Specific Requirement for the p85-p110α Phosphatidylinositol 3-Kinase during Epidermal Growth Factor-stimulated Actin Nucleation in Breast Cancer Cells*

(Received for publication, November 15, 1999, and in revised form, December 8, 1999)

Karen Hill, Susan Weltitz, Jinghua Yu, James T. Murray, Shu-Chin Yip, John S. Coneellisi, Jeffrey E. Segall‡§ and Jonathan M. Backer¶¶

From the Departments of Molecular Pharmacology and of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, New York 10461

We have studied the role of phosphatidylinositol 3-kinases (PI 3-kinases) in the regulation of the actin cytoskeleton in MTLn3 rat adenocarcinoma cells. Stimulation of MTLn3 cells with epidermal growth factor (EGF) induced a rapid increase in actin polymerization, with production of lamellipodia within 3 min. EGF-stimulated lamellipodia were blocked by 100 nM wortmannin, suggesting the involvement of a class Ia PI 3-kinase. MTLn3 cells contain equal amounts of p110α and p110β, and do not contain p110δ. Injection of specific inhibitory antibodies to p110α induced cell rounding and blocked EGF-stimulated lamellipod extension, whereas control or anti-p110β antibodies had no effect. In contrast, both antibodies inhibited EGF-stimulated DNA synthesis. An in situ assay for actin nucleation showed that EGF-stimulated formation of new barbed ends was blocked by injection of anti-p110α antibodies. In summary, the p110α isoform of PI 3-kinase is specifically required for EGF-stimulated actin nucleation during lamellipod extension in breast cancer cells.

PI 3-kinases are important signaling intermediates in a variety of regulated cellular processes. They are classified based on their regulation and substrate specificity. Class I enzymes produce PI[3]P, PI[3,4]P2, and PI[3,4,5]P3, whereas class II and III enzymes produce PI[3]P and PI[3,4]P2, or only PI[3]P, respectively. Class Ia enzymes exhibit the greatest diversity of the known PI 3-kinases, with multiple isoforms of both the regulatory (p85) and catalytic (p110) subunits. Differential phosphorylation of p85α and p85 and differential activation of p85α- and p85β-associated PI 3-kinase have been reported (3, 4). Knockouts of p85α further suggest that the p85α and p85β are not redundant (5, 6). Distinct class Ia catalytic subunit isoforms also have different functions. Both p110α and p110β play a role in mitogenesis, although p110α is required for responses to a broader range of growth factors (7, 8). Recently, distinct signaling properties for p110 isoforms have been demonstrated in macrophages (9).

We examined the specific function of p110α and p110β in MTLn3 cells, a metastatic variant of the 13762NF rat mammary adenocarcinoma. MTLn3 cells undergo motility in an EGF gradient (10, 11). This response involves the actin-dependent extension of a lamellipod in the direction of increasing EGF concentrations, with a zone of newly polymerized F-actin at the leading edge (12). Using isoform-specific inhibitory antibodies against p110α and p110β, we now show that EGF-stimulated lamellipodia were blocked by p110α, but not p110β. Significantly, anti-p110α antibodies blocked the formation of new barbed ends during an in situ actin nucleation assay. These studies provide direct evidence that p110α is required for the regulation of actin nucleation by EGF.

MATERIALS AND METHODS

Cells—MTLn3 adenocarcinoma cells and GRC-LR73 cells were grown as described previously (11, 13).

Production and Characterization of Antisera—A C-terminal peptide from human p110α (14) (CKVNWMAHTVRKDYRS) was coupled to keyhole limpet hemocyanin and used to immunize New Zealand White rabbits (Covance, Denver, PA). Antibodies were purified using the same peptide coupled to Ultralink-iodoacetyl resin (Pierce, Rockford, IL). Antibody specificity was tested by immunoprecipitation of proteins from MTLn3 cells, labeled with [35S]methionine (13). The antibodies were tested for their inhibition of recombinant p110α or p110β, as described (13). Antibodies for microinjection were diaлизed into phosphate-buffered saline and concentrated to 4 mg/ml.

Western Blotting—MTLn3 cells were lysed and subjected to four successive rounds of immunoprecipitation with IgG, anti-p110α or anti-p110β antibody followed by Western blotting with a p85α antibody.

BrdUrd Assays—GRC-LR73 cells were starved for 2 d, injected with antibodies as indicated, and stimulated with 100 nM insulin for 16 h. Alternatively, MTLn3 cells were starved for 24 h, injected with antibodies as indicated, and stimulated with 10 ng/ml EGF for 12 h. In both cases, cells were then incubated with BrdUrd for 1 h, fixed, and stained as described previously (13).

Lamellipod Extension and Actin Nucleation Assays—MTLn3 cells were starved for 3 h in medium containing 0.375% bovine serum albumin, and injected as indicated. After a 1 h recovery, the cells were stained with 5 nM EGF for 3 min. For lamellipod extension assays, the cells were fixed in 3.75% formaldehyde for 10 min at 37 °C, permeabilized and stained as described previously (13). For actin nucleation assays, cells were permeabilized with 0.25% saponin in the presence of 0.45 μM rhodamine-α-actin for 1 min, fixed in 3.7% formaldehyde for 5 min, and processed as described previously (12).

Image Acquisition—Images were acquired using a Nikon Eclipse 400 fluorescence microscope with Nikon CFI Plan Apo 60 × 1.4 numerical aperture optics and a Cohu CCD camera linked to a Scion VGS frame grabber. Figures were assembled using Adobe Photoshop.

RESULTS

Lamellipod Extension in MTLn3 Cells—The rat mammary adenocarcinoma cell line MTLn3 undergoes a rapid morphological change in response to stimulation with EGF (11). The cells

*This work was supported by a Scholar Award from the Irma T. Hirschl Trust and National Institutes of Health Grant GM55692 (to J. M. B.) and by a grant from the United States Army (to J. E. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Established Scientist of the American Heart Associate (New York Affiliate).

§ To whom correspondence should be addressed: Dept. of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Tel.: 718-430-2153; Fax: 718-430-3749; E-mail: backer@aecon.yu.edu.

¶¶ The abbreviations used are: PI 3-kinase, phosphatidylinositol 3-kinase; EGF, epidermal growth factor; FITC, fluorescein isothiocyanate; GEF, GTP exchange factor.
flattens and extends a broad lamellipod, with the deposition of new filamentous actin at the periphery of the cell (12). To characterize the role of PI 3-kinases in this process, we incubated cells with wortmannin prior to stimulation by EGF. Increasing doses of wortmannin inhibited the EGF-stimulated increase in surface area, with maximal inhibition at 100 nM wortmannin (data not shown). This dose inhibits class I and class III PI 3-kinases, but not class II PI 3-kinases or wortmannin-sensitive PI 4-kinases (1). Because inhibitory antibodies to class III PI 3-kinase do not inhibit insulin stimulated membrane ruffling in CHO cells (13), we focused on the role of class I PI 3-kinases in the EGF-stimulated cytoskeletal responses.

Characterization of Anti-p110α and Anti-p110β Antibodies—Antibodies that specifically inhibit the class Ia PI 3-kinase p110β. The antibody immunoprecipitated a single major 110-kDa band from MTLn3 cells labeled to equilibrium with [35S]methionine/cysteine (Fig. 1A). A weak band at 85 kDa is presumably the p85 regulatory subunit, which contains few methionines and cysteines relative to p110β. The anti-p110α antibodies inhibited recombinant p110α but not recombinant p110β, whereas anti-p110β antibodies inhibited p110β but not p110α (Fig. 1B). The dose responses for isoform-specific inhibition of p110α and p110β by the two antibodies were similar (Fig. 1C). Thus, the antibodies are appropriate for use as isoform-specific inhibitors in microinjected cells.

We next used the antibodies to determine the relative abundance of p110 isoforms in MTLn3 cells. Four successive immunoprecipitations from parallel MTLn3 lysates were performed with anti-p110α or anti-p110β antibodies followed by analysis with a pan-anti-p85 (Fig. 1D). Quantitation of the Western blot showed that MTLn3 cells have similar levels of p110α and p110β (data not shown).

EGF-stimulated Lamellipod Extension Requires p110α but Not p110β—Quiescent MTLn3 cells were injected with rabbit IgG, anti-p110α, or anti-p110β antibodies. After a 1-h recovery period, the cells were stimulated with EGF for 3 min, fixed, and stained with anti-rabbit antibodies, to identify injected cells, and with rhodamine-phalloidin, to visualize F-actin. Fig. 2, A and B, shows that in both control and IgG-injected cells, EGF stimulates the production of a broad lamellipod with an actin-rich edge. In contrast, microinjection of inhibitory anti-p110α antibodies had a pronounced effect on the cytoskeleton (Fig. 2, C and D), as anti-p110α-injected cells were highly condensed with few lamellipodia. Despite the fact that MTLn3 cells contain similar amounts of p1110α and p110β, injection of inhibitory anti-p110β antibodies did not cause condensation of the quiescent cells, and anti-p110α-injected cells produced lamellipodia in response to EGF (Fig. 2, E and F). To quantitate the differential response to EGF in cells injected with anti-p110α and p110β, we counted the number of cells injected with each antibody and determined the fraction of these cells that extended lamellipodia in response to EGF (Fig. 2G). Wherever injection of anti-p110α antibodies caused only a slight inhibition, injection of anti-p110α antibodies blocked lamellipod extension by 80%. This inhibition was similar to that produced by injection of recombinant p85α, which acts as an inhibitor of numerous p110-mediated responses (15). Thus, MTLn3 cells show a differential requirement for p110α and p110β during EGF-stimulated lamellipod extension.

Effect of Anti-p110 Antibodies on BrdUrd Incorporation—To test the role of p110α and p110β in other cellular functions, we measured the effect of the antibodies on insulin-stimulated DNA synthesis in GRC-LR73 cells, an insulin-responsive CHO cell line (13, 16). Insulin causes a 5-fold increase in BrdUrd incorporation in these cells, and microinjection of inhibitory antibodies to p110α blocks this stimulation (Fig. 3A). Inhibitory antibodies to p110β also reduced insulin-stimulated BrdUrd incorporation, to nearly the same extent. We next examined EGF-stimulated BrdUrd incorporation in MTLn3 cells (Fig. 3B). Despite culture in reduced serum for 24 h, MTLn3 cells maintain a high basal rate of BrdUrd incorporation; similar results were observed after 2–3 days of serum deprivation. Addition of EGF caused a 60–80% increase in BrdUrd incorporation, which was inhibited by microinjection of antibodies to either p110α or p110β. These data clearly show that both the anti-p110α and p110β antibodies are inhibitory in intact cells. Moreover, they suggest that, unlike EGF-stimulated cytoskeletal regulation, maximal insulin- or EGF-stimulated DNA synthesis requires both p110α and p110β.

p110α Is Required for EGF Stimulation of New Barbed Ends—EGF-stimulated lamellipod extension requires the formation of new barbed ends at the cell edge, with subsequent incorporation of actin monomers into the growing actin filaments. To directly test the role of p110α in the EGF-stimulated formation of new barbed ends, we conducted an in situ nucleation assay in which rhodamine-actin is added to EGF-stimulated saponin-permeabilized cells (12). As previously shown, EGF caused a marked increase in the incorporation of rhodamine-actin, particularly at the leading edge of the flat, ruffle-free lamellipod that is extended after EGF treatment (Fig. 4, A and B). Injection of control IgG (Fig. 4, A and B, arrows) or anti-
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FIG. 2. Effect of anti-p110 antibodies on EGF-stimulated lamellipod extension. Control MTLn3 cells or antibody-injected cells were incubated in the absence or presence of EGF for 3 min, fixed, and stained with FITC-anti-rabbit IgG (to identify injected cells) or with rhodamine-phalloidin (to visualize F-actin). Injected cells are marked with white arrows. A, IgG-injected cells, no EGF; B, IgG-injected cells plus EGF; C and D, anti-p110α-injected cells plus EGF; E and F, anti-p110β-injected cells plus EGF; and G, the percentage of control or injected cells that produced lamellipodia was counted. The data represent the mean ± S.E. of percentages from four separate experiments.

p110β (data not shown) had no effect on lamellipod extension or rhodamine-actin incorporation. In contrast, injection of inhibitory anti-p110α antibodies causes a significant decrease in rhodamine-actin incorporation in both quiescent and EGF-stimulated cells (Fig. 4, C and D). We scored the number of cells that incorporated rhodamine-actin into the leading edge of an EGF-stimulated lamellipod. Injection of anti-p110α antibody caused an 80% decrease in the percentage of cells with actin incorporation at the leading edge (Fig. 4E).

DISCUSSION

We have defined differential roles for class Ia PI 3-kinase isoforms during EGF-stimulated rearrangements of the actin cytoskeleton in breast cancer cells. Whereas both p110α and p110β are required for maximal mitogenic responses, we have demonstrated a specific requirement for p110α during EGF-stimulated lamellipod extension. Moreover, we have shown that inhibition of p110α blocks the formation of new barbed ends in EGF-stimulated cells, providing the first direct evidence that a specific isoform of PI 3-kinase is responsible for the regulation of actin nucleation. Our data provide a mechanistic basis for previous studies showing that growth factor-induced ruffling is blocked by PI 3-kinase inhibitors (17, 18) and directly implicate p110α in the acute regulation of the actin cytoskeleton in breast cancer cells.

There is not at present a clear explanation for the distinct intracellular functions of different class Ia PI 3-kinases. The in vitro lipid and protein kinase activities of the class Ia isoforms are similar. Furthermore, p110α and p110β form heterodimers with the same p85 regulatory subunits (1, 2). Both p110α (19) and p110β (data not shown) are labile as monomers at 37 °C, but are stabilized by association with p85 regulatory. It is therefore likely that, in intact cells, both isoforms function exclusively as dimers with p85 because dissociation would lead to rapid inactivation and degradation. However, p110α and p110β are only 42% identical and could form additional direct interactions with cellular effectors or targeting proteins (1). Thus, p110β binds directly to activated Rab5 in vitro and is found in clathrin-coated vesicles, whereas p110α is not (20). Similarly, a differential association of p110α with cytoskeletal components in MTLn3 cells could explain its role in EGF-stimulated lamellipod extension.

Injection of anti-p110α antibodies causes a rounding up of MTLn3 cells similar to that seen in cells treated with cytochalasin D (data not shown). Barbed end number is also reduced in anti-p110α-injected cells, demonstrating an inhibition of actin nucleation. Thus, our data directly implicate the p85-p110α PI 3-kinase in the formation of new barbed ends at the leading edge of the cell. Although the mechanism involved is not yet clear, activation of Rho-family GTPases appears to be central in growth factor-mediated actin reorganization (21, 22). PI 3-kinase has been linked to Rac activation in platelet-derived growth factor-stimulated cells (23), where the products of PI 3-kinase presumably signal to Rac through a protein analogous to Vav, a PI 3-kinase-dependent GTP exchange factor (GEF) in hematopoietic cells (24). Direct regulation of CDC42 by PI 3-kinase products has not been demonstrated. However, the facioscapulohumeral dystrophy protein (PCD-1) and its homologues are GEFs for CDC42 and contain both FYVE and PH domains that may bind 3-phosphoinositides (25). Activated Rac and CDC42 may affect the cytoskeleton through the effector kinase PAK-1, which phosphorylates and activates LIM kinase (26). LIM kinase in turn phosphorylates and inhibits the actin severing protein cofilin (27), leading to a decrease in the turnover of actin filaments. Cofilin may also contribute to generation of new barbed ends through severing actin filaments (28). Alter-
natively, activated CDC42 has been shown to form a complex with N-WASP and Arp2/3, which increase actin nucleating activity of Arp2/3 (22). It is not yet clear if PI 3-kinases signal with N-WASP and Arp2/3, which increase the actin nucleating nucleation.

It is interesting to compare our studies with the recently published work of Vanhaesebroeck et al. (9) who show that membrane ruffling and motility in macrophages is blocked by inhibitory antibodies to p110α, but not by inhibitory antibodies to p110β, a unique role for p110α in macrophages is not surprising because this isoform is restricted to hematopoietic cells and p110α cannot be detected in MTLn3 cells by Western blot analysis (data not shown). However, the difference in the responses of MTLn3 cells versus macrophages to inhibitory anti-p110α and anti-p110β antibodies is unexpected. The difference is not because of variability in the antibody preparations because our anti-p110α antibodies do not inhibit CSF-1-stimulated cytoskeletal rearrangements in macrophages (data not shown), and the anti-p110β antibodies produced by Vanhaesebroeck and co-workers do not block EGF-stimulated lamellipod extension in MTLn3 cells (data not shown).

The differential utilization of p110 isoforms in the two systems may reflect distinct signaling mechanisms used by the different receptors (EGF versus CSF-1). In this regard, it will be interesting to compare the coupling of EGF and CSF-1 receptors to class Ia PI 3-kinase isoforms in the same cell.

Alternatively, the differential utilization of p110 isoforms could reflect the specific cell backgrounds in the two experimental systems. The finding of a specific requirement for p110α during EGF-stimulated motile responses in breast cancer cells suggest that p110α may be an important target for the development of anti-metastatic pharmaceuticals in the treatment of breast cancer.

Acknowledgments—We thank Dr. Bart Vanhaesebroeck (Ludwig Institute for Cancer Research, London), for anti-p110α antibodies and for sharing unpublished data, and Dr. Michael Waterfield (Ludwig Institute for Cancer Research, London), for the p110α and p110β constructs. We thank Jeffrey Wykoff, Michael Cammer, and the Analytical Imaging Facility at AECOM for assistance with image acquisition.

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FIG. 4. Effect of anti-p110 antibodies on EGF-stimulated actin nucleation. Quiescent MTLn3 cells were injected with control IgG or anti-p110 antibodies. The cells were stimulated without or with EGF for 3 min and then incubated with buffer containing 0.025% saponin and 0.45 μm rhodamine-labeled actin for 1 min. The cells were then fixed and stained with FITC-anti-rabbit IgG (to identify injected cells). Injected cells are marked with white arrows. A, IgG-injected cells, no EGF; B, IgG-injected cells plus EGF; C, anti-p110α-injected cells, no EGF; D, anti-p110α-injected cells plus EGF; and E, the percentage of injected cells showing rhodamine-actin incorporation at the leading edge were counted. The data represent the mean ± S.E. of percentages from four experiments.