and the C terminus of TAPP, overlay filter binding assays were performed. A soluble GST fusion protein containing the C terminus of TAPP1 (clone 4) bound strongly to a hexahistidine (H6)-fusion protein of γ1-syntrophin and somewhat less strongly to the PDZ domains of α1- and β2-syntrophin (Fig. 2A). Longer exposures revealed weak binding to the PDZ domain of β1-syntrophin (not shown) but not to any of the other PDZ domains tested including PDZ-1, -2, and -3 of PSD-95 and
PDZ-3 of Chapsyn-110 (Fig. 2A). Thus, these in vitro binding data indicate that TAPP1 interacts preferentially with the PDZ domain of syntrophins.

In previous studies, we demonstrated that synthetic peptides corresponding to C-terminal PDZ binding motifs of various proteins are sufficient for strong binding to recombinant PDZ domains subjected to SDS-PAGE and transferred onto nitrocellulose filters (24, 26, 30). To determine if the C terminus of TAPP1 is sufficient for interaction with the PDZ domain of γ1-syntrophin, a biotinylated synthetic peptide corresponding to the C-terminal 10 amino acids of TAPP1 was assayed for binding to various recombinant PDZ domains in overlay assays. Surprisingly, no binding was observed to any of the PDZ domains tested. In parallel blots, the C-terminal 10 amino acids of diacylglycerol kinase-α1 bound to syntrophin PDZ domains and to PDZ-3 of PSD-95 and Chapsyn-110, ruling out the possibility that the recombinant proteins had lost their ability to bind PDZ ligands. Thus, these results demonstrate that the C-terminal 10 amino acids of TAPP1 are not sufficient for binding to syntrophin PDZ domains.

Most known PDZ domain interactions occur by the recognition of short C-terminal peptide motifs and require that the peptide ligands have a free carboxylic acid group (28), but in some cases internal peptide motifs that are well removed from the C terminus can mediate interactions with PDZ domains (27, 31).

To further investigate the function of the C-terminal PDZ binding motif of TAPP1, we constructed a GST fusion protein mutant in which the C-terminal 5 amino acids of TAPP1 were deleted (ΔC-term). As shown in Fig. 2D, removal of these residues completely abrogated binding to the γ1-syntrophin PDZ domain in overlay assays. Thus, our results demonstrate that the extreme C terminus of TAPP1 is necessary, but not sufficient, for binding to the syntrophin PDZ domains and suggest additional residues upstream are important for mediating strong binding or for maintaining the proper conformation of the C terminus.

PDZ Interactions Regulate the Subcellular Localization of TAPP1—PDZ-containing adapter proteins act as scaffolds for the assembly of protein complexes at the plasma membrane (2). Therefore, we reasoned that γ1-syntrophin might recruit TAPP1 to the plasma membrane of cells. We first tested whether the PDZ interaction occurs within the context of the full-length γ1-syntrophin protein expressed in mammalian cells. A GST fusion protein containing the C terminus of TAPP1 efficiently “pulled down” T7-tagged full-length γ1-syntrophin from lysates of transfected COS-7 cells (Fig. 2C), whereas GST alone did not. Thus, the PDZ interaction is sufficient to mediate the interaction with full-length syntrophin protein. We next assessed whether γ1-syntrophin and TAPP1 colocalize in transfected cells. When expressed alone in NIH-3T3 cells, epitope-tagged TAPP1 was distributed diffusely throughout the cytoplasm and was often present on internal membrane structures (Fig. 3A). TAPP1 showed modest association with the cell cortex and was also concentrated in F-actin-rich membrane ruffles along the lateral margins of transfected cells (Fig. 3A). γ1-Syntrophin was similarly distributed in singly transfected cells (Fig. 3B). In cotransfected cells, both proteins were colocalized in the cytoplasm and were concentrated in ruffles at the leading edge of lamellipodia (Fig. 3, C and C’).

To further investigate the role of the PDZ interaction, we constructed a full-length protein missing the last 5 amino acids (TAPP1ΔCT), which are required for binding (see Fig. 2D) and compared its localization to the full-length TAPP1. When expressed alone in COS-7 cells, variable amounts of TAPP1 were present in the nucleus (Fig. 4A, arrows). When TAPP1ΔCT was expressed, it was localized primarily in the nucleus (Fig. 4, B and B’), suggesting that interactions mediated by the PDZ binding motif of TAPP1 contribute to the regulation of its subcellular localization.

TAPP1 and Syntrophins Are Components of Circular Dorsal Ruffles—A recent study demonstrated that the activities of three signaling molecules, PI3K, Rac1, and Rab5, converge to regulate the formation of circular ruffles downstream of PDGF stimulation (32). Since TAPP1 lies downstream of PI3K activation and is recruited to the plasma membrane by PDGF stimulation (13, 15), we hypothesized it might play a role in the formation of circular ruffles. As a first test of this idea, we used immunofluorescence microscopy to determine if TAPP1 is present in circular dorsal ruffles. Serum-starved NIH-3T3 fibroblasts were stimulated with PDGF then were fixed and processed for immunolabeling with anti-TAPP1 antibodies and AlexaFluor 488-conjugated phalloidin. As shown in Fig. 5 (A and A’, arrows), there was bright, punctate TAPP1 immunoreactivity associated with actin-rich circular ruffles. TAPP1 was also accumulated in peripheral ruffles along the leading edge of emerging lamellipodia and was sometimes evident at non-ruf-
lar ruffles in NIH-3T3 cells, we used polyclonal antibodies specific for α1-, β1-, and β2-syntrophin isoforms. Both α1- and β2-, but not β1-syntrophin localized to circular ruffles, consistent with the in vitro binding results of Fig. 2A (Fig. 5, C–E). These results suggest TAPP1 and specific syntrophin isoforms are recruited to circular ruffles in response to PDGF stimulation.

Expression of TAPP1 Blocks PDGF-induced Circular Ruffle Formation—To determine if TAPP1 expression affects the formation of circular ruffles, serum-starved NIH-3T3 fibroblasts were transfected with HA-tagged TAPP1, stimulated with PDGF, then processed for immunolabeling with anti-HA antibodies and with AlexaFluor 488-conjugated phalloidin. Surprisingly, we found that cells expressing TAPP1 lacked circular ruffles (Fig. 6, A and A′), despite robust circular ruffle formation in neighboring cells expressing little or no TAPP1. Quantification of this effect revealed that only ~7% of the TAPP1-transfected cells had circular ruffles compared with ~48% of untransfected cells (Fig. 6F). Expression of TAPP1 had no discernable effect on peripheral ruffle formation (Fig. 6F), consistent with previous studies showing that they can form independently of circular dorsal ruffles (32). Moreover, TAPP1 localized to PDGF-induced peripheral ruffles (Fig. 7, lower panels). Thus, these results suggest TAPP1 expression selectively inhibits circular ruffling.

Syntrophin Expression Rescues Circular Ruffle Formation in TAPP1-expressing Cells—To identify possible roles for syntrophins in the formation of circular ruffles, we transfected NIH-3T3 cells with either Myc-tagged α1- or γ1-syntrophin. In contrast to TAPP1, expression of γ1-syntrophin did not appreciably affect either peripheral or circular ruffling (Fig. 6F). Moreover, γ1-syntrophin was localized to both types of PDGF-induced ruffles (Fig. 6, B and B′ and Fig. 7, upper panels). Similar results were obtained for α1-syntrophin (Fig. 6, C and C′, arrows and data not shown).

We next determined whether syntrophins could modulate the effect of TAPP1 on circular ruffling. In cells coexpressing γ1-syntrophin and TAPP1, both proteins were colocalized in dorsal circular ruffles (Fig. 6, D and D′, arrows). Moreover, the number of cotransfected cells with peripheral or circular ruffles was not significantly different from the respective untransfected controls (Fig. 6F). Again, similar results were obtained with α1-syntrophin (Fig. 6, E and E′, arrows). These data suggest syntrophins are able to recruit TAPP1 to dorsal circular ruffles.

Ruffle Closure Appears Normal in TAPP1-expressing Cells—Circular dorsal ruffles are transient structures that form only once, then constrict and disappear after 5–20 min (21). We considered the possibility that the lack of circular ruffles in TAPP1-expressing cells reflected an increase in the closure rate of ruffles, such that fewer were observed at later times. To examine this possibility, circular ruffling was assessed in transfected NIH-3T3 cells stimulated for brief periods of time (1–5 min) with PDGF. In unstimulated cells, TAPP1 and γ1-syntrophin were mostly cytoplasmic, as described above. As early as 1 min after PDGF addition, both TAPP1 and γ1-syntrophin were recruited to the plasma membrane. In most cases, the proteins were specifically localized to the cell’s leading edge or to peripheral membrane ruffles, but in some cells the proteins were found at many places on the circumference (Fig. 7, top, 2′, arrows). These regions were generally enriched in F-actin (not shown). By 3–4 min, cells expressing γ1-syntrophin had large curved ruffles, which were well removed from the periphery (Fig. 7, top, 3′, arrows). In contrast, circular ruffles were rarely observed in cells expressing TAPP1; and ruffles were generally confined to the cell periphery (Fig. 7,
FIG. 6. Effect of TAPP1 expression on PDGF-induced membrane ruffling. A–E, representative images of NIH-3T3 cells singly transfected with constructs encoding HA-TAPP1 (A and A'), Myc-γ1-syntrophin (B and B'), or Myc-alpha-1-syntrophin (C and C') or cotransfected with TAPP1 and γ1-syntrophin (D and D'), or TAPP1 and α1-syntrophin (E and E'). The cells were serum-starved overnight, treated with 5 ng/ml PDGF for 5 min, then fixed and labeled with antibodies specific for each epitope tag followed by AlexaFluor 594-conjugated secondary antibodies. Actin was visualized using AlexaFluor 488-conjugated phalloidin. Arrowheads indicate circular ruffles. In E and E', arrowheads indicate large vesicles. A and A', note the lack of circular ruffles in TAPP1-expressing cells despite many untransfected cells with ruffles in the same field. F, quantification of membrane ruffling. The graph shows the percentage of cells with edge ruffles (open bars) and circular ruffles (filled bars) for each of the indicated constructs. Ruffling in PDGF-treated, untransfected cells (Untransf) is shown for comparison. Membrane ruffling was quantified as described under “Experimental Procedures.” The data are the average of three independent experiments. Error bars indicate S.D. Scale bars, 20 μm.

FIG. 7. Time course of PDGF-induced translocation of γ1-syntrophin and TAPP1 into membrane ruffles. NIH-3T3 cells transfected with Myc-γ1-syntrophin (top panels) or HA-TAPP1 (bottom panels) were serum-starved overnight then treated with PDGF for 5 min. The cells were fixed and stained with epitope tag-specific antibodies followed by AlexaFluor 594-conjugated secondary antibodies. Shown are representative images of untreated cells (0') or cells treated for various times (2', 3', or 5') with PDGF. Arrows indicate membrane staining, edge ruffles, and circular ruffles. Scale bars, 20 μm.

bottom, 3', arrows). By 5 min of PDGF stimulation, large circular ruffles were observed in cells expressing γ1-syntrophin but not in TAPP1-expressing cells. These results suggest that reduced circular ruffling in TAPP1-expressing cells is not due to an increase in their closure rate but rather that circular ruffles fail to form.

Dystrophin-associated Proteins Are Components of Dorsal Circular Ruffles—To determine if the DGC has a role in actin cytoskeletal changes underlying circular ruffle formation, we screened PDGF-treated NIH-3T3 cells with a panel of well characterized antibodies to different DGC proteins by immunofluorescence microscopy. We identified dystrophin, α- and β-dystroglycan, utrophin, and dystrobrevin as components of PDGF-induced circular ruffles (Fig. 8). All these proteins showed considerable overlap with actin (Fig. 8, arrows). These results suggest the DGC plays a role in the formation or maintenance of circular ruffles.

DISCUSSION

Cell migration in response to PDGF stimulation is characterized by a rapid reorganization of the actin cytoskeleton, which gives rise to several types of plasma membrane specializations including lamellipodia, peripheral membrane ruffles, and dorsal circular ruffles (21). These transient, dynamic structures require spatially and temporally controlled changes in actin organization. There is increasing evidence that local changes in the actin cytoskeleton such as these are initiated by the production of phosphoinositides at discreet regions of the plasma membrane (1). Thus, considerable effort has been made to define the phosphoinositides that regulate different actin-based structures and to identify the downstream signaling proteins involved.

Several studies have shown that wortmannin, a specific inhibitor of PI3K, blocks PDGF-induced circular ruffling, implying that its main lipid product PI(3,4,5)P₃ is necessary for circular ruffle formation (34–36). In support of this idea, the PH domain of Akt has been used as a probe to show that PI(3,4,5)P₃ accumulates within PDGF-induced circular ruffles (36). Signaling via PI(3,4,5)P₃ is antagonized by the action of PTEN and SHIP phosphatases which remove the 3- and 5-inositol phosphates, respectively, to generate PI(4,5)P₂. This can stimulate Akt, which binds to PI(3,4)P₂ and stimulates cell migration (13, 15). Here, we demonstrate that TAPP1 is recruited to PDGF-induced circular ruffles in NIH-3T3 cells, which implies that PI(3,4)P₂ accumulates in circular ruffles and has a role in their formation, maintenance or closure.
TAPP1 Regulates Circular Ruffling

We found that overexpression of TAPP1 potently blocked the formation of PDGF-induced circular ruffles, but not peripheral ruffles. This specificity argues against an overall inhibitory effect on actin cytoskeleton dynamics and is consistent with recent studies that suggest circular and peripheral ruffles are induced by distinct but overlapping signaling mechanisms (32, 36). Peripheral ruffles are induced by a linear signaling cascade from receptor-tyrosine kinases via Ras to Rac (39, 40), while at least two additional, independent signals from PI3K and Rab5 are required for the formation of circular ruffles (32). Pretreatment of cells with wortmannin prior to stimulating with PDGF prevents the translocation of TAPP1 to the plasma membrane suggesting it is a direct target of PI3K signaling (13). Thus, our finding that exogenous TAPP1 expression specifically inhibits circular ruffling supports the idea that peripheral and circular ruffles are distinct structures that are regulated by different signaling mechanisms and that TAPP1 acts downstream of PI3K signaling in the regulation of actin remodeling.

We can envisage several possible mechanisms that can account for the inhibition of circular ruffling by TAPP1 overexpression. We favor the idea that the absence of stoichiometric amounts of syntrophins prevents exogenous TAPP1 from being properly targeted to ruffles, because coexpression of syntrophin with TAPP1 prevented the inhibition of circular ruffling. If correct, this would suggest TAPP1 positively regulates ruffle formation. By correctly targeting TAPP1, syntrophins might promote the interaction of TAPP1 with additional effector proteins required for circular ruffling. In the absence of such an interaction, TAPP1 might sequester and effectively inactivate these proteins. Alternatively, mislocalized TAPP1 could sequester PI(3,4)P₂, removing it from its site of action or preventing it from activating other downstream effectors. Sequestering PI(3,4)P₂ in this manner could also decrease the local concentrations of PI(3,4,5)P₃ by shifting the equilibrium in favor of dephosphorylation by SHIPs.

Another interesting possibility is suggested by a study by Kimber et al. (13), who showed that TAPP1 interacts via its C terminus with the first PDZ domain of the protein tyrosine phosphatase PTPL1 and that both proteins translocate to the plasma membrane upon stimulation of PI(3,4)P₂ production. Overexpression of TAPP1 might recruit the available PTPL1 to the plasma membrane, which would lead to dephosphorylation of the PDGF receptor and adapter proteins, thereby inactivating the PI3K pathway. Coexpression with syntrophins could compete with the binding of PTPL1 to TAPP1 and prevent this inactivation. In this scenario, TAPP1 would negatively regulate ruffle formation. Thus, it is not clear yet whether native TAPP1 positively or negatively regulates circular ruffle formation, but both alternatives are possible given the currently available data.

A recent electron microscope labeling study using the second PH domain of TAPP1 as a probe for PI(3,4)P₂ has shown that this lipid, in addition to being present on the plasma membrane, is present on internal membranes, including the endoplasmic reticulum, multivesicular bodies and the nuclear envelope (20). This suggests that different pools of TAPP1 reside within these compartments or that TAPP1 translocates along various endocytic pathways. Our results suggest interactions mediated by the C-terminal PDZ binding motif of TAPP1 contribute to the regulation of its intracellular localization. Moreover, yeast two-hybrid and biochemical assays indicate that the C terminus of TAPP1 binds preferentially to the PDZ domain of γ₁-, α₁-, and β₂-syntrophins and not to other closely related PDZ domains. Both α₁- and β₂-syntrophin are components of circular ruffles, suggesting these isoforms target TAPP1 to this actin-rich membrane domain. However, a previous study showed that the C terminus of TAPP1 interacts with the multi-PDZ domain-containing scaffold protein MUPP1 (41). It was not determined in that study whether MUPP1 affects the subcellular localization of TAPP1, but it is interesting to speculate that different PDZ-containing scaffold proteins direct TAPP1 to distinct intracellular membrane domains.

The DGC forms a transmembrane axis through which the ECM is tightly linked to the actin cytoskeleton. The central components of this axis are the peripheral membrane protein α-DG, which binds laminin and other ECM proteins with laminin-like G domains (42–44), and the transmembrane protein β-DG, which anchors α-DG to the cell surface. β-DG binds to the C terminus of dystrophin, which in turn, binds via its N terminus to cortical actin. This study demonstrates that DGC components are enriched in PDGF-induced circular ruffles, suggesting they participate in the regulation of actin organiza-

![Fig. 8. Dystrophin-associated proteins are components of dorsal circular ruffles.](image) NIH-3T3 cells were stimulated with PDGF, fixed, and double-stained for various dystrophin glycoprotein complex proteins (A–E) and actin (A'–E'). Arrows indicate circular ruffles. A and A', labeling for dystrophin with mAb MANDYS-8 revealed punctate staining that overlapped with actin in circular ruffles. B, B' and C, C', similarly, antibodies specific for α- and β-dystroglycan labeled circular ruffles, but the staining appeared more uniform than for dystrophin. D and D', punctate utrophin staining of circular ruffles was weak, but consistent. Utrophin staining was stronger along the cell cortex and at ruffles, but the staining appeared more uniform than for dystrophin. E and E', mAb 13H1 against dystrobrevins showed diffuse, punctate labeling, which was clearly enriched in circular ruffles.
tion downstream of PDGF signaling. An interesting possibility is that changes in actin organization induced by PDGF stimulation are transmitted via the DGC to the ECM to coordinate regulation of intracellular and extracellular structural changes. In this regard, it is interesting to note that the ECM-degrading enzyme matrix metalloproteinase-2 localizes to the tips of circular ruffles (36). These structures have been suggested to be the topological equivalent of the leading edge of cells undergoing three-dimensional migration through the ECM. Thus, the DGC might help to organize signaling components that remodel the actin cytoskeleton during three-dimensional migration.

Changes in actin organization are tightly linked to the production of signaling phosphoinositides, which recruit PDZ-domain-containing proteins such as TAPP1 to sites of receptor activation at the plasma membrane. Syntrophins and the DGC may stabilize the association of TAPP1 with the membrane or act as a scaffold to promote the formation of a signaling complex that includes TAPP1 and other effectors. Interestingly, earlier studies showed that as many as four syntrophins can associate with the DGC at once (23, 45), so they may recruit multiple signaling proteins with PDZ binding motifs to PDGF-induced circular ruffles. Indeed, we previously reported that multiple signaling proteins with PDZ binding motifs to PDGF-associate with the DGC at once (23, 45), so they may recruit actin cytoskeleton during three-dimensional migration. Syntrophins and the DGC might also help to organize signaling components that remodel the actin cytoskeleton during three-dimensional migration.

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