Osteopontin Stimulates Cell Motility and Nuclear Factor κB-mediated Secretion of Urokinase Type Plasminogen Activator through Phosphatidylinositol 3-Kinase/Akt Signaling Pathways in Breast Cancer Cells

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We have recently reported that osteopontin (OPN) induces nuclear factor κB (NFκB)-mediated promatrix metalloproteinase-2 activation through 1xB/IKK signaling pathways and that curcumin (diferulolylmethane) down-regulates these pathways (Philip, S., and Kundu, G. C. (2003) J. Biol. Chem. 278, 14487–14497). However, the molecular mechanism by which upstream kinases regulate the OPN-induced NFκB activation and urokinase type plasminogen activator (uPA) secretion in human breast cancer cells is not well defined. Here we report that OPN induces the phosphatidylinositol 3′-kinase (PI 3-kinase) activity and phosphorylation of Akt in highly invasive MDA-MB-231 and low invasive MCF-7 cells. The OPN-induced Akt phosphorylation was inhibited when cells were transfected with a dominant negative mutant of the p85 domain of PI 3-kinase (p85), indicating that PI 3′-kinase is involved in Akt phosphorylation. OPN enhances the interaction between 1xB/ kinase (IKK) and phosphorylated Akt. OPN also induces NFκB activation through phosphorylation and degradation of IκBα by inducing the IKK activity. However, both pharmacological (wortmannin and LY294002) and genetic (Δp85) inhibitors of PI 3′-kinase inhibited OPN-induced Akt phosphorylation, IKK activity, and NFκB activation through phosphorylation and degradation of IκBα. OPN also enhances uPA secretion, cell motility, and extracellular matrix invasion. Furthermore, cells transfected with Δp85 or the super-repressor form of IκBα suppressed the OPN-induced uPA secretion and cell motility, whereas cells transfected with p110CAAX enhanced these effects. Pretreatment of cells with PI 3-kinase inhibitors or NFκB inhibitory peptide (SN-50) reduced the OPN-induced uPA secretion, cell motility, and invasion. To our knowledge, this is first report that OPN induces NFκB activation and uPA secretion by activating PI 3′-kinase/Akt/IKK-mediated signaling pathways and further demonstrates a functional molecular link between OPN-induced PI 3′-kinase-dependent Akt phosphorylation and NFκB-mediated uPA secretion, and all of these ultimately control the motility of breast cancer cells.

Cell migration and extracellular matrix invasion are two of the major steps in embryonic development (1, 2) and wound healing and cancer cell metastasis (3, 4). However, the exact molecular mechanisms that regulate these processes are not well defined. In the past several investigators have shown that urokinase type plasminogen activator (uPA)3 plays a major role in the regulation of cancer cell motility, extracellular matrix (ECM) invasion, and metastasis by degrading the ECM proteins (5–7). Current investigations have focused on the understanding of molecular mechanism(s) by which osteopontin (OPN), an ECM protein, regulates uPA secretion and controls motility in human breast cancer cells.

OPN is a non-collagenous, sialic acid-rich, and glycosylated phosphoprotein (8, 9). This protein has a functional thrombin cleavage site and is a substrate for tissue transglutaminase (9). It has an N-terminal signal sequence, a highly acidic region consisting of nine consecutive aspartic acid residues, and a GRGDS cell adhesion sequence predicted to be flanked by the β-sheet structure (10). OPN binds with several integrins and CD44 variants in an RGD sequence-dependent and -independent manner. This protein is involved in normal tissue-remodeling processes such as bone resorption, angiogenesis, wound healing, and tissue injury as well as certain diseases such as restenosis, atherosclerosis, tumorigenesis, and autoimmune diseases (11–13). OPN expression is up-regulated in several cancers and reported to be associated with tumor progression and metastasis (14–16). OPN causes cell adhesion and migration, ECM invasion, and cell proliferation by interacting with its receptor α,β3 integrin in various cell types (12). Integrins are non-covalently associated, heterodimeric, cell-surface glycoproteins with α- and β-subunits. Integrins are a superfAMILY of transmembrane glycoproteins found predominantly on the surface of leukocytes that mediate cell-cell and cell-substratum interactions. Until today, ~12 α-subunits, 8 β-subunits, and 20 αβ-heterodimers were documented in the literature (17).

Cell migration, a major step in cancer metastasis, is often associated with the activation of phosphatidylinositol (PI) 3-kinase (7, 18). PI 3′-kinase consists of a catalytic subunit p110 (α, β, and δ or p110γ) and a regulatory subunit p85 (α, β, p55y, and p101) (19). PI 3′-kinase is activated by a number of growth factors. One of the downstream target molecule of PI

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3 The abbreviations used are: uPA, urokinase type plasminogen activator; uPAR, uPA receptor; OPN, osteopontin; PI 3′-kinase, phosphatidylinositol 3′-kinase; PIP, phosphatidylinositol phosphate; IRS, IκB kinase; NFκB, nuclear factor-κB; IκBα, inhibitor of nuclear factor-κB; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; pNP, p-nitrophenyl phosphate; Luc, luciferase; ECM, extra-cellular matrix; TNF, tumor necrosis factor; PKB, protein kinase B; CMV, cytomegalovirus; ASOPN, OPN-specific phosphorothioate-linked antisense oligonucleotide.
3'-kinase is Akt. Akt is a serine threonine kinase and also known as protein kinase B (PKB) or RAC-PK (related to A and C protein kinase). Akt is a cellular homolog of v-akt oncogene (20). Akt is activated by factors that stimulate PI 3'-kinase activity in cells such as thrombin, platelet-derived growth factor, and insulin (21). The activation of Akt is also occurred by stress factor, and that is independent of the PI 3'-kinase-mediated pathway (22, 23). In PI 3'-kinase-dependent pathway, phosphatidylinositol 3,4-bisphosphate, a product of PI 3'-kinase directly binds to the pleckstrin homology domain of Akt and leads to its activation (24, 25). However, the complete activation of Akt requires its phosphorylation on serine and threonine residues (26). Akt regulates cell cycle progression, growth factor-mediated cell survival, and cell migration. It promotes cell survival by phosphorylation and inactivation of Bad and Caspase-9 (27, 28). Several metastatic tumors express higher level of Akt (29). Previous data indicated that the PI 3'-kinase/Akt pathway is critically involved in anchoragedependent growth of tumor cells, which is one of the important steps of cancer metastasis (30). The mechanism by which OPN regulates PI 3'-kinase activity and controls cell motility in human breast cancer cells is not well defined.

The NFkB family consists of several members, including p65, p50, RelB, and c-Rel molecules (31). The activity of NFkB is tightly controlled by its inhibitor, the IκB family of proteins (32). These inhibitory proteins bind to NFkB dimers, hiding their nuclear localization sequence resulting in cytoplasmic retention of NFkB (33). Upon stimulation, IκB is phosphorylated and degraded through the ubiquitination and proteasome-mediated pathway, permitting activation and nuclear import of NFkB where it binds to cognate sequence in promoter regions of multiple genes. There are other less explored pathways by which NFkB translocates into the nucleus through tyrosine phosphorylation of IκB (34). IKK (IκB kinase) is a multisubunit protein kinase, the activation of which phosphorylated IκB. Constitutive activation of NFkB has been detected in lymphomas, melanomas, and breast cancers (35-39). The signaling pathways by which OPN regulates Akt phosphorylation followed by activations of IκK and NFkB in human breast cancer cells are not clearly understood.

uPA is a member of serine protease that interacts with the uPA receptor (uPAR) and facilitates the conversion of inert plasminogen into widely acting serine protease plasmin and activation of metalloproteinases (40, 41). These proteases then degrade the surrounding matrix components (collagen, fibronectin, and laminin) and allow cancer cells to migrate to the distant sites. uPA is also involved in cell adhesion and chemotaxis (42, 43). It is well documented that uPA plays a significant role in tumor growth and metastasis (6,7). NFkB-responsive element is present in the promoter region of uPA, which plays a key role in cancer metastasis. However, the molecular mechanism by which OPN induces NFkB-mediated uPA secretion and regulates cell migration and ECM invasion in breast cancer cells is not well documented.

In this study, we demonstrate that OPN induced the PI 3'-kinase activity and phosphorylation of Akt in human breast cancer cells. OPN also enhanced nuclear translocation of p65 subunit of NFkB, NFkB-DNA binding, and NFkB transactivating through phosphorylation and degradation of IκBα by inducing the IKK activity. Moreover, OPN induced uPA secretion, ECM invasion, and cell motility in these cells. The OPN-induced NFkB transactivation, uPA secretion, and cell motility were suppressed when both these cells were transfected with super-repressor form of IκBα or Δp85 and enhanced when cells were transfected with p110CAAX suggesting that PI 3'-kinase is involved in these processes. Pretreatment of cells with PI 3'-kinase inhibitors (wortmannin and LY294002), α,β integrin antibody, or NFkB inhibitory peptide (SN-50) reduced the OPN-induced uPA secretion, cell motility, and invasion. Taken together, these data demonstrate that OPN enhances the cell motility and induces NFkB-mediated uPA secretion through the PI 3'-kinase/Akt/IKK-mediated signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Materials**—The rabbit polyclonal anti-phospho-Akt1 (Ser-473), anti-p65 subunit of NFkB (anti-p65), anti-NFkB p65 X TransCruz, anti-IκBα, anti-IκKα/β, anti-actin, mouse monoclonal anti-phosphotyrosine antibody, anti-phospho IκBα, goat polyclonal anti-Akt1/2, and IκBα mouse monoclonal protein were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-uPA antibody and normal rabbit IgG were obtained from Oncogene. Mouse monoclonal anti-human α,β integrin antibody was from Chemicon International. LY294002, SN-50, and SN-50M were obtained from Calbiochem. Wortmannin was from Sigma. LipofectAMINE Plus, GRGDSF, and GRGESP were obtained from In-vitrogen. The dual luciferase reporter assay system and NFkB consensus oligonucleotide were from Promega. Boyden-type cell migration chambers were obtained from Corning and BioCoat Matrigel™ invasion chambers were from Collaborative Biomedical. γ-32P]-ATP was purchased from Board of Radiation and Isotope Technology (Hyderabad, India). The human OPN was purified from milk as described previously (44) and used throughout these studies. All other chemicals were analytical grade.

**Cell Culture**—The MDA-MB-231 and MCF-7 cells were purchased from ATCC (Manassas, VA). Both MDA-MB-231 and MCF-7 cells were cultured in Dulbecco’s modified Eagle’s medium. The medium was supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine in a humidified atmosphere of 5% CO2 and 95% air at 37°C.

**DNA Transfection**—HA-Delta.p85 in pCMV (Δp85) and Myc-p110.CAAX in pSG5 (p110CAAX) cDNA constructs were generous gifts from Dr. Alex Toker (Tufts University, Boston, MA). Both MCF-7 and MDA-MB-231 cells were split 12 h prior to transfection in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. These cells were transiently transfected with HA-Delta.p85 (Δp85) or Myc-p110.CAAX cDNA using LipofectAMINE Plus according to the manufacturer’s instructions. Briefly, Δp85 or p110CAAX cDNA (8 μg) was mixed with Plus reagent, and then cDNA reagent Plus was incubated with LipofectAMINE. The LipofectAMINE Plus cDNA complexes were then added to the cells and incubated further at 37°C for 12 h. The control cells received LipofectAMINE Plus alone. The cell viability was detected by a trypan blue dye exclusion test. After incubation, the medium was removed, and the cells were refed with fresh medium and maintained for an additional 12 h. In another experiments, these cells were individually transfected with super-repressor form of IκBα-DNA fusion construct to a FLA transfectant in an expression vector (pCMV) (a kind gift from Dr. Dean Ballard, Vanderbilt University School of Medicine) under the same conditions as described above. These transfected cells were used for the NFkB activity by luciferase reporter gene assay, detection of uPA expression by Western blot analysis and cell migration. The Δp85-transfected cells were also used for Akt phosphorylation, IKK activity, and LebEs phosphorylation studies.

**Western Blot Analysis**—To delineate the role of OPN in regulation of Akt phosphorylation, both MCF-7 and MDA-MB-231 cells were treated with 5 μM OPN at 37°C for 0–90 min. In separate experiments, cells were individually pretreated with PI 3'-kinase inhibitor (0–100 nM wortmannin or 0–10 μM LY294002), α,β integrin antibody (0–20 μg/ml), or with RGD peptide (0–10 μM GRGDS or GRGESP) for 1 h and then treated with 5 μM OPN at 37°C for 30 min in MCF-7 cells and 15 min in MDA-MB-231 cells. Cells were lysed in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM iodoacetamide, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 2 mM EDTA) containing 25 mM NaF and 2 mM Na3VO4. The cleared lysates were collected by centrifugation at 12,000 × g for 15 min at 4°C. The protein concentration in the lysate was measured by Bio-Rad protein assay. The lysates containing equal amounts of total proteins were resolved by SDS-PAGE. The proteins were electrotransferred from gel to nitrocellulose membrane. The membrane was incubated with rabbit polyclonal anti-Akt antibody and incubated further with horseradish peroxidase-conjugated IgG. The membrane was washed and detected by the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences) according to the manufacturer’s instructions. The membrane was reprobed with goat polyclonal anti-Akt antibody to...
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ensure equal protein loading. In separate experiments, both these cells were individually transfected with a dominant negative mutant of the p85 domain of PI 3-kinase (Δp85) or the activated form of PI 3-kinase (p110CAAX) in the presence of LipofectAMINE Plus and then stimulated with OPN. Cells were lysed in lysis buffer. The lysates containing equal amounts of total proteins were resolved by SDS-PAGE and detected by Western blot analysis as described previously.

To check the effect of OPN in regulation of IkBα serine/threonine phosphorylation and degradation in breast cancer cells, both these cells were either treated with 5 μM OPN for 0–90 min or pretreated with LY294002 (10 μM) or wortmannin (100 nm) for 1 h and then treated with 5 μM OPN for 30 min in MCF-7 and 15 min in MDA-MB-231 cells at 37 °C. In these experiments, cells were transfected with pMSCV-IKKα (6) or pMSCV-IKKβ (7) and then treated with OPN as described above. Cells were lysed in lysis buffer containing 25 mM NaF and 2 mM Na3VO4. The equal amounts of total proteins in the lysates were separated by SDS-PAGE and detected by Western blot using mouse anti-phospho-IκBα antibody. The same blots were reprobed with rabbit anti-IκBα antibody. As loading controls, the expression of actin was also analyzed by reprobing the blots with anti-actin antibody and detected by using the ECL detection system.

To investigate the role of OPN on uPA secretion in both MCF-7 and MDA-MB-231 cells, both these cells were individually treated with various concentrations of OPN (0–5 μM) for 24 h. In separate experiments, cells were pretreated with anti-αv,β3 integrin antibody (0–20 μg/ml) or p85 cDNA (10–100 μg/ml) or GRGESP (GRGESP) or p85 inhibitory peptide (0–100 μg/ml) for 30 min, and then treated with 5 μM OPN for additional 24 h at 37 °C. These cells were lysed, and cell lysates containing equal amounts of total proteins were subjected to Western blot analysis using mouse monoclonal anti-uPA antibody. In separate experiments, both these cells were individually transfected with dominant negative mutant of PI 3-kinase (Δp85), the activated form of PI 3-kinase (p110CAAX), or the super-repressor form of IκBα in the presence of LipofectAMINE Plus and then treated with 5 μM OPN for 24 h. Cells were lysed in lysis buffer. The level of uPA in these lysates was detected by Western blot analysis. As loading controls, the expression of actin was also detected by reprobing the blots with rabbit anti-actin antibody.

Immunoprecipitation—To check whether OPN regulates the interaction between IKK and phosphorylated Akt, MCF-7, and MDA-MB-231 cells were treated with 5 μM OPN at 37 °C for 30 and 15 min, respectively. In another experiments, these cells were pretreated with LY294002 (10 μM) or wortmannin (100 nm) for 1 h or transfected with Δp85 cDNA and then treated with 5 μM OPN as described above. Cells were lysed in lysis buffer (20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 15% v/v glycerol, 1 mM EDTA) containing phosphatidylinositol (0.25 mg/ml), Na3VO4 and clarified by centrifugation at 12,000 × g for 1 h. The kinase reactions were stopped by addition of SDS-sample buffer. The samples were resolved by SDS-PAGE, dried, and autoradiographed. The remaining half of the immunoprecipitated samples were subjected to SDS-PAGE and analyzed by Western blot using anti-IKKα antibody. In separate experiments, both these cells were either transfected with Δp85 cDNA or pretreated with LY294002 (10 μM) or wortmannin (100 nm) then treated with OPN (5 μM). These cells were used for detection of IKK activity under the same conditions as described previously.

Nuclear and Cytoplasmic Extracts and Western Blot—Both MCF-7 and MDA-MB-231 cells were treated with 5 μM OPN for 4 h at 37 °C. The nuclear extracts were prepared as described (46). Briefly, the cells were scraped, washed with phosphate-buffered saline (pH 7.4), resuspended in hypotonic buffer (10 mM Hepes (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol), and allowed to swell on ice for 10 min. Cells were homogenized in a Dounce homogenizer. The nuclei were separated by spinning at 3,000 × g for 5 min at 4 °C. The supernatant was used as cytoplasmic extract. The nuclear pellet was extracted in nuclear extraction buffer (100 mM Hepes (pH 7.9), 0.4 M KCl, 1.5 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 25% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT) for 30 min on ice and centrifuged at 12,000 × g for 30 min. The supernatant was used as a nuclear extract. The protein concentrations in the supernatants of both nuclear and cytoplasmic extracts were measured by the Bio-Rad protein assay. The nuclear and cytoplasmic extracts (30 μg) were resolved by SDS-PAGE, and the level of p65 was detected by Western blot analysis using rabbit anti-p65 antibody.

EMSA—EMSA was performed as described previously (46). Both MCF-7 and MDA-MB-231 cells were treated with 5 μM OPN for 0–6 h. The nuclear extracts (10 μg) were incubated with 16 fmol of NFκB-p50 labeled double-stranded oligonucleotide (5’-AGT TGA GGC GAC TTT CCC AGG C-3’) in binding buffer containing 50 mM Hepes (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT, 1% Nonidet P-40, 5% glycerol, and 50 mM NaCl containing 2 μg of poly(dI-dC) DNA. The DNA-protein complex was resolved on a native polyacrylamide gel and analyzed by autoradiography. For supershift assay, the OPN-treated nuclear extracts were incubated with anti-p65 antibody for 30 min at room temperature and analyzed by EMSA.

NFκB Luciferase Reporter Gene Assay—The semiconfluent cells (MCF-7 and MDA-MB-231) grown in 24-well plates were transiently transfected with a luciferase reporter construct (pNFκB-Luc) containing five tandem repeats of the NFκB binding site (a generous gift from Dr. Rainer de Martin, University of Vienna, Vienna, Austria) using LipofectAMINE Plus reagent (Invitrogen). The transfection efficiency was normalized by cotransfecting the cells with pRl vector (Promega) containing a full-length Renilla luciferase gene under the control of a constitutive promoter. After 24 h of transfection, the cells were treated with varying concentrations of OPN (0–5 μM) for 6 h or pretreated with anti-αv,β3 integrin antibody (20 μg/ml), wortmannin (100 nm), and LY294002 (10 μM) for 1 h and then treated with 5 μM OPN for an additional 6 h at 37 °C. In other experiments, both MCF-7 and MDA-MB-231 cells were individually transfected with HA-Delta.p85, Myc-p110CAAX, super-repressor form of IκBα, and ASOPN (OPN-specific phosphorothioate-linked antisense oligonucleotide) in the presence of pNFκB-Luc and then treated with OPN (5 μM) for 6 h. Cells were harvested, and cell lysates were freshly prepared with nonionic detergent (1% Nonidet P-40). The activities were measured by luminometer (Lab Systems) using the dual assay system assay according to the manufacturer’s instructions (Promega).

Changes in luciferase activity with respect to control were calculated.

Cell Migration Assay—The migration assay was conducted using...
Transwell cell culture chamber according to the standard procedure as described previously (44, 46). Briefly, the confluent monolayer of MCF-7 or MDA-MB-231 cells were harvested with trypsin-EDETA and centrifuged at 800 × g for 10 min. The cell suspension (5 × 10^5 cells/well) was added to the upper chamber of the prehydrated polycarbonate membrane filter. The lower chamber was filled with fibroblast condition medium, which acted as a chemostatant. Purified OPN (0–5 μg) was added to the upper chamber. In another experiments, MCF-7 or MDA-MB-231 cells were individually pretreated with anti-α3β1 integrin antibody (0–20 μg/ml), GRGDSP or GRGESP (0–10 μM), PI 3'-kinase inhibitors (0–100 nM wortmannin or 0–10 μM LY294002), NFκB inhibitor peptides (0–100 μg/ml SN-50 or SN-50M), and monoclonal anti-μA/μB antibody (0–20 μg/ml) at 37 °C for 6 h. In other experiments, cells were individually transfected with super-repressor form of IκBα, p55, or p110CAAX and used for migration assay. OPN (5 μg) was used in the upper chamber. After treatment, these cells were incubated in a humidified incubator in 5% CO2 and 95% air at 37 °C for 6 h. The non-migrated cells on the upper side of the filter were scraped, and the filter was washed. The migrated cells in the reverse side of the filter were fixed with methanol and stained with Giemsa. The migrated cells on the filter were counted under an inverted microscope (Olympus). The experiments were repeated in triplicate. Preimmune IgG served as nonspecific control.

**Chemoinvasion Assay**—The chemoinvasion assay was performed using Matrigel™-coated invasion chamber as described (44, 46). Briefly, the confluent monolayers of MCF-7 or MDA-MB-231 cells were individually pretreated with anti-α3β1 integrin antibody (20 μg/ml), PI 3'-kinase inhibitors (100 nM wortmannin or 10 μM LY294002), or NFκB inhibitory peptide (100 μg/ml SN-50) at 37 °C for 6 h. In other experiments, cells were individually transfected with super-repressor form of IκBα, p55, or p110CAAX and used for migration assay. OPN (5 μg) was used in the upper chamber. After treatment, these cells were incubated in a humidified incubator in 5% CO2 and 95% air at 37 °C for 6 h. The non-migrating cells on the upper side of the filter were scraped and the filter was washed. The migrated cells in the reverse side of the filter were fixed with methanol and stained with Giemsa. The migrated cells on the filter were counted under an inverted microscope (Olympus). The experiments were repeated in triplicate. Preimmune IgG served as nonspecific control.

**RESULTS**

**OPN Induces PI 3'-Kinase Activity and Akt Phosphorylation**—Because we have reported earlier that OPN induces NFκB activity through phosphorylation and degradation of IκBα by activating IKK (46), we sought to determine whether any upstream kinases such as PI 3'-kinase/Akt play any role in OPN-induced NFκB activation by inducing IKK activity in breast cancer cells. Accordingly, both MCF-7 and MDA-MB-231 cells were treated with 5 μM OPN in basal medium for 0–60 min at 37 °C. The lysates containing an equal amount of total proteins were immunoprecipitated with anti-phosphotyrosine antibody. The immunoprecipitated samples were used for the PI 3'-kinase assay. The PIP was separated by TLC and visualized by autoradiography. The maximum PI 3'-kinase activity was found at 15–30 min in both MCF-7 and MDA-MB-231 cells (Fig. 1, A and B, lanes 1–5). Pretreatment of cells with anti-α3β1 integrin antibody suppressed the OPN-induced PI 3'-kinase activity in both these cells (panels A and B, lane 6) suggesting that OPN induces PI 3'-kinase activity through the integrin-mediated pathway. The bands were analyzed densitometrically (Kodak Digital Science), and the fold changes were calculated.

To ascertain the role of OPN on Akt phosphorylation, both these cells were treated with 5 μM OPN for 0–90 min at 37 °C, the cell lysates containing equal amounts of total proteins were resolved by SDS-PAGE, and the level of phosphorylated Akt was detected by Western blot analysis using anti-phospho-Akt antibody (Ser-473). The data revealed that the maximum level of OPN-induced Akt phosphorylation occurred at 30 min in MCF-7 cells and at 15 min in MDA-MB-231 cells (Fig. 1, C and D, upper panels, lanes 1–6) and also suggested that Ser-473 residue is crucial for phosphorylation of Akt. The same blots were reprobed with anti-Akt antibody and the data showed that there was no change in expression of non-phospho Akt in both these cells upon treatment with OPN confirming the equal loading of the samples (lower panels, lanes 1–6). To check whether OPN-induced Akt phosphorylation occurred through the PI 3'-kinase-mediated pathway, both these cells were either transfected with Δp85 or CAAxP110 of PI 3'-kinase in the presence of LipofectAMINE Plus or pretreated with PI 3'-kinase inhibitor (wortmannin or LY294002) and then treated with OPN. To further delineate whether α3β1 integrin or the RGD/RGE peptide is involved in OPN-induced Akt phosphorylation; cells were pretreated with anti-α3β1 integrin antibody or with RGD/RGE peptide (GRGDSP or GRGESP) and then treated with OPN. The level of phosphorylated Akt was detected by Western blot analysis. The data demonstrated that PI 3'-kinase inhibitor (wortmannin or LY294002), integrin antibody, and RGD (GRGDSP) but not RGE (GRGESP) peptide suppressed the OPN-induced Akt phosphorylation in these cells (Fig. 1, E and F, upper panels, lanes 1–12). Similarly, Δp85 inhibited but CAAxP110 enhanced the OPN-induced Akt phosphorylation suggesting that PI 3'-kinase is involved in Akt phosphorylation (Fig. 1, G and H, upper panels, lanes 1–4), and it occurred through the integrin-mediated pathway. The level of non-phospho Akt was unchanged in transfected or treated cells (Fig. 1, E–H, lower panels). Western blot data were quantified by densitometric analysis, and the fold changes were calculated. These results further suggested that OPN binds with α3β1 integrin receptor and regulates Akt phosphorylation through the PI 3'-kinase-dependent pathway.

**OPN Stimulates the Interaction between Phosphorylated Akt and IKK**—To determine if OPN has any role in regulating the interaction between phosphorylated Akt and IKK, both these cells were treated with 5 μM OPN. The cells were lysed and immunoprecipitated with goat polyclonal anti-Akt antibody. Half of the immunoprecipitated samples were analyzed by Western blot analysis using anti-IKKα/β antibody, and the remaining half of the samples were immunoblotted by anti-phospho-Akt antibody. The same blots were reprobed with anti-Akt antibody to ensure that the basic level of Akt was the same. The results indicated that OPN induces the interaction between IKKα/β (Fig. 2, A and B, upper panel, lane 2) and phosphorylated Akt (middle panel, lane 2) compared with control (lane 1). LY294002 and wortmannin suppressed the OPN-induced interaction between IKKα/β and phosphorylated Akt in both MCF-7 and MDA-MB-231 cells (upper and middle panels, lanes 3 and 4). The cells transfected with Δp85 followed by treatment with OPN showed significant inhibition of this interaction (lane 5). As expected, the level of non-phospho Akt remained unchanged in both these cells (lower panel).

To further confirm this interaction, cells were treated with OPN; the lysates were immunoprecipitated with anti-IKKα/β antibody, and the levels of Akt, phospho-Akt, or IKK were detected by Western blot analysis using their specific antibodies. The results showed that OPN induces the interaction between IKK and Akt (Fig. 2, C and D, upper and middle panels, lane 2) compared with control (lane 1) in these cells. LY294002, wortmannin, or Δp85 inhibited this interaction (upper and middle panels, lanes 3–5) in these cells. As expected, the level of IKKα/β remained identical (lower panel). As control, both IKK- and Akt-specific bands were detected when cell lysates were used for direct Western blot analysis using their specific antibodies (Fig. 2, A–D, lane 7). As expected, no IKKα/β- or Akt-specific band was found when cell lysates were immunoprecipitated with normal goat/rabbit IgG (lane 6). All these bands were quantified densitometrically, and the fold changes were calculated.
OPN Enhances the IκBα Phosphorylation by Inducing IKK Activity—To delineate whether OPN has any effect on phosphorylation of IκBα through modulating the activation of IKK in breast cancer cells, both these cells were treated with 5 μM OPN. The cells were lysed and immunoprecipitated with anti-IKKα/β antibody. Half of the immunoprecipitated samples were used for kinase assay using recombinant IκBα as substrate. The radiolabeled, phosphorylated IκBα-specific band was detected in OPN-treated cells, demonstrating that OPN induces the IKK activity (Fig. 3, A and B, upper panels, lane 2). The IKK activity was dramatically reduced when cells were either transfected with Δp85 (lane 3) or pretreated with LY294002 (lane 4) or wortmannin (lane 5), suggesting that OPN induces IKK activity through the PI 3-kinase-dependent pathway in breast cancer cells. A low level of IKK activity was detected in untreated cells (lane 1). The protein bands were quantified by densitometric analysis, and the -fold changes are calculated. The results shown here represent three experiments exhibiting similar effects. [Fig. 1. OPN induces PI 3-kinase activity (A and B) and Akt phosphorylation (C-H) in MCF-7 and MDA-MB-231 cells. A and B, PI 3-kinase activity. Both MCF-7 (A) and MDA-MB-231 (B) cells were treated with 5 μM OPN for 0–90 min. Equal amounts of total proteins from the untreated or treated lysates of MCF-7 or MDA-MB-231 cells were immunoprecipitated with mouse monoclonal anti-phosphotyrosine antibody, and the immunocomplexes were assayed for their ability to phosphorylate PI to PIP using [γ-32P]ATP at 30 °C for 10 min. The PIP was resolved by TLC and autoradiographed (lanes 1–5). In separate experiments, cells were pretreated with anti-α,β3 integrin antibody (20 μg/ml) and treated with OPN, and cell lysates were used for PI 3-kinase assay. Note that maximum OPN-induced PI 3-kinase activity was observed at 30 min in MCF-7 and at 15 min in MDA-MB-231 cells. α,β3 integrin antibody suppressed the OPN-induced PI 3-kinase activity in both these cells. C and D, serum-starved cells were treated with 5 μM OPN for 0–90 min; lysates containing equal amounts of total proteins were resolved by SDS-PAGE and subjected to Western blot analysis using anti-phospho Akt antibody (lanes 1–6, upper panels). The same blots were reprobed with anti-Akt antibody (lanes 1–6, lower panels). Arrows indicate the phospho- and non-phospho-specific Akt bands. E and F, both these cells were individually pretreated with two different concentrations of α,β3 integrin antibody, GRGDSP, GRGESP, LY294002, and wortmannin and then treated with 5 μM OPN. The cells were lysed, and lysates containing equal amounts of total proteins were analyzed by Western blot using anti-phospho or anti-non-phospho Akt antibodies. Lane 1, control; lane 2, 5 μM OPN; lane 3, 10 μg/ml α,β3 integrin antibody and 5 μM OPN; lane 4, 20 μg/ml α,β3 integrin antibody and 5 μM OPN; lane 5, 5 μM GRGDSP and 5 μM OPN; lane 6, 10 μM GRGDSP and 5 μM OPN; lane 7, 5 μM GRGESP and 5 μM OPN; lane 8, 10 μM GRGESP and 5 μM OPN; lane 9, 5 μM LY294002 and 5 μM OPN; lane 10, 10 μM LY294002 and 5 μM OPN; lane 11, 10 μM wortmannin and 5 μM OPN, and lane 12, 100 nM wortmannin and 5 μM OPN. G and H, cells were transiently transfected with a dominant negative p85 domain of PI 3-kinase (Δp85) or activated form of p110 domain of PI 3-kinase (p110CAAX) in the presence of LipofectAMINE Plus and then treated with 5 μM OPN. The levels of phospho Akt and Akt in the cell lysates were detected by Western blot analysis. Lane 1, control; lane 2, 5 μM OPN; lane 3, transfected with Δp85 and treated with 5 μM OPN; and lane 4, transfected with p110CAAX and treated with 5 μM OPN. Upper panel shows the phospho-specific Akt, and the lower panel indicates non-phospho-specific Akt bands. All of these bands in A–H were quantified by densitometric analysis, and the -fold changes are calculated. The results shown here represent three experiments exhibiting similar effects.]

**Fig. 1.** OPN induces PI 3-kinase activity (A and B) and Akt phosphorylation (C-H) in MCF-7 and MDA-MB-231 cells. A and B, PI 3-kinase activity. Both MCF-7 (A) and MDA-MB-231 (B) cells were treated with 5 μM OPN for 0–90 min. Equal amounts of total proteins from the untreated or treated lysates of MCF-7 or MDA-MB-231 cells were immunoprecipitated with mouse monoclonal anti-phosphotyrosine antibody, and the immunocomplexes were assayed for their ability to phosphorylate PI to PIP using [γ-32P]ATP at 30 °C for 10 min. The PIP was resolved by TLC and autoradiographed (lanes 1–5). In separate experiments, cells were pretreated with anti-α,β3 integrin antibody (20 μg/ml) and treated with OPN, and cell lysates were used for PI 3-kinase assay. Note that maximum OPN-induced PI 3-kinase activity was observed at 30 min in MCF-7 and at 15 min in MDA-MB-231 cells. α,β3 integrin antibody suppressed the OPN-induced PI 3-kinase activity in both these cells. C and D, serum-starved cells were treated with 5 μM OPN for 0–90 min; lysates containing equal amounts of total proteins were resolved by SDS-PAGE and subjected to Western blot analysis using anti-phospho Akt antibody (lanes 1–6, upper panels). The same blots were reprobed with anti-Akt antibody (lanes 1–6, lower panels). Arrows indicate the phospho- and non-phospho-specific Akt bands. E and F, both these cells were individually pretreated with two different concentrations of α,β3 integrin antibody, GRGDSP, GRGESP, LY294002, and wortmannin and then treated with 5 μM OPN. The cells were lysed, and lysates containing equal amounts of total proteins were analyzed by Western blot using anti-phospho or anti-non-phospho Akt antibodies. Lane 1, control; lane 2, 5 μM OPN; lane 3, 10 μg/ml α,β3 integrin antibody and 5 μM OPN; lane 4, 20 μg/ml α,β3 integrin antibody and 5 μM OPN; lane 5, 5 μM GRGDSP and 5 μM OPN; lane 6, 10 μM GRGDSP and 5 μM OPN; lane 7, 5 μM GRGESP and 5 μM OPN; lane 8, 10 μM GRGESP and 5 μM OPN; lane 9, 5 μM LY294002 and 5 μM OPN; lane 10, 10 μM LY294002 and 5 μM OPN; lane 11, 10 μM wortmannin and 5 μM OPN, and lane 12, 100 nM wortmannin and 5 μM OPN. G and H, cells were transiently transfected with a dominant negative p85 domain of PI 3-kinase (Δp85) or activated form of p110 domain of PI 3-kinase (p110CAAX) in the presence of LipofectAMINE Plus and then treated with 5 μM OPN. The levels of phospho Akt and Akt in the cell lysates were detected by Western blot analysis. Lane 1, control; lane 2, 5 μM OPN; lane 3, transfected with Δp85 and treated with 5 μM OPN; and lane 4, transfected with p110CAAX and treated with 5 μM OPN. Upper panel shows the phospho-specific Akt, and the lower panel indicates non-phospho-specific Akt bands. All of these bands in A–H were quantified by densitometric analysis, and the -fold changes are calculated. The results shown here represent three experiments exhibiting similar effects.
To check the effect of OPN on IκBα phosphorylation and degradation, both MCF-7 and MDA-MB-231 cells were treated with 5 μM OPN for 0–90 min and lysed. The lysates containing equal amounts of total proteins were resolved by SDS-PAGE, and phosphorylated IκBα was detected by Western blot analysis using anti-phospho-IκBα antibody. The maximum level of OPN-induced IκBα phosphorylation was detected in 30 min in MCF-7 cells (Fig. 4A, upper panel) and in 15 min in MDA-MB-231 cells (Fig. 4B, upper panel). The level of phospho-IκBα was reappeared in 90 min in both MCF-7 and MDA-MB-231 cells (upper panels of A and B). The blots were reprobed with anti-IκBα antibody, and the data indicated that the maximum OPN-induced degradation was observed in 30 min in both MCF-7 and MDA-MB-231 cells (middle panels of A and B). After that,
OPN Modulates PI 3'-Kinase and NFκB-mediated uPA Secretion

Fig. 4. OPN induces IκBα phosphorylation and degradation. A–D, both MCF-7 (A and C) and MDA-MB-231 (B and D) cells were either treated with 5 μM OPN for 0–90 min (A and B) or pretreated with PI 3'-kinase inhibitor (10 μM LY294002 or 100 nM wortmannin) for 1 h and then treated with 5 μM OPN (C and D) or transfected with Δp85 and then treated with 5 μM OPN (C and D) as described under “Experimental Procedures.” Cell lysates containing equal amounts of total proteins were analyzed by Western blot using anti-phospho IκBα antibody. The same blots were reprobed with anti-IκBα or anti-actin antibody. Note that maximum IκBα phosphorylation occurred at 30 min in MCF-7 and at 15 min in MDA-MB-231 cells, whereas maximum degradation was observed at 30 min in both these cells. PI 3'-kinase inhibitor blocks the OPN-induced IκBα phosphorylation and degradation in MCF-7 and MDA-MB-231 cells. The blots were analyzed densitometrically, and the values were normalized to actin. The relative values of phospho-IκBα or IκBα in terms of -fold changes are indicated. The data shown represent three experiments exhibiting similar effects.

IκBα synthesis was reactivated possibly by NFκB in 60 min (middle panels). The reduced level of phosphorylated IκBα at 60 min in MCF-7 and MDA-MB-231 cells indicates that the rate of degradation exceeded the rate of IκBα phosphorylation at this time point (upper panels of A and B).

In separate experiments, cells were pretreated with LY294002 (10 μM) or wortmannin (100 nM) for 1 h and then treated with OPN (5 μM) for 30 min in MCF-7 and for 15 min in MDA-MB-231 cells. In other experiments, both these cells were transfected with Δp85 in the presence of LipofectAMINE Plus and then treated with OPN (5 μM). The cells were lysed, and equal amounts of total proteins from the lysates were resolved by SDS-PAGE and analyzed by Western blot using anti-phospho-specific IκBα or anti-IκBα antibody as described above. The results indicated that both PI 3'-kinase inhibitor (LY294002 or wortmannin) and Δp85 inhibited OPN-induced IκBα phosphorylation and degradation in these cells (upper and middle panels of C and D) suggesting that OPN induces IκBα phosphorylation and degradation through the PI 3'-kinase-dependent pathways. As loading controls, all these blots were reprobed with anti-actin antibody (lower panels of A–D). The bands were quantified by densitometry, and the values were normalized with respect to actin expression. The -fold changes, as compared with control, were calculated.

OPN Induces Translocation of the p65 Subunit of NFκB into the Nucleus.—To check whether OPN induces the NFκB translocation in low invasive (MCF-7) and highly invasive (MDA-MB-231) breast cancer cells, both these cells were treated with 5 μM OPN for 4 h at 37°C. The nuclear and cytoplasmic fractions were prepared from the untreated and treated cells. The levels of p65 in these fractions were analyzed by Western blot using rabbit anti-p65 antibody (Fig. 5, A and B). In the OPN-untreated cells, the p65 was localized mostly in the cytoplasm (lane 1) instead of the nucleus (lane 2), whereas in the OPN-treated cells, it was translocated from the cytoplasm (lane 3) to the nucleus (lane 4). The Western blot data were quantified densitometrically, and the -fold changes as compared with control were calculated.

OPN Stimulates NFκB-DNA Binding and NFκB Transactivation.—Cells were treated with 5 μM OPN for 0–6 h, and nuclear extracts were prepared and used for EMSA using 32P-labeled NFκB oligonucleotides. Fig. 5 (C and E) showed the maximum NFκB-DNA binding in 4 h in both MCF-7 (panel C, lanes 1–4) and MDA-MB-231 (panel E, lanes 1–4) cells. Whether the band (panels C and E) obtained by EMSA in OPN-treated cells is indeed NFκB, the nuclear extracts were incubated with anti-p65 antibody and then analyzed by EMSA. Fig. 5 (D and F) showed the shift of the NFκB-specific band to a higher molecular weight when the nuclear extracts were treated with anti-p65 antibody, suggesting that the OPN-activated complex consists of the p65 subunit in MCF-7 (panel D, lanes 1 and 2) and MDA-MB-231 (panel F, lanes 1 and 2) cells.

To detect whether OPN stimulates NFκB transcriptional activity and whether αvβ3 integrin and PI 3'-kinase are in-
Fig. 5. OPN enhances nuclear accumulation of p65 subunit of NFκB, NFκB-DNA binding, and NFκB activation. A and B, nuclear translocation of p65 into nucleus by Western blot analysis. Both MCF-7 (A) and MDA-MB-231 (B) cells were treated with or without OPN (5 μM) for 4 h. The nuclear and cytoplasmic extracts from untreated and treated cells were immunoblotted with rabbit polyclonal anti-p65 antibody. In the untreated cells, p65 was detected in the cytoplasm (lane 1) but not in the nucleus (lane 2). In the OPN-treated cells, p65 translocated from cytoplasm (lane 3) into the nucleus (lane 4). The levels of p65 were quantified by densitometric analysis, and the -fold changes are calculated. C and E, electrophoretic mobility shift assay. MCF-7 (C) and MDA-MB-231 (E) cells were treated with 5 μM OPN for 0–6 h. The nuclear extracts were prepared and analyzed by EMSA as described under “Experimental Procedures.” The arrow indicates the NFκB-specific bands. Similarly, lane 2, control; lane 3, with 5 μM OPN and anti-p65 antibody. The cell lysates were used to measure the luciferase activity. The values were normalized to Renilla luciferase activity. The -fold changes were calculated, and mean ± S.E. of triplicate determinations are plotted.

volved in this process, a luciferase reporter gene assay was performed. MCF-7 and MDA-MB-231 cells were transected with pNFκB luciferase reporter construct (pNFκB-Luc) in the presence of LipofectAMINE Plus. Transfected cells were either treated with OPN (0–5 μM) or pretreated with anti-αvβ3 integrin antibody (20 μg/ml) or IL-8 (200 nM) or wortmannin (100 nM) and then treated with OPN (5 μM). The transfection efficiency was normalized by cotransferring the cells with pRL vector. Changes in luciferase activity with respect to control were calculated. The -fold changes were calculated, and the means of triplicate determinations were plotted. The data demonstrated that OPN stimulated the NFκB transcriptional activity in a dose-dependent manner and that αvβ3 integrin antibody and PI 3′-kinase inhibitor (LY294002 and wortmannin) suppressed the OPN-induced NFκB activity (Fig. 5, G and H). Similarly, OPN, a super-repressor form of IL-8, and ASOPN inhibited and CAAxP110 enhanced the OPN-induced NFκB activity in these cells (panels G and H). These data further suggested that both catalytic (p110) and regulatory (p85α) subunits of PI 3′-kinase are involved in OPN-induced NFκB transactivation through the αvβ3 integrin-mediated pathway.

**OPN Induces PI 3′-Kinase and NFκB-dependent uPA Secretion**—To delineate whether OPN plays any role in uPA secretion, both MCF-7 and MDA-MB-231 cells were treated individually with varying concentrations of OPN (0–5 μM). The cells were lysed, and the lysates containing equal amounts of total
proteins were resolved by SDS-PAGE and analyzed by Western blot using mouse monoclonal anti-uPA antibody. The data indicated that OPN enhanced the uPA secretion in a dose-dependent manner in both MCF-7 and MDA-MB-231 cells (Fig. 6, A and B, lanes 1–5).

To examine whether OPN-induced uPA secretion occurred through αvβ3 integrin/PI 3'-kinase/NFκB-mediated pathways, both these cells were individually treated with anti-αvβ3 integrin antibody, RGD peptide (GRGDSP or GRGESP), PI 3'-kinase inhibitors (LY294002 or wortmannin), and NFκB inhibitory peptide (SN-50 or SN-50M (0–100 μM)) for 24 h (C and D). The level of uPA in the lysates was detected by Western blot analysis using anti-uPA antibody. The arrow indicates the uPA-specific band. The same blots were reprobed with anti-actin antibody. The constitutive expression of uPA was significantly higher in MDA-MB-231 cells compared with MCF-7 cells. Note that OPN induced uPA secretion (A and B, lanes 1–5). αvβ3 integrin antibody, GRGESP peptide, PI 3'-kinase inhibitors, and NFκB inhibitory peptide suppressed OPN-induced uPA secretion (C and D, lanes 1–16). GRGESP and SN-50M had no effect on OPN-induced uPA secretion (C and D, lanes 1–16). The bands were quantified by densitometric analysis, and fold changes were calculated. The results shown represent three experiments exhibiting similar effects.

In separate experiments, cells were also individually transfected with Δp85, CAAXp110, or a super-repressor form of IκBα in the presence of LipofectAMINE Plus and then treated with OPN as described above. The results indicated that both Δp85 (Fig. 7, A and B, lane 3) and that super-repressor form of IκBα (C and D, lanes 3) suppressed and CAAXp110 (A and B, lane 4) enhanced OPN-induced uPA secretion compared with OPN-treated (A–D, lane 2) cells. The low level of the uPA-specific band was detected in untreated cells (A–D, lane 1). As loading controls, all these blots were reprobed with anti-actin antibody (Fig. 7, A–D, lower panels).

In all of these experiments, the uPA-specific bands were quantified by densitometric analysis, and the values of fold changes were determined (Figs. 6 and 7). These results suggested that OPN induces uPA secretion via the αvβ3 integrin-mediated pathway and further demonstrated that PI 3'-kinase and NFκB are involved in this process.

PI 3'-Kinase, NFκB, and uPA Play Crucial Roles in OPN-induced αvβ3 Integrin-mediated Cell Migration and Chemoinvasion.
vasion—Because OPN regulates the PI 3'-kinase-dependent NFκB activation and uPA secretion in MCF-7 and MDA-MB-231 cells, we sought to determine whether OPN-regulated PI 3'-kinase/NFκB-dependent uPA expression has any role in breast cancer cell migration and invasion. Accordingly, both these cell lines were individually transfected with anti-α,β3 integrin antibody, GRGDSP, GRGESP, LY294002, wortmannin, SN-50, SN-50M, and anti-uPA antibody and used for the migration assay. The results indicated that OPN induces the cell migration in a dose-dependent manner in both these cells (Fig. 8, A and B). α,β3 integrin antibody, GRGDSP, LY294002, wortmannin, SN-50, and anti-uPA antibody suppressed the OPN-induced cell migration in MCF-7 cells (Figs. 8C and 9A) and MDA-MB-231 cells (Figs. 8D and 9B). GRGESP and SN-50M had no effect on OPN-induced cell migration (Figs. 8C and D) and 9 (A and B)). In other experiments, cells were transiently transfected with Δp85, CAAXp110, or the super-repressor form of IκBα in the presence of LipofectAMINE Plus and then used for migration assay. The data showed that both Δp85 and the super-repressor form of IκBα inhibited and CAAXp110 enhanced the OPN-induced cell migration in MCF-7 cells (Fig. 9C) and MDA-MB-231 cells (Fig. 9D).

Similarly, both these cells were pretreated with anti-α,β3 integrin antibody, LY294002, wortmannin, and SN-50 and used for chemoinvasion assay. The results showed that OPN induced ECM invasion and that α,β3 integrin antibody, LY294002, wortmannin, and SN-50 suppressed the OPN-induced ECM invasion in MCF-7 cells (Fig. 10A) and MDA-MB-231 cells (Fig. 10B). These data demonstrated that OPN-induced cell migration, ECM invasion, and uPA secretion are regulated by PI 3'-kinase, Akt, and NFκB in MCF-7 and MDA-MB-231 cells.

DISCUSSION

In a recent study (46), we have demonstrated that OPN stimulated NFκB activity through phosphorylation and degradation of IκBα by inducing the IKK activity in murine melanoma cells. However, it was not clear whether any upstream kinases (e.g. PI 3'-kinase/Akt/PKB) are involved in these OPN-induced activation processes. Moreover, the molecular mechanisms by which OPN regulates IKK-mediated NFκB activity and controls cell motility and whether uPA is involved in OPN-induced cell motility in human breast cancer cells is not well defined. Accordingly, we have investigated the role of OPN on PI 3'-kinase/Akt-mediated activation of NFκB in human breast cancer cell lines. In this report, we have shown that OPN stimulated PI 3'-kinase activity and subsequently phosphorylated Akt in breast cancer cells. The OPN-induced Akt phosphorylation was suppressed when cells were transfected with a dominant negative mutant of the p85 domain of PI 3-kinase (Δp85) and enhanced when cells were transfected with an activated form of PI 3-kinase (p110CAAX) indicating that PI 3-kinase is involved in Akt phosphorylation. Moreover, pretreatment of these cells with anti-α,β3 integrin antibody followed by treatment with OPN suppressed the OPN-induced PI 3'-kinase activity and Akt/PKB phosphorylation suggesting that OPN regulates the PI 3'-kinase/Akt activity through α,β3 integrin-mediated pathways. OPN enhances the interaction between IκBα kinase (IKK) and phosphorylated Akt in these cells. OPN also induces NFκB activation through phosphorylation and degradation of IκBα by inducing the IKK activity in breast cancer cells. However, both wortmannin and LY294002 inhibited OPN-induced Akt phosphorylation, IKK and NFκB activities, and phosphorylation and degradation of IκBα demonstrating that PI 3'-kinase plays crucial roles in these processes. OPN also enhances the uPA secretion and cell motility in both MCF-7 and MDA-MB-231 cells. Pretreatment of cells with pharmacological (wortmannin and LY294002) and genetic (Δp85) inhibitors of PI 3-kinase, α,β3 integrin antibody, and NFκB inhibitory peptide (SN-50) reduced the OPN-induced uPA secretion, cell motility, and ECM invasion. These data
showed that OPN induces αβ3 integrin-mediated cell motility, invasion, and uPA secretion by activating PI 3′-kinase/Akt/NFκB signaling pathways in breast cancer cells.

Cell-matrix interactions play an important role in tissue remodeling, cell survival, and tumorigenesis (44). OPN, an extracellular matrix protein, plays a significant role in cell migration, adhesion, and metastasis. OPN plays crucial roles in normal tissue remodeling processes such as bone resorption, angiogenesis, wound healing, and tissue injury as well as certain diseases such as restenosis, atherosclerosis, tumorigenesis, and autoimmune diseases (11–13). It has been shown that overexpression of OPN is associated with various cancers and their metastatic potentials (14–16). OPN regulates cell migration, ECM invasion, and cell proliferation by interacting with its receptor αβ3 integrin in various cell types (12). Several studies have indicated the correlation between uPA expression and metastatic potential and suggested that uPA plays a major role in controlling cell migration and ECM invasion of various cancer cells (48, 49). Therefore we sought to determine whether OPN regulates uPA secretion and controls cell migration and ECM invasion in highly invasive and low invasive breast cancer cells. In this study we have demonstrated that OPN induces uPA secretion, ECM invasion, and cell migration in both highly invasive and low invasive breast cancer cells.

It has been documented that PI 3′-kinase plays a significant role in NFκB activation in different cell types, including breast cancer cells (7, 50). PI 3′-kinase inhibitor has no effect on interleukin-1-dependent IkBα degradation, nuclear accumulation of NFκB, and NFκB-DNA binding (50). Tumor necrosis factor α-induced NFκB activation is not affected by PI 3′-kinase inhibitors (LY294002 and wortmannin). Our recent data indicated that Syk, a non-receptor protein-tyrosine kinase downstream of PI 3′-kinase transactivation by inhibiting the interaction between the tyrosine-phosphorylated IκBα and the p85 subunit of PI 3′-kinase (7). Previous reports have also shown that pervanadate and tumor necrosis factor α induced NFκB activation in Jurkat cells, and only pervanadate-induced activation of NFκB is inhibited by wortmannin (51). It has been documented that OPN protects cells from apoptosis by activating the PI 3′-kinase/Akt pathway (52). But the molecular mechanisms by which OPN regulates PI 3′-kinase/Akt-mediated NFκB activation and controls uPA secretion, cell motility, and ECM invasion using breast cancer cells are not well defined. Earlier data showed that Akt is involved in the vascular endothelial growth factor-mediated endothelial cell migration (53). We have demonstrated here that OPN stimulates the PI 3′-kinase activity and Akt phosphorylation in MCF-7 and MDA-MB-231 cells. Using genetic (Δp85 or CAAXp110) or pharmacological (LY294002 or wortmannin) inhibitors of PI 3′-kinase, we have shown that both catalytic and regulatory subunits of PI 3′-kinase are involved in OPN-induced Akt phosphorylation, PI 3′-kinase, and NFκB activation and controls uPA secretion, cell motility, and ECM invasion. Previous reports have indicated that p110 isoforms of PI 3′-kinase played significant roles in cell migration, and differential activation of specific p110 isoforms is responsible for particular signaling events in different cell types (7). Recently, Sliva et al. (18) reported that the regulatory p85α subunit of PI 3′-kinase is essential for enhanced migration of metastatic tumor cells, because overexpression of a dominant negative regulatory subunit (p85DN) drastically reduced the cell migration. Our data also revealed that OPN induces the interaction between IKK and phosphorylated Akt and IKK activity in breast cancer cells. PI 3′-kinase

\[ \text{Fig. 8. OPN enhances PI 3′-kinase-dependent } \alpha\beta_3 \text{ integrin-mediated breast cancer cell migration. A–D, the migration assay was performed either by using untreated cells (5 × 10^5 cells/well) or cells pretreated with different doses of } \alpha\beta_3 \text{ integrin antibody (0–20 μg/ml), GRGDSP or GRGESP peptide (0–10 μM), or PI 3′-kinase inhibitor (0–10 μM LY294002 or 0–100 nM wortmannin for 6 h. The purified human OPN was added in the upper chamber. Note that OPN stimulates the cell migration in a dose-dependent manner (A, MCF-7 cells; B, MDA-MB-231 cells). The OPN-induced cell migration was inhibited by } \alpha\beta_3 \text{ integrin antibody, PI 3′-kinase inhibitors, and GRGDSP but not by GRGESP (C, MCF-7 cells; D, MDA-MB-231 cells). The results are expressed as the mean ± S.E. of three determinations.} \]
inhibitor (genetic or pharmacological) down-regulates the OPN-induced interaction and IKK activity in these cells. The levels of PI 3'-kinase and IKK activities were significantly higher in MDA-MB-231 cells compared with MCF-7 cells. We have shown earlier that the constitutive activation of PI 3'-kinase was higher in MDA-MB-231 cells compared with MCF-7 cells (7). Moreover, we have shown that the expression of cell surface αvβ3 integrin is also higher in MDA-MB-231 cells compared with MCF-7 cells (data not shown). This data is consistent with the data reported by other group (47).

Several reports have indicated that NFκB is involved in the control of a large number of normal cellular processes such as inflammatory and immune responses, developmental processes, cell growth, and apoptosis. In addition, NFκB is activated in several pathological conditions like arthritis, inflammation, asthma, neurodegenerative diseases, heart diseases, and cancers (46). In a previous report (44), we have demonstrated that OPN induces the activation of promatrix-metalloproteinase-2 through NFκB-mediated pathways in B16F10 cells. OPN-induced activation of NFκB occurred through phosphorylation and degradation of IκBα by inducing the IKK activity in these cells (46). However, the molecular mechanism by which OPN induces IKK activity and subsequently activates NFκB in breast cancer (MCF-7 and MDA-MB-231) cells is not well defined. In the present report, we have shown that OPN induces IKK activity through PI 3'-kinase-dependent phosphorylation of Akt/PKB in MCF-7 and MDA-MB-231 cells. OPN induces phosphorylation and degradation of IκBα in a time-dependent manner, and both pharmacological (LY294002 and wortmannin) and genetic (Δp85) inhibitors of PI 3'-kinase blocked the OPN-induced phosphorylation and degradation of IκBα in these cells. OPN also enhances nuclear accumulation of NFκB, NFκB-DNA binding, and NFκB transcriptional activity. The OPN-induced NFκB activation occurred through phosphorylation and degradation of IκBα. These data clearly suggested that PI 3'-kinase plays crucial role in OPN-induced NFκB activation through the αvβ3 integrin-mediated pathway in breast cancer cells.

Previous data have demonstrated that OPN induces cell motility, invasiveness, and uPA expression in human mammary epithelial cells (54, 55). Moreover, earlier data reported that OPN-induced migration of human mammary epithelial cells involves activation of EGF receptor and multiple signal transduction pathways (56). It has been shown that uPA plays a major role in regulating matrix metalloproteinases activation (40, 41). However, the molecular mechanism of OPN-regulated cell motility, invasiveness, and uPA expression in breast cancer cells is not well established. Here, we have reported that OPN induces cell motility and invasion through secretion of uPA in both highly invasive (MDA-MB-231) and low invasive (MCF-7)
cells. The αβ3 integrin antibody and GRGDSP but not GRGESP peptide inhibit OPN-induced cell migration, invasion, and uPA secretion, indicating that OPN induces uPA secretion through αβ3 integrin-mediated pathways in these cells. Both pharmacological (LY294002 and wortmannin) and genetic (dominant negative mutant of p85, Δp85) inhibitors of PI 3-kinase suppressed the OPN-induced Akt phosphorylation and cell migration, demonstrating that both catalytic and regulatory subunits of PI 3-kinase are involved in these processes. NFκB inhibitory peptide (SN-50) also inhibited OPN-induced uPA secretion, cell migration, and invasion indicating that OPN regulates uPA secretion and enhances invasiveness and cell migration through the NFκB-mediated pathway in breast cancer cells.

In summary, we have demonstrated for the first time that OPN enhances cell motility and ECM invasion and induces the PI 3-kinase activity in MCF-7 and MDA-MB-231 cells. OPN also stimulates phosphorylation of Akt in these cells. The OPN-induced Akt phosphorylation was suppressed when the cells were transfected with Δp85 and enhanced when cells were transfected with p110CAAX, indicating that PI 3-kinase is involved in Akt phosphorylation. OPN enhances the interaction between IKK and phosphorylated Akt in these cells. OPN also induces NFκB activation through phosphorylation and degradation of IκBα by inducing the IKK activity. However, both PI 3-kinase inhibitor (wortmannin or LY294002) and Δp85 inhibited OPN-induced Akt phosphorylation, IKK activity, NFκB activation, and phosphorylation of IκBα. Moreover, OPN enhances invasiveness and cell motility through secretion of uPA in these cells. Furthermore, cells transfected with either the super-repressor form of IκBα or Δp85 suppressed the OPN-induced NFκB activity, uPA secretion, and cell motility, whereas cells transfected with p110CAAX enhanced these effects. Pretreatment of cells with αβ3 integrin antibody, PI 3-kinase inhibitors, or NFκB inhibitory peptide (SN-50) reduced the OPN-induced uPA secretion, cell motility, and ECM invasion. Finally these data demonstrated that OPN induces the PI 3-kinase-dependent Akt phosphorylation, NFκB activity, and uPA secretion and all these factors, ultimately controlling the induction of cell motility and invasiveness of breast cancer cells. Taken together, OPN induces NFκB activation and uPA secretion by activating PI 3-kinase/Akt/IKK-mediated signaling pathways. These findings may be useful in designing novel therapeutic interventions that block the OPN-regulated PI 3-kinase-dependent Akt phosphorylation and NFκB activation resulting in reduction of uPA secretion and consequent blocking of cell motility, invasiveness, and metastatic spread of breast cancer.

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