N-terminal Domains of the Class IA Phosphoinositide 3-Kinase Regulatory Subunit Play a Role in Cytoskeletal but Not Mitogenic Signaling*

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Phosphoinositide (PI) 3-kinases are required for the acute regulation of the cytoskeleton by growth factors. We have shown previously that in the MTLn3 rat adenocarcinoma cell line, the p85/p110α PI 3-kinase is required for epidermal growth factor (EGF)-stimulated lamellipod extension and formation of new actin barbed ends at the leading edge of the cell. We have now examined the role of the p85α regulatory subunit in greater detail. Microinjection of recombinant p85α into MTLn3 cells blocked both EGF-stimulated mitogenic signaling and lamellipod extension. In contrast, a truncated p85(1–333), which lacks the SH2 and iSH2 domains and does not bind p110, had no effect on EGF-stimulated mitogenesis but still blocked EGF-stimulated lamellipod extension. Additional deletion analysis showed that the SH3 domain was not required for inhibition of lamellipod extension, as a construct containing only the proline-rich and breakpoint cluster region (BCR) homology domains was sufficient for inhibition. Although the BCR domain of p85 binds Rac, the effects of the p85 constructs were not because of a general inhibition of Rac signaling, because sorbitol-induced JNK activation in MTLn3 cells was not inhibited. These data show that the proline-rich and BCR homology domains of p85 are involved in the coupling of p85/p110 PI 3-kinases to regulation of the actin cytoskeleton. These data provide evidence of a distinct cellular function for the N-terminal domains of p85.

The regulation of cellular motility is important in a variety of physiological processes, ranging from wound healing to the metastatic behavior of transformed cells. We have used a metastatic breast cancer cell model, the MTLn3 cell, to study the role of phosphoinositide 3’-kinases in EGF1-stimulated motility. The acute regulation of the actin cytoskeleton by EGF requires the p85/p110α isoform of PI 3-kinase. MTLn3 breast cancer cells express similar levels of p85/p110α and p85/p110β. However, EGF-stimulated lamellipod extension is blocked by microinjection of inhibitory antibodies to p110α but not p110β.

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¶ The abbreviations used are: EGF, epidermal growth factor; PI, phosphoinositide; PRD(s), proline-rich domain(s); BCR, breakpoint cluster region; GST, glutathione S-transferase; BrdUrd, deoxybromouridine; JNK, c-Jun N-terminal kinase; FITC, fluorescein isothiocyanate; ANOVA, analysis of variance nPRD, N-terminal PRD; cPRD, C-terminal PRD.
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MATERIALS AND METHODS

Recombinant Proteins—GST-N17Rac was purchased from Cytoskeleton, Inc. (Denver, CO). Recombinant p85 constructs used in this study are summarized in Fig. 1. p85ΔΔcPRD (deletion of residues 146–299) was provided by Dr. Christopher Rudd, Harvard University. Deletion of the PRDs from full-length p85 was accomplished by the method of Kunkel et al. (27) using oligonucleotides that deleted residues 84–96 (nPRD), 303–314 (cPRD), or both. These constructs were then amplified by polymerase chain reaction using forward primers encompassing bases 1–21 of human p85a and reverse primers encompassing bases 1041–1021 and subcloned into pGEX2T (Amersham Pharmacia Biotech). Finally, the 82–333 fragment was amplified by polymerase chain reaction and subcloned into pGEX2T. Recombinant proteins were produced in BL-21 Escherichia coli and purified by affinity chromatography on glutathione-Sepharose (Amersham Pharmacia Biotech). Proteins were extensively dialyzed against phosphate-buffered saline and concentrated to 3 mg/ml using Centricon concentrators (Millipore). Protein purity and final concentration was assayed by SDS polyacrylamide gel electrophoresis and Coomassie Blue staining. The proteins were mixed with 1M sorbitol for 30 min, fixed with 10% paraformaldehyde for 30 min at room temperature, and permeabilized with methanol on dry ice for 10 min. After blocking in 1% bovine serum albumin/5% donkey serum, the cells were stained with anti-active JNK antibodies (Promega) followed by Cy3 anti-donkey antisera, to measure JNK activation, or FITC anti-mouse antibody, to identify injected cells. The data are representative of 2 separate experiments.

Inhibition of EGF-stimulated Lamellipod Extension by Recombinant p85—Overexpression of p85 has been shown previously to inhibit membrane ruffling in Ras-transformed fibroblasts (29). Although some of this inhibition could be due to the titration of intracellular phosphotyrosine residues by the p85 SH2 domains (30), the N-terminal half of p85 also contains numerous protein-protein interaction domains (SH3, proline-rich, and BCR homology domains). Thus, p85 overexpression could also inhibit ruffling by saturating the intracellular targets of these domains.

To study the function of these N-terminal domains of p85, we made GST fusions of wild-type p85 or p85 containing mutations in the conserved FLRV motifs in both p85 SH2 domains (29). Although some of this inhibition could be due to the titration of intracellular phosphotyrosine residues by the p85 SH2 domains (30), the N-terminal half of p85 also contains numerous protein-protein interaction domains (SH3, proline-rich, and BCR homology domains). Thus, p85 overexpression could also inhibit ruffling by saturating the intracellular targets of these domains.

RESULTS AND DISCUSSION

Inhibition of EGF-stimulated Lamellipod Extension by Recombinant p85—Inhibition of lamellipod extension was assessed by injecting recombinant p85 constructs into MTLn3 cells. After a 2-h recovery, the cells were stimulated with EGF for 3 min, fixed, and stained with rhodamine-phalloidin and FITC anti-mouse antibodies (to identify injected cells). The percentage of injected cells that extended lamellipodia was counted. Ctl, control. B, as above, except the cells were incubated without serum for 4 h, injected for 10 min, and stimulated after an additional 10 min. All values are the mean ± S.E. of four determinations. ANOVA analysis demonstrated significance at p < 0.0001; statistical significance of differences between individual means and IgG-injected cells (Tukey HSD test) are indicated.

Anti-mouse antibody, to identify injected cells. The data are representative of 2 separate experiments.

Imaging—Images were acquired using a Nikon Eclipse 400 fluorescence microscope with Nikon CFI Plan Apo 60 × 1.4 numerical aperture optics and a Cahu charge-coupled device camera linked to a Scion Y5G frame grabber. Figures were assembled using Adobe PhotoShop.

Statistical Methods—Statistical analysis was performed using ANOVA and Tukey HSD tests, using software from Dr. Richard Lowry, Vassar College.

RESULTS AND DISCUSSION

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To study the function of these N-terminal domains of p85, we made GST fusions of wild-type p85 or p85 containing mutations in the conserved FLRV motifs in both p85 SH2 domains. We have shown previously that the R358A/R659A mutations abolish phosphopeptide binding by the p85 SH2 domains (29). We reasoned that even in the absence of functional SH2 domains, these constructs could interfere with interactions between p85 and intracellular targets and block PI 3-kinase-dependent signaling. Purified GST-p85 or GST-p85(R358A/R659A) were microinjected into quiescent MTLn3 cells. After a 2-h recovery, the cells were stimulated with EGF for 3 min, fixed, stained, and scored for lamellipod extension. Both constructs caused a significant inhibition of lamellipod extension; microinjection of the mutant GST-p85(R358A/R659A) inhibited lamellipod extension by ~52%, relative to control, whereas GST-p85 inhibited lamellipod extension by 77% (Fig. 2). These data suggest that SH2-independent interactions between p85 and intracellular proteins are required for EGF-stimulated lamellipod extension.

p85-p110 binding is extremely stable (31), and the exchange

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**Fig. 2.** Inhibition of lamellipod extension by p85 does not require functional SH2 domains. *A*, MTLn3 cells were incubated without serum for 2 h and then injected with rabbit IgG, GST-p85, or GST-p85(R358A/R659A) as indicated. After an additional 2 h, cells were stimulated with EGF (5 nM) for 3 min, fixed, and stained with rhodamine-phalloidin and FITC anti-mouse antibodies (to identify injected cells). The percentage of injected cells that extended lamellipodia were counted. Ctl, control. B, as above, except the cells were incubated without serum for 4 h, injected for 10 min, and stimulated after an additional 10 min. All values are the mean ± S.E. of four determinations. ANOVA analysis demonstrated significance at p < 0.0001; statistical significance of differences between individual means and IgG-injected cells (Tukey HSD test) are indicated.

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**Fig. 1.** p85ΔΔcPRD-derived constructs used in this study.
of p110 between different p85 molecules is presumably slow. Nonetheless, we wanted to rule out the possibility that endogenous p110 was being sequestered by mutant p85 during the 2-h period after injection. We therefore repeated the experiments but stimulated the cells with EGF 10 min after microinjection. Once again, GST-p85(R358A/R659A) inhibited lamellipod extension nearly as well as wild-type p85 (Fig. 2B).

N-terminal Domains of p85 Are Involved in Cytoskeletal but Not Mitogenic Signaling—The data in Fig. 2 show that recombinant p85 inhibited lamellipod extension even in the absence of functional SH2 domains. This suggested that the N-terminal domains of p85 might also be critical for coupling of p85/p110 to the regulation of the actin cytoskeleton. To test this directly, we constructed a truncated actin p85-1–333, which lacks the SH2 and SH2 domains and cannot bind p110 (Fig. 1). When microinjected into MTLn3 cells, GST-p85-1–333 inhibited EGF-stimulated lamellipod extension almost as well as full-length p85 (Fig. 2B).

Microinjection of GST-p85 had no effect on lamellipod extension (50% of GST-injected cells responded to EGF, as compared with 62% of uninjected cells). However, microinjection of GST-p85 and GST-p85-1–333 reduced EGF-stimulated lamellipod extension to 25 and 33%, respectively (Fig. 3A).

We next tested whether the N-terminal fragment of p85 could inhibit other PI 3-kinase-dependent signaling pathways. We have shown previously that EGF-stimulated BrdUrd incorporation in MTLn3 cells is dependent on class IA PI 3-kinases (2). Basal BrdUrd incorporation in serum-starved MTLn3 cells was ~40% and was unaffected by microinjection of recombinant proteins (data not shown). In contrast, EGF-stimulated BrdUrd incorporation is almost completely blocked by microinjection of recombinant p85 (Fig. 3B). However, microinjection of p85-1–333 had no significant effect on EGF-stimulated BrdUrd incorporation. The BrdUrd incorporation assay requires a longer post-injection incubation (12 h) than is used in the lamellipod extension assays. To rule out the possibility that the BrdUrd results reflected the degradation or inactivation of p85-1–333, we conducted lamellipod extension experiments using the same protocol as in the BrdUrd assays. Quiescent cells were microinjected with GST, GST-p85-1–333, or GST-nSH2 domains (a positive control for inhibition of lamellipodia) and then incubated for 12 h prior to stimulation with EGF for 3 min (Fig. 3C). Once again, the p85-1–333 fragment markedly inhibited lamellipod extension. Thus, our data show that GST-p85-1–333, which lacks SH2 domains but contains SH3, PRD, and BCR homology domains, can selectively interfere with EGF-stimulated cytoskeletal signaling but not EGF-stimulated DNA synthesis.

We have shown previously that MTLn3 cells injected with inhibitory antibodies to p110a are highly condensed and stain brightly with rhodamine-phalloidin (2). Cells injected with full-length GST-p85 had a similar condensed morphology (Fig. 4C). The effect of microinjected GST-p85-1–333 was less pronounced. Although GST-p85-1–333-injected cells did not extend lamellipodia in response to EGF, their morphology was somewhat variable. A few cells resembled GST-p85-injected cells, but many were similar in morphology to unstimulated control cells (Fig. 4, D–F). The less pronounced morphological changes in GST-p85-1–333-injected cells, as well as failure of GST-p85-1–333 to block EGF-stimulated BrdUrd incorporation, are consistent with the hypothesis that GST-p85-1–333 inhibits a subset of p85/p110-dependent signaling processes.

Deletional Mapping of N-terminal Domains Involved in Cytoskeletal Signaling—To determine which regions of p85-1–333 were required for lamellipod extension, we prepared seven constructs (Fig. 1). The effect of microinjection of recombinant proteins on lamellipod extension was determined as described above. All values are the mean ± S.E. of three to five determinations. ANOVA analysis demonstrated significance at p < 0.01; statistical significance of differences between individual means and GST-injected cells (Tukey HSD test) are indicated.

FIG. 3. A truncated p85-1–333 inhibits lamellipod extension but not BrdUrd incorporation. A, MTLn3 cells were incubated without serum for 2 h and then injected with GST, GST-p85, or GST-p85-1–333 as indicated. After an additional 2 h the cells were stimulated for 3 min with 10 nM EGF, and lamellipod extension was determined as described above. Ctl, control. B, MTLn3 cells were rendered quiescent in medium containing 1% fetal bovine serum for 24 h and then injected with GST, GST-p85, or GST-p85-1–333 as indicated. The cells were then incubated in the absence or presence of 2 nM EGF for 12 h, followed by BrdUrd for an additional 2 h. The cells were fixed and stained with anti-BrdUrd antibodies or FITC anti-rabbit antibodies (to identify injected cells), and the percentage of cells that incorporated BrdUrd was counted. C, MTLn3 cells were rendered quiescent in medium containing 1% fetal bovine serum for 24 h and then injected with GST, GST-nSH2 domains, or GST-p85-1–333 as indicated. The cells were incubated for 12 h and then stimulated with 10 nM EGF for 3 min. Lamellipod extension was determined as described above. All values are the mean ± S.E. of three to five determinations. ANOVA analysis demonstrated significance at p < 0.01; statistical significance of differences between individual means and GST-injected cells (Tukey HSD test) are indicated.

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Deletional analysis of p85[1–333]. Quiescent MTLn3 cells were injected with GST-p85-(82–333), GST-p85-(1–333), GST-p85ΔPRD, or GST-p85ΔcPRD, incubated without or with EGF for 3 min, and stained with rhodamine-phalloidin and FITC anti-rabbit antibodies (to identify injected cells). Arrows indicate injected cells.

Morphology of cells injected with GST-p85 or GST-p85-(1–333). Quiescent MTLn3 cells were not injected (A) or injected with GST (B), GST-p85 (C), or GST-p85-(1–333) (D, E, and F). Cells were stimulated with EGF for 3 min (B–F), fixed, and stained with rhodamine-phalloidin and FITC anti-rabbit antibodies (to identify injected cells). Arrows indicate injected cells.

Domains of p85 Involved in Cytoskeletal Signaling

p85 Constructs Do Not Inhibit Rac-dependent Signaling in MTLn3 Cells—EGF-stimulated lamellipod extension is blocked by dominant negative Rac, consistent with data from other systems (32). The BCR homology domain of p85 binds activated Rac and CDC42 (33, 34). Because the nPRD-BCR-cPRD domain is sufficient to block lamellipod extension, we considered the possibility that p85-(1–333) was acting as a generalized Rac inhibitor, through the sequestration of activated Rac. Therefore, the activation of the JNK kinase, which is known to be Rac-dependent (35, 36). MTLn3 cells were treated with sorbitol for 30 min and then fixed and stained with a phospho-specific anti-JNK antibody. As reported previously in other systems (37), sorbitol treatment lead to the activation of JNK, whereas it was completely blocked by microinjection of N17-Rac (data not shown). Thus, the Rac-dependent activation of JNK was not inhibited by GST-p85-(82–333), suggesting that the effects of this construct on EGF-stimulated lamellipod extension were not because of a general sequestration of endogenous activated Rac.

In this paper, we have defined a subset of PI 3-kinase-dependent responses that are inhibited by microinjection of N-terminal domains of p85. The p85-(1–333) and p85-(82–333) constructs do not contain the SH2 and iSH2 domains and therefore should not disrupt endogenous p85/p110 binding or activation of endogenous p85/p110 by phosphorysorine-containing proteins. We therefore presume that these constructs interfere with the targeting of endogenous p85/p110 molecules to sites involved in cytoskeletal regulation. Disruption of this targeting apparently does not interfere with the activation of mitogenic signaling pathways, which may be less spatially organized. Consistent with this latter idea, we have demonstrated previously an increase in DNA synthesis by anti-p85 antibodies that activate p85/p110 dimers but that would be unlikely to cause the targeting of p85/p110 to specific intracellular sites (28).

Although the nPRD-BCR-cPRD fragment of p85 is sufficient to inhibit lamellipod extension, the intracellular targets of these domains of p85 are not yet known. Although the BCR homology domain binds to Rac/CDC42 (33, 34), microinjected p85 fragments do not block JNK activation and are therefore not acting as a global Rac inhibitor. However, the p85-derived constructs could disrupt the targeting of a subset of activated Rac to specific regions of the cell.

In addition to Rac and CDC42, a number of cytoskeletal regulatory proteins interact with p85. Cas and focal adhesion kinase bind to the SH2 domains of p85 (38, 39) and should not therefore be affected by p85-(82–333). Similarly, cbl binds to the SH3 domain of p85 and should be unaffected by p85-(82–333) (40). Tyrosine-phosphorylated ezrin also binds to the p85 SH2 domains, but additional binding occurs through the N

Fig. 5. Deletional analysis of p85[1–333]. A, quiescent MTLn3 cells were injected with GST, GST-p85-(82–333), GST-p85ΔPRD, GST-p85ΔcPRD, or GST-p85ΔPRDΔcPRD, incubated without or with EGF for 3 min, and stained as described for Fig. 2. All values are the mean ± S.E. of three determinations. ANOVA analysis demonstrated significance at p < 0.001; statistical significance of differences between individual means and GST-injected cells (Tukey HSD test) are indicated. Ctl, control.

* p < .01

Contrast, deletion of either the nPRD or the cPRD, or both PRDs, eliminated the inhibitory effect on lamellipod extension (Fig. 5). These relatively small deletions of 11–12 amino acids were unlikely to cause significant structural perturbation, because their deletion from full-length p85 had no effect on binding to p110 or phosphopeptide activation of p85/p110 dimers (data not shown). Preliminary results also show that a construct lacking the BCR domain was unable to inhibit EGF-stimulated lamellipod extension (data not shown). Although we cannot yet state with certainty that the BCR and PRD domains are necessary for inhibition of lamellipod extension, our data show that the nPRD-BCR-cPRD fragment is sufficient for inhibition of lamellipod extension.
terminus of ezrin to an unknown region of p85. The GTP exchange factor Pak interacting exchange factor and the actin-binding protein profilin also bind to p85 at unknown sites and could be affected by microinjection of p85(82–333) (41, 42). Finally, p85-(82–333) could act by binding to SH3 domains in Src and related kinases (43, 44), thereby disrupting their interactions with endogenous p85.

In summary, we have demonstrated that different regions of the p85 regulatory subunit are involved in distinct PI 3-kinase-dependent responses. Whereas the SH2 domains of p85 have pleiotropic effects on multiple pathways, the BCR and proline-rich domains of p85 are coupled to cytoskeletal signaling but not DNA synthesis. These data suggest that different isoforms of class IA regulatory subunit, particularly the full-length (p85) versus short forms (p55/p50), are involved in different subsets of PI 3-kinase-dependent signaling events.

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