Phosphatidylinositol 3-Kinase and NF-κB Regulate Motility of Invasive MDA-MB-231 Human Breast Cancer Cells by the Secretion of Urokinase-type Plasminogen Activator*

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Cell migration is a fundamental aspect of the neoplastic cell metastasis. Here, we show that phosphatidylinositol (PI) 3-kinase is constitutively active and controls cell motility of highly invasive breast cancer cells by the activation of transcription factor, NF-κB. The urokinase-type plasminogen activator (uPA) promoter contains an NF-κB binding site, and uPA expression in MDA-MB-231 cells is induced by the constitutively active NF-κB. Thus, motility was inhibited by overexpression of a dominant negative p85α regulatory subunit of PI 3-kinase (p85DN), as well as by pretreatment of cells with specific inhibitors of the p110 catalytic subunit of PI 3-kinase, wortmannin, and LY294002. The involvement of gene transcription in cell motility was suggested because treatment with actinomycin D and cycloheximide, which inhibit transcription and new protein synthesis, respectively, abolished endogenous migration of MDA-MB-231 cells. Although wortmannin, LY294002, or overexpression of p85DN did not significantly reduce DNA binding activity of NF-κB in nuclear extracts, wortmannin, LY294002, and the overexpression of p85DN or IκBα inhibited constitutive activation of NF-κB in a reporter gene assay. Highly invasive MDA-MB-231 cells constitutively secreted uPA in amounts significantly higher than poorly invasive MCF-7 cells. Furthermore, inhibition of NF-κB markedly attenuated endogenous migration, and inhibition of PI 3-kinase and NF-κB reduced secretion of uPA. Our data suggest a link between constitutively active PI 3-kinase, NF-κB, and secretion of uPA, which is responsible for the migration of highly invasive breast cancer cells. Thus, constitutively active PI 3-kinase controls cell motility by the regulation of expression of uPA through the activation of NF-κB.

Tumor invasion and metastases are multifaceted processes involving adhesion, proteolytic degradation of tissue barriers, and cell migration. Induction of cellular motility has been demonstrated with multiple growth factors and in some instances has been linked to the activation of phosphatidylinositol (PI) 3-kinase (1–4). PI 3-kinase is a key intermediate in many cellular responses induced by a vast array of divergent agonists, responses that result from the activation of downstream targets by proteins and lipids regulated by or generated from PI 3-kinase (5, 6). Several classes of PI 3-kinase, consisting of a catalytic subunit p110α (α, β, and δ) and regulatory subunit p85α (α, β, and p55γ) or consisting of the catalytic subunit p110γ and the regulatory subunit p101, have been described (for review see Ref. 7). Recent studies have demonstrated that the PI 3-kinase regulatory subunit p85α is critical for normal B cell development and proliferation (8, 9), whereas the catalytic subunits p110α, p110β, and p110γ are involved in chemotactic responsiveness (10–13).

A large number of stimuli can activate a family of transcription factors termed NF-κB/Rel (14, 15). These transcription factors are composed of homo/heterodimers of p50, RelA, RelB, and c-Rel (for review see Ref. 14). The activity of NF-κB is controlled by NF-κB inhibitors, IκBs, a family of proteins, which bind to NF-κB dimers, hiding their nuclear localization sequence resulting in cytoplasmic retention of NF-κB (15, 16). NF-κB also forms complexes with IκBα, which contains a nuclear export sequence, and the whole NF-κB-IκBα complex can be removed from the nucleus by exportin-mediated transport to the cytoplasm (17). Constitutive activation of NF-κB has been detected in some lymphomas, melanomas, and breast cancers (18–22). A direct link between activation of NF-κB and PI 3-kinase by the association of the tyrosine phosphorylated IκBα and the regulatory subunit of PI 3-kinase, p85α, has recently been demonstrated (23). In addition, another mechanism of NF-κB activation involving the catalytic (p110) subunit has been recently suggested (23). Interestingly, IL-1 stimulated the PI 3-kinase-dependent phosphorylation and transactivation of NF-κB without nuclear translocation of NF-κB, suggesting the alternative NF-κB activation pathway not involving IκBα (24).

Urokinase-type plasminogen activator (uPA) is a serine protease that cleaves the extracellular matrix and stimulates the conversion of plasminogen to plasmin (25). Plasmin can directly mediate invasion by degrading matrix proteins such as collagen IV, fibronectin, and laminin or indirectly by activating matrix metalloproteinases 2, 3, and 9 and uPA (26–29). Furthermore, uPA is also involved in cell adhesion and chemotaxis (25, 30, 31). It is well documented that uPA plays a crucial role in tumor metastasis, and overexpression of uPA in breast can

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1 The abbreviations used are: PI, phosphatidylinositol; NF-κB, nuclear factor κB; IκB, inhibitor κB; uPA, urokinase-type plasminogen activator; CAT, chloramphenicol acetytransferase; DMEM, Dulbecco’s modified Eagle medium; GEMSA, gel electrophoretic mobility shift assay.
cers is a strong indicator of poor prognosis (32, 33). Therefore, elucidation of signaling pathways responsible for the increased migratory potential of cancer cells will help to find new targets for the reduction of uPA secretion.

The present study was undertaken to characterize the role of the constitutively active PI 3-kinase, NF-κB, and uPA in the motility of human breast cancer cells. We demonstrate that the highly invasive human breast cancer cell line MDA-MB-231 expresses increased levels of PI 3-kinase activity and NF-κB DNA binding activity, as compared with levels expressed by poorly invasive MCF-7 cells. The motility of MDA-MB-231 cells is inhibited by PI 3-kinase inhibitors as well as by overexpression of a dominant negative PI 3-kinase regulatory subunit, p85DN. Here we also show that treatment with wortmannin and Ly294002 and overexpression of p85DN inhibit the constitutive transactivation of NF-κB, as assessed using a reporter gene assay. The motility of MDA-MB-231 cells was also reduced by the inhibition of NF-κB, and the secretion of uPA was decreased by the inhibition of PI 3-kinase and NF-κB. Taken together, our data suggest that secretion of uPA is tightly regulated by constitutively activated PI 3-kinase and NF-κB and is responsible for increased motility of highly invasive breast cancer cells.

**Experimental Procedures**

**Cell Culture**—MDA-MB-231 and MCF-7 cells were purchased from American Type Culture Collection (Manassas, VA) and were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with penicillin (50 units/ml), streptomycin (50 units/ml), and 10% fetal bovine serum.

**Plasmid Constructs**—PI 3-kinase dominant negative Myc-tagged p85a pCMV6-Myc-p85aDN (p85DN) and pCMV6 plasmids were gifts from Drs. L. C. Cantley and B. Duckworth (Harvard Medical School, Boston, MA). Plasmid pCMV-IκBα was purchased from CLONTECH (Palo Alto, CA). The NF-κB-CAT reporter construct and the β-galactosidase expression vector pCH110 were gifts from Dr. H. Nakshatri (Indiana University School of Medicine, Indianapolis, IN) and were described previously (21).

**Cell Migration Assay**—MDA-MB-231 cells were harvested and preincubated with specific inhibitors, as indicated in the text. Chemokine s was assessed in Transwell chamber filters (8-μm pore size) in DMEM containing 10% fetal bovine serum after 3 h of incubation, as previously described (34). In some experiments, the cells were transfected with p85DN or IκBα plasmids, respectively, and harvested after 48 h. Migration assays were performed after 4 h of incubation, as described above. After fixing and staining, the number of migrating cells was determined microscopically by enumeration at 20× magnification from at least four random fields (34). The data points represent the averages ± S.D. of four individual filters within one representative experiment repeated at least twice.

**DNA Transfection**—MDA-MB-231 (1 × 10^6 cells/100-mm dish) were split the day prior to transfection in DMEM containing 10% fetal calf serum. Transient transfections were performed with the Effectene reagent (Qiagen, Valencia, CA) according to the manufacturer’s instructions with various plasmid combinations as indicated (transfection efficiency was usually around 70–80%, as assessed by the standard procedure with β-galactosidase staining).

**PI 3-kinase Assay**—MDA-MB-231 or MCF-7 cells (5 × 10^6) were washed three times with ice-cold phosphate-buffered saline containing 5 mM NaF and 1 mM Na3VO4. The cells were resuspended in lysis buffer (10 mM Tris-HCl, pH 8.0, 157 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml aprotinin) and kept on ice for 15 min. The cell lysates were centrifuged for 10 min at 14,000 rpm to remove detergent-insoluble material. The protein concentration was determined by using the Bio-Rad protein assay kit. In some experiments, MDA-MB-231 cells were pretreated with PI 3-kinase inhibitors, wortmannin and LY294002, as indicated in the text. PI 3-kinase assays were performed as described with slight modifications (35). Briefly, the cell lysates (1 mg/ml) were immunoprecipitated with polyclonal p85a antibody (2 μg) followed by overnight incubation with protein G- plus/protein A-agarose. Immunocomplexes were recovered by centrifugation and washed twice with lysis buffer, twice with 10 mM Tris-HCl pH 7.4, and twice with 10 mM Hepes, pH 7.4. PI 3-kinase activity was assayed in a total volume of 0.05 ml of assay buffer (25 mM Hepes, 10 mM MgCl2, 1 mM EDTA containing 0.25 mg/ml of phosphatidylinositol, 100 mM ATP, and 15 μCi of [γ-32P]ATP). The reactions were carried out for 10 min at 30 °C and terminated by the addition of acidified chloroform-methanol (2:1). The lipids were extracted as previously described (36) and separated on a thin-layer plate consisting of a solvent system containing chloroform, methanol, 20% aqueous sodium phosphate, visualized by autoradiography, excised, and quantified by scintillation counting.

**Immunoprecipitation and Western Blot Analysis**—MDA-MB-231 cells were transfected with control plasmid pCMV6 or dominant negative Myc-tagged p85a (pCMV6-Myc-p85aDN), as described above. After 48 h, the cell lysates were prepared and immunoprecipitated, and Western blot analysis with anti-IκBα antibody (Upstate Biotechnology, Lake Placid, NY) was conducted as described (37). The expression of IκBα in MDA-MB-231 was determined in the whole cell extracts (25 μg) subjected to SDS-PAGE and Western blot analysis with anti-IκBα antibody (Upstate Biotechnology). The homogeneity of expression was confirmed by reprobing blots with anti-actin antibody (Oncogene Research Products, Cambridge, MA).

**Gel Electrophoretic Mobility Shift Assay (GEMSA)**—Nuclear extracts were prepared as previously described (38). GEMSA was performed with [32P]labeled NF-κB according to the manufacturer’s instruction (Promega, Madison, WI). Oligonucleotide probes containing consensus sequences for NF-κB and AP-1 binding sites, and recombinant human NF-κB protein (p50) were purchased from Promega.

**Chloramphenicol Acetyltransferase Assay**—MDA-MB-231 cells were transfected with 1 μg of NF-κB-CAT reporter construct, 3 μg of β-galactosidase expression vector pCH110 (for the normalization of transfection efficiency), and different amounts of p85DN or IκBα plasmids, as indicated in the text. In some experiments, cells transfected with the NF-κB-CAT reporter construct and the β-galactosidase expression vector pCH110 were treated with specific inhibitors as described below. The cells were harvested 48 h after transfection, the cell extracts were prepared, and β-galactosidase activity was measured as described elsewhere (39). Normalized amounts (equal numbers of β-galactosidase units) of cell extracts were used in liquid CAT assay with [14C]chloramphenicol as described (39). The data points represent the averages ± S.D. of three to six independent transfection experiments.

**uPA Secretion**—DMEM from MCF-7 or MDA-MB-231 cells untreated or treated with specific inhibitors were collected after 48 h. The medium was concentrated 10-fold by using a Microcon YM-10 filter (Amicon, Cambridge, MA). Secretion of uPA was detected by Western blot analysis of conditioned medium with anti-uPA antibody (Oncogene Research Products, Cambridge, MA).

**Results**

**PI 3-Kinase Is Constitutively Active in MDA-MB-231 Cells**—We have previously shown that, in addition to agonist-dependent receptor-mediated migration, constitutive activation of specific signaling pathways is responsible for the enhanced migration of invasive MDA-MB-231 cells, pathways that are not active in poorly invasive MCF-7 cells (34). To assess the involvement of PI 3-kinase in the metastatic motility of breast cancer cells, we compared basal PI 3-kinase activity in nonmetastatic MCF-7 cells with that of highly invasive MDA-MB-231 cells. Cell extracts were prepared, and p85a immunoprecipitates were assayed for PI 3-kinase activity. As seen in Fig. 1A, MDA-MB-231 cells displayed a 3.6-fold increase in the level of endogenous PI 3-kinase activity over that observed in MCF-7 (34). We next carried out experiments using effect of PI 3-kinase inhibitors wortmannin and LY294002 on the endogenous activation of PI 3-kinase in MDA-MB-231 cells. The cells were pretreated for 1 h with wortmannin (100 nm) or LY294002 (10 μM), the cell extracts were prepared, and p85a immunoprecipitates were assayed for PI 3-kinase activity as described above. As seen in Fig. 1C, both wortmannin and LY294002 significantly inhibited constitutive activation of PI 3-kinase in MDA-MB-231 cells. Thus, PI 3-kinase is constitutively active in highly invasive breast cancer cell line MDA-MB-231, consistent with the hypothesis that PI 3-kinase is involved in increased metastatic potential of these cells.
Both the Catalytic (p110) Subunit and Regulatory (p85α) Subunits of PI 3-Kinase Are Responsible for the Enhanced Motility of MDA-MB-231 Cells—To determine which of the PI 3-kinase subunits was responsible for enhanced cell motility, MDA-MB-231 cells were treated with specific inhibitors of PI 3-kinase, wortmannin and LY294002. Wortmannin has previously been shown to form a complex with the p110 subunit and therefore inhibits the catalytic activity of p110 (40). Another PI 3-kinase inhibitor, LY294002, does not covalently react with p110 but instead targets the ATP-binding site of p110, resulting in catalytic inactivation (41). Pretreatment of cells with wortmannin (1 h, 10–100 nm) and LY294002 (1 h, 1–10 μM) significantly reduced constitutive migration of MDA-MB-231 cells (Fig. 2A). Thus, the catalytic subunit (p110) of PI 3-kinase is necessary for enhanced endogenous cell motility.

Next, we investigated the role of the regulatory subunit (p85α) of PI 3-kinase in enhanced constitutive migration. MDA-MB-231 cells were transfected with a dominant negative construct of PI 3-kinase, Myc-p85DN, and migration was measured 48 h after transfection. The expression of Myc-tagged p85DN in MDA-MB-231 cells was verified by immunoprecipitation and Western blot analysis with Myc antibody (Fig. 2B). As seen in Fig. 2C, increased concentrations of transfected p85DN (0.5–5.0 μg DNA) significantly inhibited constitutive migration of MDA-MB-231 cells. These results suggest that in addition to the catalytic p110 subunit, regulatory p85α subunits also play an important role in constitutive migration of MDA-MB-231 cells. Thus, enhanced migration of these cancer cells can be abolished either by the inhibition of the catalytic or regulatory subunits of PI 3-kinase.

Constitutively Active PI 3-Kinase Controls Transactivation of NF-κB—As demonstrated above, constitutively active PI 3-kinase is responsible for the enhanced motility of MDA-MB-231 cells. Recently, it has been shown that both regulatory (p85α) and catalytic (p110) subunits of PI 3-kinase are involved in the activation of NF-κB by a tyrosine phosphorylation-dependent pathway (23). In addition, constitutive DNA binding activity and transactivation of NF-κB has been reported in MDA-MB-231 cells (21). To compare the DNA binding activity of NF-κB with the migratory potential of breast cancer cells, we prepared nuclear extracts from highly and poorly invasive MDA-MB-231 and MCF-7 cells, respectively. Gel shift analysis confirmed constitutive NF-κB DNA binding activity in nuclear extracts from MDA-MB-231 cells, the specificity of which was determined by competitive and supershift assays with recombinant human p50 subunit of NF-κB (Fig. 3A, lanes 1–4). NF-κB DNA binding activity was significantly increased in MDA-MB-231 cells as compared with the activity in MCF-7 cells (Fig. 3A, lanes 1 and 5). Therefore, we postulated that inhibition of constitutively active PI 3-kinase would decrease endogenous transactivation of NF-κB and also constitutive DNA binding activity of NF-κB. MDA-MB-231 cells were transiently transfected with a reporter CAT plasmid containing multiple NF-κB binding sites (NF-κB-CAT) and increased concentrations of p85DN (0.25–5.0 μg of DNA). As seen in Fig. 3B, overexpression of p85DN repressed constitutive transactivation of NF-κB, suggesting that p85α directly controls the transactivation of NF-κB in MDA-MB-231 cells. To test whether the catalytic p110 subunit of PI 3-kinase is also involved in the constitutive transactivation of NF-κB, MDA-MB-231 cells were transiently transfected with a reporter NF-κB-CAT plasmid and treated with wortmannin or LY294002. Exposure of MDA-MB-231 cells to 1–100 nm wortmannin or 1–10 μM LY294002 for 24 h inhibited constitutive transactivation of NF-κB, as assessed by the CAT reporter gene assay (Fig. 3C). To compare constitutive transactivation of NF-κB with the NF-κB DNA binding activity, the cells were transfected with p85DN or treated with wortmannin and LY294002, and nuclear extracts were subjected to gel shift analysis. Surprisingly, neither transfection with p85DN (Fig. 3D) nor treatment of MDA-MB-231 cells with wortmannin or LY294002 affected the DNA binding activity of NF-κB (Fig. 3E). These results suggest that both regulatory (p85α) and catalytic (p110)
NF-κB Controls Migration of MDA-MB-231 Cells—To confirm the role of protein synthesis in cell migration in this system, MDA-MB-231 cells were pretreated with actinomycin D and cycloheximide for 1 h, and migration was determined as described above. As seen in Fig. 4A, both actinomycin D (1 and 10 μg/ml) and cycloheximide (1 and 10 μg/ml) significantly inhibited endogenous migration of MDA-MB-231 cells in a dose-dependent manner, suggesting that cell motility is, in fact, dependent on the transcription and de novo protein synthesis. We further investigated whether the inhibition of NF-κB suppresses migration of MDA-MB-231 cells. MDA-MB-231 cells were treated with specific NF-κB inhibitor PPM-18 (42). Cell migration assays revealed that 1 h of pretreatment with PPM-18 (0.01–1 μM) markedly suppressed cell motility in a dose-dependent manner (Fig. 4B). These results are consistent with our hypothesis that constitutive transactivation of NF-κB is responsible for the enhanced motility of invasive breast cancer cells.

Overexpression of IκBα Inhibits Cell Motility by a NF-κB-Dependent Mechanism—To identify whether NF-κB-dependent motility is regulated by the natural NF-κB inhibitor IκBα, MDA-MB-231 cells were transfected with pCMV-IκBα expression vector and control pCMV vector. After 48 h, cell migration was determined as described above. Increased concentration of overexpressed IκBα (0.25–5 μg of DNA) significantly inhibited migration of MDA-MB-231 cells (Fig. 5A). To confirm that the inhibition of migration caused by the overexpression of IκBα is directly linked to the transactivation of NF-κB, MDA-MB-231, the cells were transfected with pCMV-IκBα and pCMV expression vectors and reporter NF-κB-CAT construct. As seen in Fig. 5B, overexpression of IκBα (0.25–5 μg DNA) extensively repressed the NF-κB-CAT activity. These results show that NF-κB regulates the motility of MDA-MB-231 cells.

PI 3-Kinase Inhibition of Migration and Transactivation of NF-κB Is IκBα-independent—As demonstrated above, overexpression of IκBα abolishes cell motility and NF-κB transactivation in MDA-MB-231 cells. Therefore, we investigated whether the inhibition of PI 3-kinase would directly increase the levels of IκBα and, by sequestering NF-κB in cytoplasm, would repress cell migration. MDA-MB-231 cells were transfected with a control vector or dominant negative p85DN, and the cell lysates were subjected to SDS-PAGE and Western blot analysis with IκBα antibody. Regardless of the dramatic decrease in cell motility and constitutive NF-κB transactivation, overexpression of p85DN did not increase the levels of IκBα (Fig. 6A). Additionally, treatment of MDA-MB-231 cells with wortmannin (1, 10, and 100 nM) or LY294002 (1, 5, and 10 μM) had no significant effect on IκBα levels (Fig. 6B). These results suggest that PI 3-kinase inhibits the transactivation of NF-κB and cell motility by altering or participating in a distinct signaling pathway, which is independent of IκBα.

uPA Overexpression Is Repressed by the Inhibition of PI 3-Kinase and NF-κB—In addition to proteolytic activity of uPA necessary for cell invasion, uPA is also responsible for cell migration (30, 31). As shown above, our data demonstrate that by inhibiting constitutively active PI 3-kinase, NF-κB, and protein synthesis, we are able to inhibit cell motility of MDA-MB-231 cells. Furthermore, poorly invasive MCF-7 cells with low migratory potential have significantly decreased level of endogenous PI 3-kinase activity, and they do not contain constitutive DNA binding activity of NF-κB. Therefore, we hypothesized that uPA is overexpressed in MDA-MB-231 cells but not in MCF-7 cells and that NF-κB in the promoter region of uPA is responsible for the enhanced migration of MDA-MB-231 cells. To address this hypothesis, conditioned media from MCF-7 and MDA-MB-231 cells incubated for 48 h in the serum-

scale of PI 3-kinase control the activity of NF-κB at the transactivation level because the constitutive NF-κB DNA binding activity was not affected by the inhibition of PI 3-kinase.
free DMEM were collected, concentrated, and subjected to Western blot analysis with anti-uPA antibody. As seen in Fig. 7, MCF-7 cells do not express and secrete uPA into the medium as compared with MDA-MB-231 cells with high constitutive expression and secretion of uPA (lanes 1 and 2). Treatment with PI 3-kinase inhibitor LY294004 repressed constitutive uPA overexpression and secretion from MDA-MB-231 cells (lane 3), as well as inhibition of protein synthesis by cycloheximide (lane 4). The NF-κB inhibitor, PPM-18, suppressed the secretion of uPA only partially, suggesting that other transcription factors in the promoter region of uPA may be responsible for the constitutive expression of uPA (lane 5). Taken together, our data indicate that uPA is secreted from breast cancer cell line MDA-MB-231 and that uPA overexpression is induced by constitutive PI 3-kinase and NF-κB activity. Therefore, inhibition of PI 3-kinase and NF-κB suppresses uPA expression and secretion in breast cancer cells MDA-MB-231. Furthermore, we demonstrate that inhibition of PI 3-kinase reduced activation of NF-κB and that PI 3-kinase and NF-κB inhibition repressed cell migration. The results suggest that constitutively active PI 3-kinase, through the activation of NF-κB, induces expression and secretion of uPA, which is responsible for the augmented motility of MDA-MB-231 cells.

As previously described, activation of PI 3-kinase in the human breast cancer cell line MCF-7 by insulin-like growth factor-I resulted in cell cycle progression and tyrosine phosphorylation of IRS-1 (43, 44). Furthermore, PI 3-kinase signaling was required for the depolarization and consequent stimulation of cell motility of insulin-like growth factor-I-treated MCF-7 cells (45). In this report, we have identified significantly increased endogenous activity of PI 3-kinase in MDA-MB-231 as compared with that of PI 3-kinase activity within MCF-7 cells. The former activity was inhibited by wortmannin and LY294002, which have been shown to inhibit PI 3-kinase in multiple cell types by distinct modes of action (46). Thus, increased constitutive PI 3-kinase activation is characteristic for the highly invasive human breast cancer cell line MDA-MB-231. Furthermore, we demonstrate that inhibition of PI 3-kinase reduced activation of NF-κB and that PI 3-kinase and NF-κB inhibition repressed cell migration. The results suggest that constitutively active PI 3-kinase, through the activation of NF-κB, induces expression and secretion of uPA, which is responsible for the augmented motility of MDA-MB-231 cells.
may thereby be responsible for the increased migratory potential and consequent metastatic activity of these cells.

Using genetic and pharmacological inhibitors of PI 3-kinase, we found that both the regulatory p85 and catalytic p110 subunits of PI 3-kinase are required for the enhanced constitutive migration of MDA-MB-231 cells. The involvement of the catalytic (p110) subunit in cell motility is consistent with recently published data demonstrating the requirement of p110 in epidermal growth factor-stimulated actin nucleation during lamellipod extension in rat mammary adenocarcinoma cells (47) and p110 and p110 in macrophage colony-stimulating factor-1-activated membrane ruffling and motility of macrophages (10). Furthermore, neutrophils from mice deficient in p110 showed a reduction in movement toward a chemoattractant (11–13). Therefore, all four p110 isoforms of PI 3-kinase are involved in the cell motility, and the differential activation of specific p110 isoforms may reflect particular signaling mechanisms used by cells of different origin. Data from our laboratory recently demonstrated that PI 3-kinase-mediated calcium mobilization regulates chemotaxis in phosphatidic acid-stimulated neutrophils by a mechanism different from that in IL-8-stimulated neutrophils, where PI 3-kinase-dependent chemo-

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uPA Secretion Responsible for Breast Cancer Cell Migration

FIG. 4. Constitutively active NF-κB is responsible for the migration of MDA-MB-231 cells, and the migration is dependent on the transcription or a new protein synthesis. A, cells were pretreated for 1 h with actinomycin D (Act D) or cycloheximide (CHX, 1 and 10 μg/ml), and cell migration was determined as described for Fig. 2A. The data are the means ± S.D. of triplicate determinations. Similar results were obtained in at least two additional experiments. B, cells were pretreated for 1 h with PPM-18 (0.01, 0.05, 0.1, 0.5, and 1 μM), and cell migration was determined, as described above. The data are the means ± S.D. of triplicate determinations. Similar results were obtained in at least two additional experiments.

FIG. 5. Overexpression of IκBα inhibits migration by NF-κB dependent mechanism. A, cells were transfected with indicated amounts of control plasmid pCMV and IκBα (pCMV-IκBα). After 48 h, the cells were harvested, and migration was determined after 4 h of incubation. The data are the means ± S.D. of quadruplicate determinations. Similar results were obtained in at least two additional experiments. B, MDA-MB-231 cells were transfected with the indicated amounts of control plasmid pCMV, IκBα expression vector (pCMV-IκBα), 1 μg of NF-κB-CAT reporter construct, and 3 μg of β-galactosidase plasmid. CAT activity in an equal number of β-galactosidase units was measured 48 h after transfections. The data are the means ± S.D. from three to five independent experiments.

FIG. 6. The levels of IκBα after the overexpression of p85DN and treatment with PI 3-kinase inhibitors. A, MDA-MB-231 cells were transfected with pCMV and P85DN plasmids as described for Fig. 2A. The cell extracts were subjected to Western blot analysis with anti-IκBα antibody. The identical blot was reprobed with anti-actin antibody. These results are representative of three separate experiments. B, MDA-MB-231 cells were treated for 6 h with wortmannin (W, 1, 10, and 100 nM) or LY294002 (LY, 1, 5, and 10 μM) and analyzed as described for A. CHX, cycloheximide.
These results are representative of three separate experiments.

of uPA was detected by Western blot analysis with anti-uPA antibody. Interleukin-1-dependent degradation of IκBα was inhibited by specific PI 3-kinase inhibitors in hepatoma HepG2 cells (51). PI 3-kinase inhibitors did not show any effect on the interleukin-1-dependent degradation of IκBα, the nuclear translocation of NF-κB, and the NF-κB DNA binding (24). However, tumor necrosis factor-α-induced NF-κB activation was not affected by wortmannin or LY294002 or by the expression of dominant negative p85 PI 3-kinase (52). In addition, although pervanadate and tumor necrosis factor-α induced NF-κB activation in Jurkat T cells, only pervanadate-mediated activation of NF-κB was inhibited by wortmannin (23). We show here that overexpression of a p85DN or specific inhibitors of the p110 subunit of PI 3-kinase, wortmannin and LY294002, abrogate constitutive transactivation of NF-κB in MDA-MB-231 cells. However, the inhibition of PI 3-kinase by p85DN or wortmannin and LY294002 did not affect the constitutive DNA binding activity of NF-κB. Thus, our data suggest that although both the p85 and p110 subunits of PI 3-kinase are responsible for enhanced cell motility and constitutive transactivation of NF-κB of invasive breast cancer cells, transactivation of NF-κB is independent of NF-κB DNA binding in MDA-MB-231 cells. Inhibition of NF-κB transcription activity independent of DNA binding activity has been recently reported for the interferon-inducible factor and the glucocorticoid receptor (53, 54).

Our data demonstrate that in this system, protein synthesis is required for cell migration, because migration was inhibited by inhibitors of both transcription and translation. Furthermore, our results show that activation of NF-κB is required for enhanced migration of MDA-MB-231 cells. A specific pharmacological inhibitor of NF-κB, PPM-18, markedly decreased the motility of these cancer cells, and overexpression of IκBα, a natural NF-κB inhibitor, blocked the motility of these breast cancer cells in a manner consistent with the inhibition of NF-κB activity as assessed by the reporter gene assay. Nevertheless, overexpression of p85DN or pretreatment with wortmannin or LY2940002 did not affect the expression of IκBα, suggesting that constitutively active PI 3-kinase-dependent transactivation of NF-κB and cell migration is regulated by the alternative pathways not involving IκBα, as has been demonstrated for the activation of NF-κB by interleukin-1 (24).

Constitutive activation of NF-κB has been shown to be involved in the progression of breast cancer cells to a highly invasive phenotype (21). Proteolytic enzymes such as matrix metalloproteinases and uPA are involved in the ability of epithelial cells to migrate and invade through the subendothelial matrix, and NF-κB has been identified as one of the transcription factors responsible for the induction of matrix metalloproteinases 1, 3, and 9 and uPA (55–57). Because uPA is also responsible for the cell migration and is constitutively expressed in MDA-MB-231 cells (30, 31, 58), the mechanism of uPA secretion from MDA-MB-231 cells is of particular interest. In this study, we have identified constitutive secretion of uPA from MDA-MB-231 as compared within MCF-7 cells, which do not secrete uPA. This observation is consistent with a previous report in which the authors demonstrated significantly increased RNA level of uPA in MDA-MB-231 but not in MCF-7 cells, respectively (59). The constitutive uPA secretion from MDA-MB-231 cells was repressed by the inhibition of PI 3-kinase, protein synthesis de novo, and NF-κB inhibition. Therefore, it is possible that constitutive synthesis and secretion of uPA as a result of constitutive activity of PI 3-kinase and constitutive transactivation of NF-κB in highly invasive breast cancer cells leads to the increased cellular motility and thus the increased metastatic potential of these cells. In summary, our data suggest that the endogenous cell motility of MDA-MB-231 cells is regulated by the constitutively active PI 3-kinase via transactivation of NF-κB and induction of expression and secretion of uPA. This knowledge may be useful in the design of new therapeutic interventions aimed at the disruption of PI 3-kinase and NF-κB signaling pathways resulting in the reduction of uPA secretion and consequent inhibition of the metastatic spread of breast cancer.

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[Diagram of uPA secretion Responsible for Breast Cancer Cell Migration]
