Cell Permeant Polyphosphoinositide-binding Peptides That Block Cell Motility and Actin Assembly*

C. Casey Cunningham‡, Rolands Vegners§, Robert Bucki¶, Makoto Funaki¶, Neha Korde¶, John H. Hartwig‡, Thomas P. Stossel¶, and Paul A. Janmey||

From the ‡Hematology Division, Brigham & Women's Hospital, Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, the §Latvian Organic Synthesis Institute, Riga LV1006, Republic of Latvia, and the ¶Department of Physiology, Institute for Medicine and Engineering, University of Pennsylvania, Philadelphia, Pennsylvania 19063.

Polyphosphoinositides (PPIs) affect the localization and activities of many cellular constituents, including actin-modulating proteins. Several classes of polypeptide sequences, including pleckstrin homology domains, FYVE domains, and short linear sequences containing predominantly hydrophobic and cationic residues account for phosphoinositide binding by most such proteins. We report that a ten-residue peptide derived from the phosphatidylinositol 4,5-bisphosphate (PIP2) binding region in segment 2 of gelsolin, when coupled to rhodamine B has potent PIP2 binding activity in vitro; crosses the cell membrane of fibroblasts, platelets, melanoma cells, and neutrophils by a process not involving endocytosis; and blocks cell motility. This peptide derivative transiently disassembles actin filament structures in GFP-actin-expressing NIH3T3 fibroblasts and prevents thrombin- or chemotactic peptide-stimulated actin assembly in platelets and neutrophils, respectively, but does not block the initial [Ca2+] increase caused by these agonists. The blockage of actin assembly and motility is transient, and cells recover motility within an hour after their immobilization by 5–20 μM peptide. This class of reagents confirms the critical relation between inositol lipids and cytoskeletal structure and may be useful to probe the location and function of polyphosphoinositides in vivo.

Synthesis and turnover of phosphorylated inositol lipids or polyphosphoinositides (PPI) are critical features of cellular signaling in many different contexts. In addition to their effects on cytoskeletal rearrangements required for cell motility and shape change (1), inositol lipids are implicated in control of cellular pH (2), Ca influx (3), cell adhesion (4), vesicle traffic, and several other functions (5–7).

Despite the numerous possible pathways that are potential targets of inositol lipids, recent studies in intact cells confirm that altering the levels of specific PPI by overexpression of inositol lipid kinases or phosphatases causes profound changes in the state of cellular actin assembly (5, 8–10). Disrupting PPI-protein interactions also detaches the cytoskeleton from the cell membrane (4, 11). The binding of these and other studies in vitro show PIP2 and some other PPI bind tightly and specifically to several actin-associated proteins, including the actin filament severing protein gelsolin (reviewed in Refs. 12, 13). Studies of proteolytic fragments (14) and recombinant gelsolin deletion mutants (15) identify two sequences of 10–20 amino acids responsible for the polyphosphoinositide binding activity of intact gelsolin. Peptides based on sequences from either segment 1 of gelsolin (GS135–142) (16) or segment 2 (GS150–169) (17) bind avidly to polyphosphoinositides and compete with the parent protein for binding to PI(4)P and PIP2 (17). Both peptides are polyvalent cations with several hydrophobic residues. Their binding to PPIs is not exclusively electrostatic, because it is much stronger than that of similar peptides that have larger positive charges (17). Similar sequences are found in other phosphoinositide-bound proteins, including phospholipase Cβ (18), α-actinin, vinculin, (11, 19, 20), and cortexillin (21) and are implicated in enzymatic function (22).

Synthetic peptides based on the residues essential for PPI binding in gelsolin retain much of the affinity for PPIs compared with the parent protein but are less selective among different classes of PPIs (17). The sequence QRLFQVGRR competes well with intact gelsolin for binding PPIs and, when added to permeabilized cells, exerts effects consistent with a potent inhibition of the production or presentation of PPIs required to uncap actin filament ends and desessquerter actin monomers as required for stimulation of actin assembly at the cytoskeleton/membrane interface (23, 24). In an attempt to construct a fluorescent derivative of QRLFQVGRR that could be used to stabilize and localize intracellular PPIs after microinjection, one such peptide derivative, rhodamine B-QRLFQVGRR (PB10), exhibited properties not observed with the unlabeled peptide or derivatives based on fluorescein, pyrene, or tetramethylrhodamine (25). When PB10 was released into the culture medium from a micropipette outside of the surface of a fibroblast, the cell interior became fluorescent within seconds. Subsequent studies showed that PB10 at 1–5 μM concentrations was able to block the motility of PIP2-containing phospholipid vesicles within Xenopus extracts in which they otherwise assembled an actin-rich tail similar to that constructed by Listeria monocytogenes and other pathogens.
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(26). PBP10 also strongly stimulates the enzymatic activity of P13K in vitro (27) and promotes ATP-dependent transport in canalicular membrane vesicles (28) and in permeabilized cells that require P13K activity, even under conditions where external signals to stimulate P13K activity were lacking (29).

Although the direct binding of this class of peptide on PPIs in vitro and its effect on PPI-dependent cellular processes in intact cells has been reported, the biochemical properties of this peptide are largely unknown, and its effect on cell motility and actin assembly is not reported. In this study we show the biochemical specificity of this class of peptides, explore the generality of its ability to enter live cells, and examine its effects on the actin system in vivo.

MATERIALS AND METHODS

Peptide Synthesis and Labeling—QRLFVKGRR (gelosin residues 160–169) and other peptides were prepared by solid phase peptide synthesis on p-benzyloxybenzyl alcohol/polyethylene resin (30) using α-N(9-fluorenylmethoxycarbonyl (α-Fmoc) protection chemistry and carbodiimide/N-hydroxybenzotriazole coupling. The side chains were protected as follows: Arg (Pmc), Gln (Trt), Lys (Boc). To couple fluorophores on these peptides, ester derivatives of each fluorophore were cleaved from the solid phase support. After coupling, the peptides were cleaved from the solid support with trifluoroacetic acid and phenol (95:5, v/v). All peptides were purified by reverse-phase high performance liquid chromatography on a Silica Gel C18 column using a 20–60% acetonitrile gradient in 0.1% trifluoroacetic acid and dried.

Fluorescence—Solution fluorescence of the peptides dissolved in PBS was measured with an LS-5B spectrofluorometer (PerkinElmer Life Sciences) using an excitation wavelength of 565 nm and measuring emission at 590 nm. In experiments where fluorescent peptides were mixed with phospholipids, micelles of PIP2 or bilayer vesicles composed of other lipids were prepared by a combination of sonication and extrusion as described elsewhere (27).

Surface Pressure—The surface activity of peptides was measured using a Kibron (Helsinki, Finland) monolayer trough as described elsewhere (31). Briefly 1-ml samples of peptide, dissolved in PBS (7.5 mM sodium phosphate, 140 mM NaCl, pH 7.0), were placed in each of 15 wells in a multiple trough, and the surface pressure was measured 10 min after position of the probe at the air/liquid interface. Kinetic measurements of surface pressure starting 5 s after dilution of peptide from a 20 mM stock solution showed that a stable reading was obtained after 5 min and that this reading remained constant within 5% for at least 30 min for all pressure reported (data not shown).

Cell Culture—The human melanoma line A7 was grown at 37 °C and 5% CO2 in minimum essential medium (Life Technologies, Inc.) supplemented with 8% newborn calf serum and 2% fetal calf serum. NIH3T3 cells were grown under the same conditions in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum. A plasmid encoding EGFP fused to the N terminus of human β-actin (pEGFP-actin) was purchased from CLONTECH (Palo Alto, CA) and was transfected into NIH3T3 fibroblasts using a liposomal method (Lipofectin, Life Technologies, Inc.) with 0.5% dextrose. Actin assembly after addition of FMLP at 10−7 M was determined by densitometry of actin sedimented by centrifugation essentially as described above for platelets.

Microscopy—Fluorescence microscopy was performed on a Zeiss Axiovert 405MInverted microscope (Carl Zeiss, Inc., Thornwood, NY) with various lenses: a 40×, 0.9 numerical aperture (N.A.) Plan-neofluor; a 100×, 1.4 N.A. Planapochromatic (Oil). For deconvolution microscopy, images were acquired by a DeltaVision system (Applied Precision, Issaquah, WA) and a Zeiss Axiovert S100 2TV inverted microscope (Carl Zeiss, Inc.) with a 63×/1.4 N.A. Plan-achromat objective lens and then processed by SoftWoRx software (Applied Precision).

Cell Motility—Motility assays were performed in a modified Boyden chamber. For each assay, 106 cells suspended in buffer were loaded into the top wells of a 48-well, two compartment chamber (Nucleopore, Pleasanton, CA) separated from the bottom wells by a polycarbonate membrane with uniform pore sizes. Some aliquots of cells were incubated at room temperature with varying concentrations of peptide for 3 min prior to loading. The bottom wells had been filled with buffer containing either FMLP at 10−7 M for neutrophils, 10−6 M for platelets, or conditioned media for A7 cells. The pore size of the polycarbonate filters was 3 μm for the neutrophils and 5 μm for the NIH3T3 and A7 cells. After a 2-h incubation at 37 °C, the membranes were removed from the chambers, stained, and examined with a Nikon inverted microscope with a 40× objective. The number of cells that had migrated through the membrane was counted for each well.

For wash-out experiments, the cells were pelleted, resuspended in 10 volumes of HBSS, and left for varying periods of time at 4 °C before pelleting and resuspending in serum-free media or HBSS at cell concentrations of 2 × 106 for loading into the chamber. The rate of neutrophil motility was also measured by video tracking of randomly migrating human neutrophils stimulated with 10−5 M FMLP.

Assay of Intracellular [Ca2+]i—Ca2+ mobilization by neutrophils was measured by monitoring the change in fluorescence after excitations at 340 and 380 nm in human neutrophils loaded with 5 μM Fura 2-AM in response to 10−7 M F-Met-Leu-Phe (FMLP) as previously described (32). Platelets were loaded with Fura 2-AM by the method of Heemskerk et al. (33).

Determination of Actin Incorporation into the Triton-insoluble Cytoskeleton of Activated Platelets and Immunoblotting Analysis—Platelet suspensions in buffer A (3 × 1010/ml) were incubated with 5−250 μM Rhod-QRLFVKGRR peptide or Rhod-QRL peptide as control peptides at 37 °C for 5 min, without stirring. At appropriate times (0, 15, 30, 60, 120, and 300 min) during platelet aggregation induced with thrombin (0.1 U/ml), the platelets were lysed by addition of equal volume of lysis solution containing 1.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 120 mM PIPEs, 50 mM HEPES, 20 mM EGTA, 4 mM MgCl2, 0.1 mM dithiothreitol, 10 mM glucose, 20 μg/ml leupeptin, 150 μg/ml benzamidine, and 80 μg/ml aprotinin, pH 7.2. The lysate was kept at 4 °C for 5 min and then centrifuged at 12,000 × g for 10 min. The actin content of the Triton-insoluble cytoskeleton was determined by removing the supernatant fluid from the pellet and adding 20 μl of gel sample buffer. After boiling, the entire cytoskeleton was subjected to electrophoresis on 10% polyacrylamide gels in the presence of SDS. The relative amount of actin in each lane was determined by gel densitometry. After electrophoresis, proteins were transferred to nitrocellulose membranes (Immobilon-NC, Millipore) that were blocked by incubation in 5% nonfat dry milk in TTBS (150 mM NaCl, 50 mM Tris, 0.05% Tween 20, pH 7.4). After transfer to the membrane, proteins were probed with a monoclonal anti-β-actin antibody (Sigma A5451) used at 1:5000 dilution in TTBS. Horseradish peroxidase-conjugated secondary antibodies were used at 1:5000 dilution in TTBS and 4% nonfat dry milk. Immunoblots were developed with Kodak BioMax MR film using a horseradish peroxidase-targeted chemiluminescent substrate.
resuspended in buffer A containing 2 mM CaCl2. After introduction of platelets in a cuvette of an SLM-Aminco MC 200 fluorometer and addition of agonists, the suspension was stirred (1000 rpm) for 20 s and transient Fura 2-AM fluorescence was recorded for 10 min ($I_{max} = 340$ nm, $I_{min} = 510$ nm).

Assay of Neutrophil Granule Release—For intracellular enzyme release measurements, human neutrophils were suspended in phosphate buffer with 2 mM cytochalasin D in either the presence or absence of $10^{-7}$ 12-O-tetradecanoylphorbol-13-acetate. Aliquots of the supernatant were then assayed for the presence of each enzyme by standard techniques (34). Briefly, lysozyme activity was quantified by measuring the rate of lysis of Micrococcus lysodeikticus (Sigma Chemical Co., St. Louis, MO) by a turbidometric method. Lactate dehydrogenase was assayed based on the spectrophotometric measurement of NADPH consumption. Glucuronidase activity was assayed by spectrophotometrically measuring the release of p-nitrophenol from p-nitrophenoxy-β-glucuronide by hydrolysis. Myeloperoxidase activity was assayed by measuring the absorbance change produced by the oxidation of tetramethylbenzidine (35).

Platelet Secretion—Platelet secretion was monitored using [5-14C]hydroxytryptamine ([5-14C]HT) release assay, as described by Rogers et al. (36). Purified platelets were preincubated with [5-14C]HT (Amer sham Pharmacia Biotech, specific activity 50–62 mCi/mmol) at 0.05 mCi/ml platelet suspension ($3 \times 10^{8}$ c/mm) for 60 min at 37 °C. Platelet activation with 0.5 unit/ml of human thrombin was performed in Eppendorf tubes, during 5 min without stirring. Platelets were preincubated with various concentrations of labeled peptides (5–40 μM), during 5 min at room temperature before activation. During the platelet secretion assay a sample of platelet was incubated without the addition of thrombin to measure the amount of [5-14C]HT that was not taken up by the platelets and may have been spontaneously released during the procedure (blank). Platelet activation was stopped by the addition of acetylsalicylic acid (15 mM), and samples were placed on ice for ~5 min and spun at 12,000 × g for 5 min. 50-μl aliquots were taken from the supernatant and counted for analysis of [5-14C]HT release with an LS-6500 scintillation counter (Beckman). The percentage of 5-HT release was calculated as follows: (amount of [14C]HT – blank/total counts – blank) × 100%.

RESULTS

Peptide-lipid binding depends on both the nature of the acidic lipid and the sequence of the cationic peptide. The fluorescence of rhodamine B-QRLFQVKGRR (PBP10) is strongly decreased by addition of phosphatidylinositol 4,5-biphosphate. Fig. 1a shows that the fluorescence intensity of PBP10 is decreased by 90% at an equimolar ratio of PIP2 to PBP10, whereas PS and PC have little or no effect on fluorescence even at molar ratios greater than 4 PS to 1 PBP10 (Fig. 1a and data not shown). Phosphatidylinositol and PI(4)P are less potent than PIP2 in decreasing fluorescence of PBP10, and the order of their effect on PBP10 is similar to their ability to inhibit gelsolin activity (37). PBP10 does not appear to distinguish between phosphatidylinositol 3,4-biphosphate and PIP2, because the effects of these two isomers on PBP10 fluorescence are not significantly different (data not shown). PBP10 fluorescence is also strongly quenched by lysophosphatidic acid, the only other known inhibitor of gelsolin (38). The plot of PBP10 fluorescence versus [lysophosphatidic acid] overlays the data shown for PI(4)P and is omitted for clarity.

Interaction of rhodamine B-labeled cationic peptides with PIP2 depends strongly on the peptide sequence. Fig. 1b shows that substitution of Leu for Arg at position 161 abolishes the binding, as does truncation of the peptide at either the N terminus or the C terminus, because neither RhoB-QRLFQ nor RhoB-QVKGRR bound PIP2 with high affinity.

As with other rhodamine derivatives, but not unconjugated rhodamine B, the fluorescence of PBP10 is strongly dependent on pH as shown in Fig. 1c. Fluorescence is slightly increased by monovalent salt at constant pH, but no specific effect of divalent cations was observed. The very strong dependence of rhodamine-peptide fluorescence was also seen with most cationic peptide derivatives without regard for affinity for PIP2. However, the fluorescence of rhodamine B itself was nearly constant over the entire pH range of Fig. 1c (data not shown) and higher than that of the peptide derivatives even at low pH.

Surface Activity of PPI-binding Peptides—To determine if the membrane permeation activity of PBP10 is associated with an amphipathic character that promotes partitioning to hydrophobic interfaces, measurements of surface tension at the air/water interface were made in the presence of various concentrations of PBP10 and related compounds. In addition to its specific binding to inositol lipids, the amphipathic PBP10 peptide and other rhodamine derivatives also show strong surface activity at micromolar concentrations. Fig. 1d shows how the surface pressure at the liquid/air interface changes when increasing amounts of PBP10 are added to a solution of phospho-buffered saline. A significant increase in surface pressure occurs at peptide concentrations between 1 and 10 μM. The dependence of pressure on concentration is not linear, and the abrupt onset suggests a coordinated effect on surface tension common to surface-active compounds. The surface activity of PBP10 is stronger than that of either the unlabeled peptide of the same sequence or the rhodamine B label by itself. RhoB-QRF also shows enhanced surface activity relative to rhodamine B, but this activity is significantly weaker than that of PBP10. However, a strongly membrane-stabilizing peptide such as the antibiotic peptide LL37 is active at one order of magnitude lower concentration.

FIG. 1. In vitro characterization of PBP10. a, fluorescence intensity of 2 μM PBP10 after addition of various molar ratios of PIP2 (diamonds), PI(4)P (downward triangles), phosphatidylinositol (upward triangles), PS (filled circles), or PC (open circles) in 10 mM Tris (pH 7.0). Fluorescence emission was measured at 590 nm after excitation at 560 nm. b, fluorescence intensity after addition of various amounts of PIP2 to solutions containing 2 μM PBP10 (diamonds), rhodamine B-QRLFQVKGRR (open circles), rhodamine B-QLRFQ (upward triangles), or rhodamine B-QVKGRR (downward triangles). Error bars represent standard deviations from three independent measurements. c, optical density at 550 nm of 20 μM PBP10 in 7.5 mM phosphate buffer at various pH (open circles) or in phosphate buffer containing 140 mM NaCl (filled circles). d, surface pressure at the liquid/air interface for phospho-buffered saline (7.5 mM phosphate, 140 mM NaCl, pH 7.0) with various concentrations of PBP10 (upward triangles), rhodamine B-QRL (closed circles), rhodamine B (open circles), or QRLFQVKGRR (diamonds). All measurements represent mean and standard deviations from at least three separate samples prepared for each peptide concentration.
Inhibition of Cell Motility and Platelet Activation—Because PPI-regulated actin polymerization is implicated in cell motility, PPI-binding peptides would be expected to alter this function when present in the cytoplasm. PBP10 exerted a concentration-dependent inhibitory effect on the directed migration of cultured fibroblasts, melanoma cells, and human blood neutrophils (Fig. 3, a–c). Maximal inhibition occurred at concentrations of 25 μM and above, but motility was impaired at peptide concentrations as low as 5 μM. This effect was partially reversible. Neutrophils incubated in 10–20 μM PBP10, then washed and incubated in excess volumes of peptide-free buffer regained motile ability in a time-dependent manner. Restoration was maximal after 1 h of incubation (Fig. 3c). In contrast, non-cell-permeant peptides that bound PIP2 had no effect on either fibroblast or neutrophil motility (data not shown). Fig. 3d shows that a similar range of PBP10 concentrations (1–25 μM) very strongly inhibited platelet aggregation as measured by light transmission, whereas the same concentrations of RhoB-QRL had no effect.

Effects on Platelet Activation and Signaling—The profound effects of PBP10 on platelet activation coincide with inhibition of actin polymerization and secretion but without effects on intracellular [Ca2+]i influx. Both shape change and aggregation of platelets triggered by thrombin require increased actin polymerization, and Fig. 4a shows the inhibition of actin assembly in thrombin-stimulated platelets as a function of time after thrombin addition at constant (20 μM) PBP10. Similar concentrations of RhoB-QRL and the Arg161→Leu mutant PBP10 had no effect on actin assembly. The concentrations of PBP10 needed to inhibit actin polymerization paralleled those observed to inhibit platelet aggregation as shown in Fig. 4b.

In contrast to the nearly total block of actin assembly, PBP10 had no significant effect on the initial increase in intracellular [Ca2+]i elicited by activation of the thrombin receptor, which activates phospholipase Cβ via a trimeric GTPase resulting in release of Ca2+ from IP3-sensitive intracellular pools as well as influx of extracellular Ca2+. Fig. 4c shows that the intracellular Ca2+ increase resulting from thrombin stimulation of Fura 2-AM-loaded platelets is not inhibited by the same concentrations that inhibit platelet activation, but the later decrease in [Ca2+]i is blunted by PBP10. This effect is not due to permeabilization of the platelet by PBP10, because concentrations of this peptide below 30 μM had no effect on resting [Ca2+]i levels. Higher concentrations of PBP10 (>50 μM) did increase intracellular [Ca2+], although not to the levels achieved with either thrombin activation or treatment with the Ca2+ ionophore A23187 (data not shown). Consistent with the effects seen on actin assembly, the data in Fig. 4d show that secretion of [5-14C]hydroxytryptamine ([5-14C]HT) from thrombin-activated platelets is also inhibited by PBP10. Although addition of PBP10 alone to platelets caused either no change or a slight amount of [5-14C]HT secretion in the absence of other stimulus, when added before thrombin activation, PBP10 strongly inhibited the release of intracellular [5-14C]HT. RhoB-QRL had no effect on thrombin-stimulated [5-14C]HT release or on secretion in the absence of thrombin.

Effects of PBP10 on Intracellular Actin Assembly and [Ca2+]i Signaling in Human Neutrophils Are Similar to Those Seen with Platelets—Fig. 5 (a and b) shows that both migration speed and actin assembly normally triggered by the chemotactic peptide fMLP are strongly inhibited by PBP10 concentrations below 20 μM. Equal or greater concentrations of RhoB-QRL have no effect. The inhibition of actin assembly and motility is not due to a general cell toxicity, because intracel-
lular Ca$^{2+}$ influx stimulated by fMLP is insignificantly altered by PBP10 at the concentration where motility and actin polymerization are maximally inhibited (Fig. 5c). At concentrations greater than 40 $\mu$M, addition of PBP10 to neutrophils in Ca$^{2+}$-containing medium caused a slow increase in intracellular Ca$^{2+}$ that was reversed on addition of extracellular EGTA, suggesting that at these, but not lower, concentrations PBP10 permeabilized the cell membrane to small ions. The cytoskele-
ton-independent secretion of lysozyme by neutrophils stimulated by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate is also unaffected by 20 μM PBP10 as shown in Fig. 5d. This figure also shows that treatment of neutrophils by inhibitory concentrations of PBP10 (20 μM) does not cause membrane permeabilization, because neither lactate dehydrogenase nor myeloperoxidase release could be detected after treatment with the peptide.

**Effects of PBP10 on Cytoskeletal Actin in Fibroblasts**—To reveal the effect of PBP10 treatment on actin filaments in vivo, NIH3T3 fibroblasts that stably express EGFP-actin were incubated with PBS (the solvent for the peptides), with 25 μM RhoB-QRL control peptide, or with 25 μM PBP10. In cells incubated in PBS for 5 min, stress fibers and cortical actin were apparent (Fig. 6, A and M). Filamentous actin structures remained intact after incubating the cells with RhoB-QRL for 5 min (Fig. 6, B and N). As was observed in Fig. 2, the RhoB-QRL peptide showed a punctate pattern in the cytoplasm, which did not significantly co-localize with actin filaments (Fig. 6, F, J, R, and W). In contrast, in cells incubated with PBP10 for 5 min, stress fibers and cortical actin were completely disrupted (Fig. 6, C, E, O, and Q). Most PBP10 fluorescence was found at the plasma membrane, in cytoplasmic aggregates, in the nucleoli, and on the nuclear envelope (Fig. 6, G and S). Actin fluorescence co-localized with PBP10 in the cytoplasmic aggregates (Fig. 6, K, U, and X) and on the plasma membrane, where both actin and PBP10 appear in distinct patches shown by the arrowheads in Fig. 6 (C, G, and K).

The disruption of actin caused by PBP10 was transient, and a normal cytoskeletal structure was restored within 2 h. Both stress fibers and cortical actin staining were indistinguishable from controls after removal of the peptide from the medium (Fig. 6, D and P). Rhodamine fluorescence in cytoplasmic aggregates and dots on the plasma membrane disappeared along with staining of the nuclear envelope and nucleoli, as seen in Fig. 6, G and S. Instead, punctate staining was detected in the cytoplasm, suggesting that PBP10 was metabolized or sequestered (Fig. 6, H and T) and the rhodamine B signal no longer co-localized with EGFP-actin in these cells (Fig. 6, L and Y). Thus, these data suggest that PBP10 disrupts actin filaments that are essential for cell morphology and cell motility, but these effects are transient.

To reveal the identity of the punctate structures to which PBP10 was targeted, NIH3T3 cells were double-stained for organellar markers (LDL or transferrin) and rhodamine B-labeled peptides (RhoB-QRL control peptide or PBP10). At steady state, LDL is targeted to late endosomes or lysosomes, whereas transferrin localizes in early endosomes and recycling endosomes. As shown in Fig. 7, the RhoB-QRL peptide completely co-localized with LDL. On the contrary, PBP10 did not show apparent co-localization with LDL or transferrin. In addition, PBP10 failed to change the distribution of caveolin-1 on the plasma membrane, a marker for membrane rafts (data not shown). These data suggest that vesicular structures stained with RhoB-QRL control peptide in Fig. 6 correspond to late endosomes and lysosomes. On the other hand, the identity of the amorphous and punctate structures stained with PBP10 still remains unclear.

**DISCUSSION**

The results presented here show that a fluorescent derivative of a 10-residue peptide with sequence derived from the phosphoinositide binding region in gelsolin is capable of entering four different eukaryotic cell types and of inhibiting morphologic changes requiring actin cytoskeletal rearrangements in which regulation of actin binding proteins by PIP2 has been implicated. Visualization of the active peptide in fibroblasts shows that it distributes throughout the cytoplasm of treated cells, concentrating in patches along the plasma membrane and in regions of the nucleus resembling nucleoli. These patches do not localize with either transferrin- or LDL-containing vesicles, consistent with the idea that entry of this peptide does not require endocytosis, in contrast to the trafficking of the inactive RhoB-QRL peptide, which, like LDL, is targeted to lysosomes. The actin cytoskeleton, as visualized by EGFP-actin stably expressed in NIH3T3 cells, is disrupted by this peptide, as stress fibers and the actin-rich cortical shell disassemble. The result is a more diffuse actin distribution containing some amorphous patches of actin that co-localize with the peptide within the cytoplasm. In contrast, fluorescent peptides that...
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enter cells but lack PPI binding activity do not disrupt actin structures and localize to distinct bright dots representing late endosomes and other vesicular compartments.

The effects of the peptide are not due to toxicity or membrane permeabilization as confirmed by a number of controls. Direct measurements of intracellular [Ca\(^{2+}\)] by Fura 2-AM show no evidence for Ca\(^{2+}\) flux at the levels of peptide sufficient to inhibit motility and disrupt cellular actin assembly. Toxicity is also unlikely, because PBP10 does not prevent the specific Ca\(^{2+}\) influx stimulated by thrombin in platelets and fMLP in neutrophils. Moreover, the effects of PBP10 on the cytoskeleton of fibroblasts and the motility of neutrophils are reversed after the cells are moved to peptide-free media. Additional arguments that the effects of the PPI-binding peptide are specific and not the result of cell damage are provided from studies that show stimulation of cellular activities dependent on PI3K (29).

At higher concentrations, typically above 50 \(\mu\)M but depending on the cell type, the PBP10 peptide slightly increased intracellular [Ca\(^{2+}\)] in the absence of other stimuli in both platelets and neutrophils (data not shown).

A quantitative analysis of peptide distribution within the cell is presently confounded by the strong dependence of peptide fluorescence on pH and PPI binding that may enhance the fluorescence in acidified vesicles and mask localization to PPI-rich membrane domains. Therefore, the punctate intracellular fluorescence and patches of bright fluorescence on the plasma membrane seen for the active peptide PBP10 may represent an even more striking peptide distribution than evident from Fig. 6, suggesting that the PPI binding sequence is relatively absent from compartments requiring endocytosis and concentrated at sites enriched in both actin and PPIs.

The relation between PPI binding and the ability to cross the cell membrane is not obvious. Except for PI, inositol lipids are generally thought to be localized to the intracellular face of the plasma membrane, cytoplasmic vesicles, and the nucleus. The fact that PIP\(_2\)-binding proteins and peptides that are coupled to rhodamine B by a sulfhydryl link to an N-terminal Cys cannot enter the cell except possibly by endocytosis (25) also strongly suggests that affinity for PIP\(_2\) is not sufficient to bind or cross the plasma membrane. On the other hand, one feature common to both PIP\(_2\)-binding peptides derived from gelsolin and several classes of peptides that cross or disrupt the plasma membrane is a cationic/hydrophobic character exemplified by many antibiotic peptides.

Recent analyses by circular dichroism and NMR show that peptides based on gelsolin residues 150–169 undergo a coil to

![Image](https://example.com/image.png)
helix transition in the presence of small concentrations of PIP$_2$ and other acidic surfactants (39). The helical structure so formed produces an amphipathic molecule with positive charges arranged on one face and hydrophobic residues on the other. Although the brevity of the PBP10 sequence prevents any strong homology search, the sequence of gelsolin 150–169 bears some resemblance to the antibiotic peptides, magainins and cecropins, that insert into the plasma cell membrane of prokaryotic and eukaryotic cells (40, 41). Such an analogy is strengthened by preliminary data showing that, at the concentrations of PBP10 at which it shows surface activity and inhibits cell motility, it also exhibits antibiotic function with *Escherichia coli*. However, the cationic amphipathic character associated with membrane permeability is not sufficient to produce the changes in cytoskeleton and motility observed with PBP10, because neither magainin nor the cathelicidin peptide LL37 exert the same effect on cell function as PBP10.

An alternative possibility is that the phosphoinositide binding due to the gelsolin sequence and cationic, hydrophobic character due to the linked fluorophore cooperate to produce unique effects not mimicked by either activity alone. Rhodamine B, like other rhodamine derivatives, has been shown to enter cells passively, and has been studied for potential cytotoxic effects (42) and as a marker for acidic vesicles in muscle cells (43). The hydrophobic and weakly cationic character of rhodamine B may initiate the contact with the cell membrane, but more specific interactions mediated by the specific peptide sequence would then dictate the mode of cell entry and the resulting intracellular localization as well as effects on the cytoskeleton and other cell functions.

Both the structure and the cellular effects of the PBP10 peptide distinguish it from PH domain constructs. In *vitro*, many PH domains bind to single lipid headgroups and often with the same affinity for IP$_3$ as for the parent lipid (44). In contrast, gelsolin-related proteins and their isolated PPI binding domains bind poorly to inositol phosphates and in some cases require the lipid to be packed into micelles or bilayers at sufficiently high molar ratio (37, 45, 46). The fluorescence pattern of PBP10 in fibroblasts also differs from that of GFP-PH domain constructs (4). These results suggest that PBP10 and PH domains and, by extension, their parent proteins may bind to different pools of PPIs in the cell membrane. In addition, binding of these different ligands may have different effects on lipid distribution in complex bilayers. Recent studies of lipid monolayers show that peptides similar to PBP10 localize to domains enriched in PIP when such domains form at moderate surface pressures (47), and the binding of peptides can further reorganize the lipid packing. If, as *in vitro* studies suggest, different classes of PPI-binding peptides have different degrees of interaction with the hydrophobic domain of lipid bilayers, they would likewise have very different effects on surface pressure of monolayers and therefore on such features as local curvature or lipid demixing in bilayers. Direct compar-

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3 F. Ahmed, N. Korde, D. Weiner, and P. A. Janmey, unpublished data.
4 R. Bucki and D. Weiner, unpublished data.
ison of such effects remains to be done, and a direct competition between gelsolin and PH domains, for example, for binding PIP$_2$ has not yet been reported.

In summary, this report documents evidence that a fluorescent derivative of a 10-residue peptide derived from the sequence of a phosphoinositide binding site in gelsolin permeates the plasma membrane of a variety of cell types and causes changes in the actin cytoskeleton that may relate to the binding of this peptide to the phosphoinositide pool on cytoplasmic-disposed surfaces. Entry of the peptide into cells does not require endocytosis nor is it accompanied by an obligate permeabilization of the cell to small molecules like Ca$^{2+}$. At larger doses than required to exert effects on cell motility, the peptide can cause partial permeabilization of the cell membrane similar to the effects produced by other amphipathic cationic peptides. The relation of the peptide’s biochemical activities to its effects on cell function is not obvious and is likely to be complex. On one hand, the peptide can compete with and displace gelsolin from PIP$_2$ and may therefore dislodge or alter the functions of similar proteins that are regulated by cellular phosphoinositides. On the other hand, the peptide is a potent activator of PI3K activity in vitro (27), presumably by facilitating substrate exposure to the enzyme, and in some contexts the increased products of the PI3K pathway may be more important than competitive effects on any specific cytoskeletal target. Although elucidating the steps in signal transduction that are disrupted by PBP10 requires much future work, this peptide’s strong and reversible effects on cellular actin and motil-

**Fig. 7. The distribution of organellar markers and rhodamine B-labeled peptides.** NIH3T3 cells were incubated with either 4 μg/ml BODIPY-LDL (A, B, E, F, I, J) or 4 μg/ml Alexa488-transferrin (C, D, G, H, K, L) at 37 °C for 30 min. Subsequently cells were treated with either 25 μM RhoB-QRL control peptide (A, C, E, G, I, K) or 25 μM PBP10 (B, D, F, H, J, L) at 37 °C for an additional 5 min, and fixed in 2% paraformaldehyde. Subcellular localization of organellar markers (BODIPY-LDL or Alexa488-transferrin, A–D), rhodamine B-labeled peptides (E–H), and their superimposed images (I–L) are shown. The areas indicated by white squares in I, J, K, and L are magnified in M, N, O, and P, respectively. Scale bar, 15 μm.
ity suggest that it has potential as a tool to manipulate and help define how signals are produced and integrated to reorganize the cytoskeleton.

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