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Syk, a Protein-tyrosine Kinase, Suppresses the Cell Motility and Nuclear Factor κB-mediated Secretion of Urokinase Type Plasminogen Activator by Inhibiting the Phosphatidylinositol 3′-Kinase Activity in Breast Cancer Cells*

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Tumor growth and metastasis are multifaceted processes that mainly involve cell adhesion, proteolytic degradation of the extracellular matrix, and cell migration. Syk is a member of a tyrosine kinase family that is expressed mostly in hematopoietic cells. Syk is expressed in cell lines of epithelial origin, but its function in these cells remains unknown. Here we report that Syk is expressed in MCF-7 cells but not in MDA-MB-231 cells. The overexpression of wild type Syk kinase but not kinase-negative Syk suppressed cell motility and inhibited the activation of phosphatidylinositol (PI) 3′-kinase in MDA-MB-231 cells. In contrast, when Syk-specific antisense S-oligonucleotide but not the sense S-oligonucleotide was transfected to MCF-7 cells the level of PI 3′-kinase activity as well as cell motility were increased. The MDA-MB-231 cells transfected with wild type Syk cDNA followed by treatment with piceatannol, a Syk inhibitor, enhanced cell motility and PI 3′-kinase activity. Pervanadate, a phosphotyrosine phosphatase inhibitor, induced PI 3′-kinase activity and stimulated the interaction between the inhibitor of nuclear factor κBα (IκBα) and the p85α domain of PI 3′-kinase through tyrosine phosphorylation of the IκBα, which ultimately resulted in nuclear factor κB (NFκB) activation. Pervanadate had no effect on the activation of Syk in these cells. However, Syk suppressed the NFκB transcriptional activation and interaction between IκBα and PI 3′-kinase by inhibiting the tyrosine phosphorylation of IκBα. Syk, PI 3′-kinase inhibitors, and NFκB inhibitory peptide inhibited urokinase type plasminogen activator (uPA) secretion and cell motility in these cells. To our knowledge, this is the first report that Syk suppresses the cell motility and inhibits the PI 3′-kinase activity and uPA secretion by blocking NFκB activity through tyrosine phosphorylation of IκBα. These data further demonstrate a functional molecular link between Syk-regulated PI 3′-kinase activity 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), wound healing, and cancer cell metastasis (3, 4). However, the exact molecular mechanisms that regulate these processes are not well understood. Syk, a nonreceptor protein-tyrosine kinase, is expressed widely in hematopoietic cells (5, 6). It has tandem amino-terminal SH2[1] domains and a carboxyl-terminal kinase domain (7, 8). The SH2 domains bind phosphorylated immunoreceptor tyrosine-based activation motifs and play significant roles in signaling through immunoreceptors (9). ZAP-70 is a cytoplasmic tyrosine kinase, and both Syk and ZAP-70 share the same tandem SH2 domains at the amino terminus and play important roles in coupling antigen and Fc receptors to downstream signaling events that mediate diverse cellular responses including proliferation, differentiation, and phagocytosis (9, 10). Fc receptors are members of the family of membrane proteins, called immunoreceptors, and they are expressed in all cells of the immune system. Both Syk and ZAP-70 are regulated by β_3 integrin-dependent cell adhesion via phosphorylation-independent interaction with the cytoplasmic domain of β_3 integrin (11). The expression of Syk has also been reported in cell lines of epithelial origin (12), but its function in these cells is not well understood. Recently it has been documented that Syk is commonly expressed in normal human breast tissue, benign breast lesions, and low tumorigenic breast cancer cell lines (13).

Several cytokines, growth factors, and other agents control the regulation of cell motility. Phosphatidylinositol 3′-kinase (PI 3′-kinase) also plays significant role in regulation of cell motility (14). Two subunits are present among all of the classes of PI 3′-kinases. One is catalytic subunit p110 (α, β, δ) or p110γ, and the other is regulatory subunit p85 (α, β, p55γ, and p101) (15). The regulatory subunit of PI 3′-kinase is responsible for B cell development and proliferation (16), and the catalytic subunits are critical for chemotactic activity (17).

The activation of NFκB is regulated by a number of proinflammatory stimuli (18, 19). The NFκB family consists of several members including p65, p50, RelB, and c-Rel molecules (18). The activity of NFκB is also tightly controlled by its inhibitor, IκB family of proteins (20). NFκB forms a complex with IκBα, and the complex can be removed from the nucleus by exportin-mediated transport to the cytoplasm. Recent report indicated that constitutively active PI 3′-kinase controls the

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[1] The abbreviations used are: SH2 domain, Src homology 2 domain; ASSyk, Syk-specific antisense phosphorothioate oligonucleotide(s); FITC, fluorescein isothiocyanate; IκBα, inhibitor of nuclear factor-κB; Luc, luciferase; NFκB, nuclear factor-κB; PI 3′-kinase, phosphatidylinositol 3′-kinase; PIP, phosphatidylinositol phosphate; PMA, phorbol 12-myristate 13-acetate; pV, pervanadate; SSyk, Syk-specific sense phosphorothioate oligonucleotide(s); SykK⁻, kinase-negative Syk(s); TRITC, tetramethylrhodamine isothiocyanate; uPA, urokinase type plasminogen activator.

activation of NFκB by the association of tyrosine-phosphorylated IκBα with the regulatory subunit of PI 3′-kinase, p85α (21). The recent data also demonstrated that interleukin-1 stimulates PI 3′-kinase dependent phosphorylation and transactivation of NFκB without nuclear translocation of NFκB, indicating an alternative pathway other than IκBα-mediated pathway (22). But the molecular mechanism by which Syk, a tyrosine kinase, regulates the PI 3′-kinase dependent activation of NFκB in breast cancer cells is not well defined.

Urokinase-type plasminogen activator (uPA) is a member of the serine protease family which induces the conversion of plasminogen to plasmin (23). Plasmin regulates cell invasion by degrading matrix proteins such as fibronectin, type IV collagen, and laminin or indirectly by activating matrix metalloproteinases and uPA (24–26). Previous reports shown that uPA plays a significant role in tumor growth and metastasis. The signaling pathway by which Syk controls uPA secretion through PI 3′-kinase-dependent activation of NFκB is not clearly understood.

In this study, we demonstrate that overexpression of wild type Syk in MDA-MB-231 cells suppressed cell motility and reduced PI 3′-kinase activity. Syk-specific antisense phosphorothioate oligonucleotide (ASSyk), when transfected to MCF-7 cells, increased cell motility and up-regulated PI 3′-kinase activity. Pervanadate (pV) stimulated PI 3′-kinase activity and induced transactivation of NFκB through tyrosine phosphorylation of IκBα, whereas Syk down-regulated the NFκB activity by inhibiting tyrosine phosphorylation of IκBα in these cells. Syk, PI 3′-kinase inhibitor, and NFκB inhibitors inhibited cell motility and uPA secretion in these cells. Taken together, Syk suppressed cell motility, uPA secretion, and PI 3′-kinase-mediated NFκB activation by inhibiting the interaction between the p85α subunits of PI 3′-kinase and tyrosine-phosphorylated IκBα.

EXPERIMENTAL PROCEDURES

Materials—The rabbit polyclonal anti-Syk and anti-IκBα and mouse monoclonal anti-PI 3′-kinase, p85α antibodies were obtained from Santa Cruz Biotechnology. The rabbit polyclonal anti-phospho-IκBα and mouse monoclonal anti-uPA antibodies were purchased from Oncogene. Piceatannol, LY294002, and ST 638 (α-cyano-(3-ethoxy-4-hydroxy-5-phenylthiomethyl)cinnamide) were purchased from Calbiochem. Wortmannin and TRITC-conjugated goat anti-mouse IgG were obtained from Sigma. The PIwas from ICN. [γ-^{32}P]ATP was purchased from the Board of Radiation and Isotope Technology (Hyderabad, India). The dual luciferase reporter assay system was obtained from Promega. The rabbit anti-phosphotyrosine antibody and LipofectAMINE Plus reagent were purchased from Invitrogen. The FITC-conjugated goat anti-rabbit IgG was from Pharmingen. Boyden type cell migration chambers were obtained from Corning. All other chemicals were analytical grade.

The pV was prepared by incubating 1 M sodium orthovanadate with 33% H_2O_2 in phosphate-buffered saline (pH 7.4) at room temperature for 15 min. The pH of the solution was neutralized with 1 N HCl, and excess H_2O_2 was deactivated with catalase.

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% CO_2 and 95% air at 37 °C.

Western Blot Analysis—To detect the level of Syk expression in MCF-7 and MDA-MB-231 cells, the cells were lysed in lysis buffer (1% Triton X-100 solution containing 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 2 mM EDTA). The cleared lysates were collected by centrifugation at 12,000 × *g* for 15 min at 4 °C. The protein concentration in the lysates 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 membranes were incubated with rabbit polyclonal anti-Syk antibody (1:200) and incubated further with anti-rabbit horseradish peroxidase-conjugated IgG (1:1,000). The membrane was washed and detected by the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences) according to the manufacturer's instructions. To check the tyrosine phosphorylation of Syk, both MCF-7 and MDA-MB-231 cell lysates were immunoprecipitated individually with anti-Syk antibody. The immunoprecipitated samples were resolved by SDS-PAGE and detected by Western blot analysis using rabbit anti-phosphotyrosine antibody as described above. These blots were reprobed with anti-actin antibody as loading control.

DNA Transfection—The wild type and kinase-negative Syk (SykK$^-$) cDNAs in an expression vector (pcDNA 3.1) were a generous gift from Dr. Susette C. Mueller (Department of Oncology, Georgetown University Medical School, Washington, D. C.). The MDA-MB-231 cells were split 12 h prior to transfection in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The cells were transiently transfected with Syk cDNA using LipofectAMINE Plus according to the manufacturer's instructions. Briefly, wild type or SykK$^-$ cDNA (8 μg) was mixed with Plus reagent, and then cDNA reagent Plus was incubated with LipofectAMINE. The LipofectAMINE Plus cDNA complex was 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. These transfected cells were used for the detection of Syk and uPA expression by Western blot analysis, NFκB activity by luciferase assay, and PI 3′-kinase activity by kinase assay. These cells were also used for a migration assay. In separate experiments, MCF-7 cells were transfected with Syk-specific S-oligonucleotides according to the methods described above. Human ASSyk (5′-TGC CGC TGC TGG CCA TGC TT-3′) and SSyk (5′-AAG CAT GGC CAG CAG CGG CA-3′) with phosphorothioate linkages were synthesized (Genomechanix). These oligonucleotides were purified by column chromatography, and purity was checked by PAGE. These oligonucleotide-transfected cells were used for the detection of Syk and uPA expression by Western blot analysis, NFκB luciferase assay, and PI 3′-kinase assay. These transfected cells were also used for a cell migration assay. To check the dose-dependent response, separate transfection experiments were performed with different doses (0–10 μg) of ASSyk.

Cell Migration Assay—The migration assay was conducted using a Transwell cell culture chamber according to the standard procedure as described previously (27). Briefly, the Syk-specific S-oligonucleotide-transfected MCF-7 cells or Syk cDNA-transfected MDA-MB-231 cells were harvested with trypsin-EDTA 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-conditioned medium, which acted as chemoattractant. In a separate experiment, either wild type Syk cDNA-transfected MDA-MB-231 or nontransfected MCF-7 cells were incubated in the absence or presence of 0–20 μM piceatannol, a Syk inhibitor, at 37 °C for 30 min and used for a migration assay. In other experiments, both of these nontransfected cells were treated individually with a PI 3′-kinase inhibitor (0–100 nM wortmannin or 0–10 μM LY294002) at 37 °C for 3 h or with 250 μM pV, a tyrosine phosphatase inhibitor, at 37 °C for 30 min or in combination and used for migration assay. To check whether NFκB or uPA is involved in migration, both of these nontransfected cell lines were treated individually with 100 μg/ml SN-50, 100 μg/ml SN-50M, 10 μg/ml actinomycin-D, 50 μM curcumin, 5 ng/ml PMA, 10 μg/ml monoclonal uPA antibody at 37 °C for 6 h. The transfected cells were also treated with anti-uPA antibody and used for migration assay. After treatment, these cells were incubated in a humidified incubator in 5% CO_2 and 95% air at 37 °C for 16 h. The nonmigrated 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. These treated cells were also used for the detection of uPA by Western blot analysis.

Immunoprecipitation and in Vitro Kinase Assay—To examine the autophosphorylation of Syk in MCF-7 and MDA-MB-231 cells, the cells were immunoprecipitated with rabbit polyclonal anti-Syk antibody and subjected to the the kinase assay. Briefly, the cells were lysed in lysis buffer (1% Triton X-100 solution containing 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 2 mM EDTA), and the protein concentration in the cleared lysates was measured by Bio-Rad protein assay. The samples containing equal amounts of total proteins were immunoprecipitated with rabbit polyclonal anti-Syk antibody according to the manufacturer's instructions (Roche Molecular Biochemicals). The immunoprecipitated samples were incubated with 2 μCi of [γ-^{32}P]ATP in

kinase assay buffer (50 mM Hepes buffer (pH 8.0) containing 10 mM Na_3VO_4, 50 mM $MgCl_2$, and 5 mM $MnCl_2$) at 30 °C for 10 min. The samples were resolved by SDS-PAGE, dried, and autoradiographed. In separate experiments, the MCF-7 cells were treated in the absence or presence of 250 μM pV for 0–60 min. The cell lysates were immunoprecipitated with anti-Syk antibody, and a Syk kinase assay was performed as described above.

To check the role of Syk in the regulation of PI 3′-kinase activity, the MCF-7 cells were transfected with SSyk or ASSyk, the MDA-MB-231 cells were transfected with wild type or SykK$^-$ cDNA, and the PI 3′-kinase activity was measured. Cells were lysed in lysis buffer as described earlier and centrifuged at 12,000 × *g* for 15 min. The cleared lysates were collected, and the protein concentration was measured by Bio-Rad protein assay. The PI 3′-kinase assay was performed with slight modification (28). Briefly, cell lysates were immunoprecipitated with mouse monoclonal anti-p85α antibody, and the immunoprecipitated samples were incubated in kinase assay buffer (25 mM Hepes (pH 7.4), 10 mM $MgCl_2$, and 1 mM EDTA) containing 0.25 mg/ml phosphatidylinositol, 100 mM ATP, and 15 μCi of [γ-^{32}P]ATP and incubated at 30 °C for 10 min. The reaction was terminated by the addition of acidified chloroform:methanol (2:1). Lipids were extracted according to the procedure described previously (29) and separated on oxalate-treated plastic TLC plates using a solvent system consisting of chloroform, methanol, and 20% methylamine (65:35:10 v/v/v). The spots corresponding to the position of radioactive phosphatidylinositol phosphate (PIP) were visualized by autoradiography. In separate experiments, wild type Syk cDNA-transfected MDA-MB-231 or nontransfected MCF-7 cells were individually treated with 0–10 μM piceatannol, a Syk kinase inhibitor, at 37 °C for 30 min and used for the PI 3′-kinase assay. In other experiments, nontransfected MDA-MB-231 or MCF-7 cells were also pretreated with 250 μM pV at room temperature for 0–30 min and used for the PI 3′-kinase assay.

To check the role of pV on the tyrosine phosphorylation of IκBα and subsequent interaction between IκBα and PI 3′-kinase, both MCF-7 and MDA-MB-231 cells were treated individually with 250 μM pV tyrosine phosphatase inhibitor for 0–30 min, and the total proteins in the lysates were measured by Bio-Rad protein assay. Half of the lysates were immunoprecipitated with nonphosphorylated anti-IκBα antibody. The immunoprecipitated samples were resolved by SDS-PAGE, and the level of tyrosine-phosphorylated IκBα was detected by Western blot analysis using anti-phosphotyrosine antibody. The remaining half of the lysates was resolved by SDS-PAGE, and the serine-phosphorylated IκBα was detected by Western blot analysis using anti-phospho-IκBα (serine-specific) antibody. Similarly, both MCF-7 and MDA-MB-231 cells were treated individually in the absence or presence of 250 μM pV alone or with 100 μM ST 638, a tyrosine kinase inhibitor, along with 250 μM pV at 37 °C for 0–30 min. The cells were lysed, and the lysates were immunoprecipitated with anti-PI 3′-kinase, p85α antibody. The immunoprecipitated samples were resolved by SDS-PAGE, and the tyrosine phosphorylation of IκBα in lysates was detected by Western blot analysis using rabbit anti-phosphotyrosine antibody. The blots were reprobed with anti-actin antibody as loading control.

To check the effect of Syk on tyrosine phosphorylation of IκBα and subsequent interaction between IκBα and PI 3′-kinase, the Syk-specific S-oligonucleotide-transfected MCF-7 or Syk cDNA-transfected MDA-MB-231 cells were treated individually with 250 μM pV for 15 min, and the cells were lysed in lysis buffer. Total proteins in the lysates were measured by Bio-Rad protein assay. The cell lysates containing equal amounts of total proteins were immunoprecipitated with anti-p85α antibody. The samples were resolved by SDS-PAGE and analyzed by Western blot analysis using anti-phosphotyrosine antibody. The blots were reprobed with anti-actin antibody.

Immunofluorescence Study—Both MDA-MB-231 and MCF-7 cells were grown in monolayer on glass slides and then treated individually in the absence or presence of 250 μM pV at room temperature for a period of 0–30 min. The cells were fixed with paraformaldehyde for 10 min, blocked with 5% bovine serum albumin for 30 min, and washed with phosphate-buffered saline (pH 7.4). The fixed cells were incubated with a mixture of mouse monoclonal anti-PI 3′-kinase, p85α (1:10 dilution), and rabbit polyclonal anti-IκBα antibodies (1:20 dilution) at room temperature for 2 h. The cells were washed with phosphate-buffered saline (pH 7.4) and incubated with a mixture of FITC-conjugated anti-rabbit IgG and TRITC-conjugated anti-mouse IgG. The cells were washed, mounted with coverslips, and analyzed under confocal microscopy (Zeiss).

NFκB Luciferase Reporter Gene Assay—The semiconfluent MCF-7 cells grown in 24-well plates were transiently transfected with Syk-specific S-oligonucleotides and a luciferase reporter construct (pNFκB-Luc) containing five tandem repeats of the NFkB binding site (a generous gift from Dr. Rainer de Martin, University of Vienna, Vienna, Austria) using LipofectAMINE Plus reagent for 12 h. Similarly, the MDA-MB-231 cells were transfected with wild type or SykK$^-$ cDNA and pNFκB-Luc under the same conditions described above. 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. In separate experiments, MCF-7 cells transfected with pNFκB-Luc or MDA-MB-231 cells transfected with wild type Syk and pNFκB-Luc were treated with 0–10 μM piceatannol. In other experiments, both MCF-7 and MDA-MB-231 cells were transfected individually with pNFκB-Luc and treated with either 100 μM pV alone for 30 min, pV with 1–100 nM wortmannin or 1–10 μM LY294002 for 3 h. Cells were harvested in passive lysis buffer (Promega). The luciferase activities were measured by luminometer (Lab Systems) using the dual luciferase assay system according to the manufacturer's instructions (Promega). Changes in luciferase activity with respect to the control were calculated.

RESULTS

Detection of Tyrosine-phosphorylated Syk Expression by Western Blot Analysis and Autophosphorylation by in Vitro Kinase Assay—The expression of Syk was analyzed by SDS-PAGE followed by Western blot in MCF-7 (Fig. 1*A*, *lane 1*) and MDA-MB-231 (*lane 2*) cells. To assess the autophosphorylation activity of Syk, both of these cell lines were lysed in lysis buffer, and the lysates were immunoprecipitated with rabbit polyclonal anti-Syk antibody. The immunoprecipitated samples were incubated with [γ-^{32}P]ATP in kinase assay buffer. The samples were resolved by SDS-PAGE and autoradiographed. Fig. 1*B* shows the autophosphorylated Syk expression in MCF-7 cells (*lane 1*), but this was absent in MDA-MB-231 cells (*lane 2*). To check whether the phosphorylation of Syk is tyrosine-specific, both of the cell lysates were immunoprecipitated with anti-Syk antibody and detected by Western blot analysis using rabbit anti-phosphotyrosine antibody. The MCF-7 (Fig. 1*C*, *lane 1*) but not MDA-MB-231 (*lane 2*) cells recognized tyrosine-phosphorylated Syk expression, suggesting that tyrosine residue of Syk is involved in autophosphorylation. All of these blots were reprobed with anti-actin antibody as loading control. To check whether pV, a tyrosine phosphatase inhibitor, has any role in the autophosphorylation activity of Syk, the MCF-7 cells were treated with 250 μM pV for 0–60 min, and the cell lysates were immunoprecipitated with anti-Syk antibody. The activity of Syk in the immunoprecipitated samples was detected by kinase assay, and the results showed that pV had no effect on Syk activity in these cells (Fig. 1*D*, *lanes 1–5*).

To control the expression of Syk and to check the status of Syk-dependent downstream signaling events and cell motility, the low invasive MCF-7 cells were transfected with Syk-specific phosphorothioate-linked SSyk or ASSyk oligonucleotides in the presence of LipofectAMINE Plus. The expression of Syk was detected by Western blot analysis, and the data indicated that there was significant expression of Syk in cells transfected with LipofectAMINE Plus alone (Fig. 2*A*, *lane 1*) or SSyk transfected cells (*lane 2*), but the level of Syk was reduced drastically when cells were transfected with ASSyk (*lane 3*). Similarly, the MDA-MB-231 cells were transfected with wild type or SykK$^-$ cDNA in the presence of LipofectAMINE Plus, and expression of Syk was determined by Western blot analysis. The expression of Syk was absent in cells transfected with LipofectAMINE Plus alone (Fig. 2*B*, *lane 1*), whereas a significant level of Syk expression was observed in both wild type (*lane 2*) and SykK$^-$-transfected cells (*lane 3*) . The level of Syk was also quantified densitometrically and analyzed statistically (Fig. 2, *A* and *B*, *lower panels*). These transfected cells were used for cell migration assays and for the detection of downstream signaling molecules.

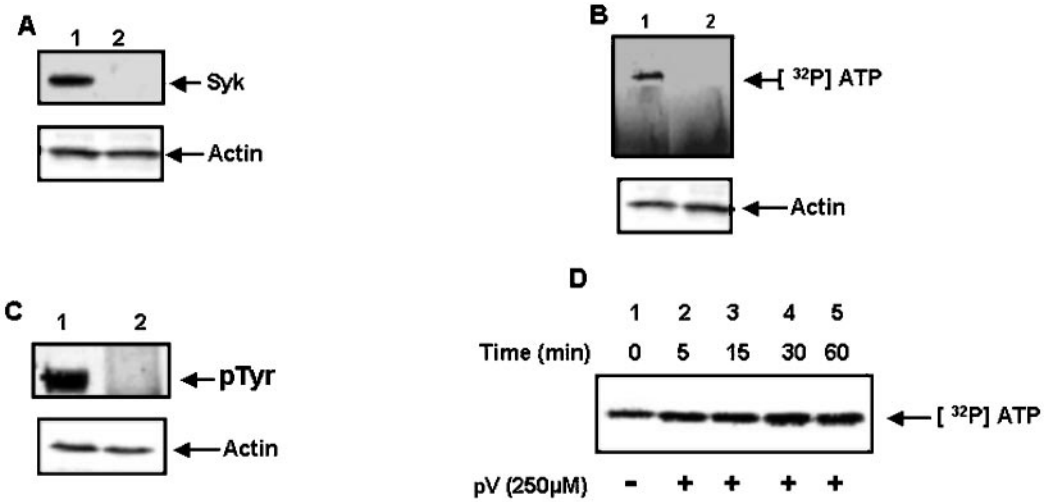

FIG. 1. **Expression and autophosphorylation of Syk in breast cancer cells.** *A*, equal amounts of total proteins in cell lysates from MCF-7 and MDA-MB-231 cells were resolved by SDS-PAGE and analyzed by Western blot using rabbit polyclonal anti-Syk antibody. *Lane 1*, MCF-7 cells; *lane 2*, MDA-MB-231 cells. *B*, equal amounts of total proteins in both cell lysates were immunoprecipitated with rabbit polyclonal anti-Syk antibody, and the immunoprecipitates were incubated with 2 μCi of [γ-^{32}p]ATP in kinase assay buffer as described under "Experimental Procedures." The sample was resolved by SDS-PAGE and autoradiographed. *Lane 1*, MCF-7 cells; *lane 2*, MDA-MB-231 cells. *C*, cell lysates containing equal amounts of total proteins were immunoprecipitated with anti-Syk antibody, and the immunocomplex was resolved by SDS-PAGE and analyzed by Western blot using rabbit anti-phosphotyrosine antibody. *Lane 1*, MCF-7 cells; *lane 2*, MDA-MB-231 cells. The *arrow* indicates the Syk-specific band. As loading controls, all of these blots were reprobed with goat polyclonal anti-actin antibody (*lower panels* in *A–C*). *D*, effect of pV on autophosphorylation activity of Syk in MCF-7 cells. The cells were treated with 250 μM pV for 0–60 min, and the cell lysates containing equal amounts of total proteins were immunoprecipitated with anti-Syk antibody. The immunoprecipitates were incubated with 2 μCi of [γ-^{32}p]ATP in kinase assay buffer as described above. The sample was resolved by SDS-PAGE and autoradiographed. *Lane 1*, control; *lane 2*, pV 5 min; *lane 3*, pV 15 min; *lane 4*, pV 30 min; and *lane 5*, pV 60 min.

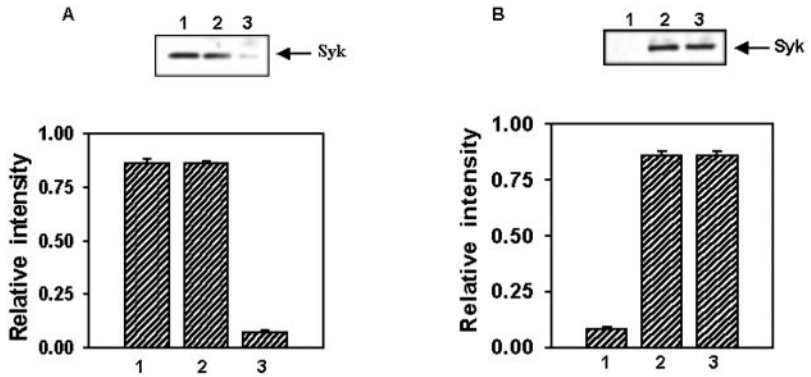

FIG. 2. **Detection of Syk in Syk-specific S-oligonucleotide-transfected MCF-7 and Syk cDNA transfected-MDA-MB-231 cells by Western blot analysis.** *A*, MCF-7 cells were transiently transfected with SSyk or ASSyk with LipofectAMINE Plus. The cell lysates containing equal amounts of total proteins were resolved by SDS-PAGE and analyzed by Western blot using anti-Syk antibody. *Lane 1*, with LipofectAMINE Plus; *lane 2*, with SSyk; *lane 3*, with ASSyk. *B*, MDA-MB-231 cells were transiently transfected with wild type or SykK$^-$ cDNA in pcDNA 3.1 with LipofectAMINE Plus. Equal amounts of total proteins in cell lysates were separated by SDS-PAGE and analyzed by Western blot using anti-Syk antibody. *Lane 1*, with LipofectAMINE Plus; *lane 2*, with wild type Syk cDNA; *lane 3*, with SykK$^-$ cDNA. The *arrow* indicates the Syk-specific band. The expression of Syk was quantified by densitometric analysis and is represented in the form of a *bar graph*. The mean value of triplicate experiments is indicated in the *lower panels* of *A* and *B*.

Syk and PI 3′-kinase Play Critical Roles in Cell Migration—To ascertain the roles of Syk and PI 3′-kinase in the regulation of cell migration, both MDA-MB-231 and MCF-7 cells were used for the migration assay. The MCF-7 cells were transfected with Syk-specific S-oligonucleotides and performed the migration assay. The data demonstrated that ASSyk-transfected cells showed a dramatic increase of cell migration (252%) compared with the cells transfected with LipofectAMINE Plus alone (100%) or SSyk-transfected cells (98%) (Fig. 3*A*). Similarly, in the highly invasive MDA-MB-231 cells, when transfected with wild type Syk cDNA, there was drastic reduction of cell migration (24%) compared with cells transfected with LipofectAMINE Plus alone (100%) or SykK$^-$-transfected cells (97%) (Fig. 3*B*). These data suggest that wild type Syk suppressed cell migration, whereas SykK$^-$ had no effect on suppression of cell migration. Pretreatment of MCF-7 cells with increasing concentrations of piceatannol (0–20 μM), a Syk inhibitor followed by migration assays, enhanced cell migration (100–258%) in a dose-dependent manner (Fig. 3*C*). To prove further the role of piceatannol on migration of wild type Syk-transfected MDA-MB-231 cells, the transfected cells were treated with different doses of piceatannol (0–10 μM), and then the migration assay was conducted. The results indicated that there was enhancement of cell migration (37–98%) when the cells were transfected with wild type Syk followed by treatment with increasing concentrations of piceatannol compared with piceatannol-untreated, wild type Syk-transfected (25%) cells (Fig. 3*D*). The SykK$^-$-transfected cells showed 98% migration, whereas the number of cells that migrated using LipofectAMINE Plus-transfected cells were considered as 100% (Fig. 3*D*). The SykK$^-$-transfected cells had no effect on suppression of cell migration in MDA-MB-231 cells. We also stud-

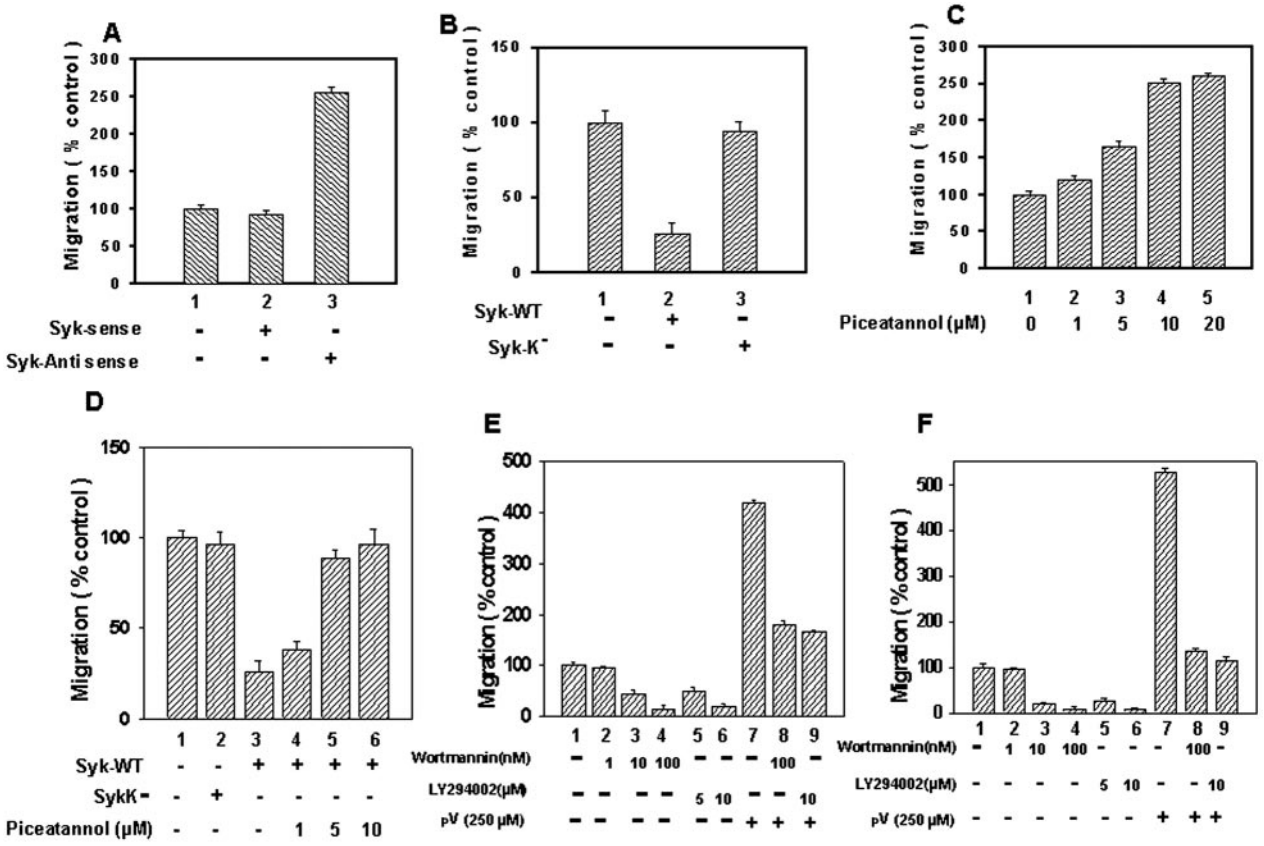

FIG. 3. *A*, effect of transfection of MCF-7 cells with Syk-specific S-oligonucleotides on cell migration. The cells were transiently transfected with SSyk or ASSyk using LipofectAMINE Plus. The transfected cells (5×10^5 cells/well) were used for cell migration assay as described under "Experimental Procedures." The cells transfected with ASSyk showed a dramatic increase of cell migration compared with cells transfected with SSyk or LipofectAMINE Plus alone. *B*, effect of transfection of MDA-MB-231 cells with wild type or SykK$^-$ cDNA on cell migration. The cells were transfected with wild type or SykK$^-$ cDNA, and the transfected cells were used for the cell migration assay. The cells transfected with wild type Syk cDNA showed a drastic reduction of cell migration compared with cells transfected with SykK$^-$ or with LipofectAMINE Plus alone. *C*, effect of piceatannol, a Syk inhibitor, on migration of MCF-7 cells. The cells were pretreated with 0–20 μM piceatannol at 37 °C for 30 min and then used for migration assay. Piceatannol enhanced the migration in a dose-dependent manner. *D*, effect of piceatannol on migration of Syk cDNA transfected MDA-MB-231 cells. The cells transfected with wild type Syk cDNA were treated with increasing concentrations (0–10 μM) of piceatannol and used for the migration assay as described above. Wild type Syk cDNA-transfected cells treated with increasing concentrations of piceatannol showed enhancement of cell migration compared with nontreated, wild type Syk cDNA-transfected cells. Cells transfected with SykK$^-$ or with LipofectAMINE Plus alone showed maximum migration. *E*, effect of pV and a PI 3′-kinase inhibitor (wortmannin or LY294002) on migration of MCF-7 cells. MCF-7 cells were treated individually with pV, wortmannin, LY294002, or with a combination of pV and wortmannin or pV and LY294002 and used for migration assay as described under "Experimental Procedures." Wortmannin and LY294002 independently reduced the migration in a dose-dependent manner, whereas pV induced the migration. pV-induced migration was also blocked by wortmannin or LY294002. *F*, effect of pV and PI 3′-kinase inhibitor (wortmannin or LY294002) on migration of MDA-MB-231 cells. MDA-MB-231 cells were treated individually with pV, wortmannin, and LY294002 under different conditions as described above and used for migration assay. Wortmannin and LY294002 reduced migration, whereas pV induced it. The pV-induced migration was also inhibited by wortmannin or LY294002. In all of these experiments, the results are expressed as the means ± S.E. of three determinations.

ied the effects of wortmannin and LY294002 (PI 3′-kinase inhibitors) on the migration of MCF-7 and MDA-MB-231 cells. Both MCF-7 and MDA-MB-231 cell lines were treated individually with different doses of wortmannin (0–100 nM) or LY294002 (0–10 μM) as described earlier and used for cell migration assays. The results indicated that both of these inhibitors independently suppressed the cell migration of MCF-7 (Fig. 3*E*) and MDA-MB-231 (Fig. 3*F*) cells in a dose-dependent manner. These data further suggested that PI 3′-kinase plays significant roles in regulating cell migration in these cells. To investigate whether pV, a tyrosine phosphatase inhibitor, regulates PI 3′-kinase dependent cell migration, both cell lines were treated with 250 μM pV in the absence or presence of 0–100 nM wortmannin or 0–10 μM LY294002 and used for the migration assay. The data demonstrated that pV induced migration in both cell lines. However, pV-induced migration is suppressed by wortmannin or LY294002 (PI 3′-kinase inhibitors) in these cells (Fig. 3, *E* and *F*).

Syk Down-regulates PI 3′-Kinase Activity in MCF-7 (Low Invasive) and MDA-MB-231 (Highly Invasive) Cells—To check the role of Syk in suppression of PI 3′-kinase activity in MCF-7 and MDA-MB-231 cells, a PI 3′-kinase assay was performed under different conditions. These cells were lysed individually in lysis buffer, and the lysates containing equal amounts of total proteins were immunoprecipitated with anti-p85α antibody. The immunoprecipitated samples were used for the kinase assay. The radioactive PIP was separated by TLC and visualized by autoradiography. The activity of PI 3′-kinase was higher in MDA-MB-231 cells (Fig. 4*A*, *lane 2*) compared with MCF-7 cells (*lane 1*). The MCF-7 cells were transfected with Syk-specific S-oligonucleotides, immunoprecipitated with anti-p85α antibody, and used for the PI 3′-kinase assay. The MCF-7 cells transfected with ASSyk showed a higher level of PI 3′-kinase activity (Fig. 4*B*, *lane 3*) compared with LipofectAMINE Plus alone (*lane 1*) or SSyk-transfected (*lane 2*) cells. These data suggested that Syk suppressed PI 3′-kinase activity in MCF-7 cells. Moreover, our previous data indicated that MCF-7 cells transfected with ASSyk showed enhancement of

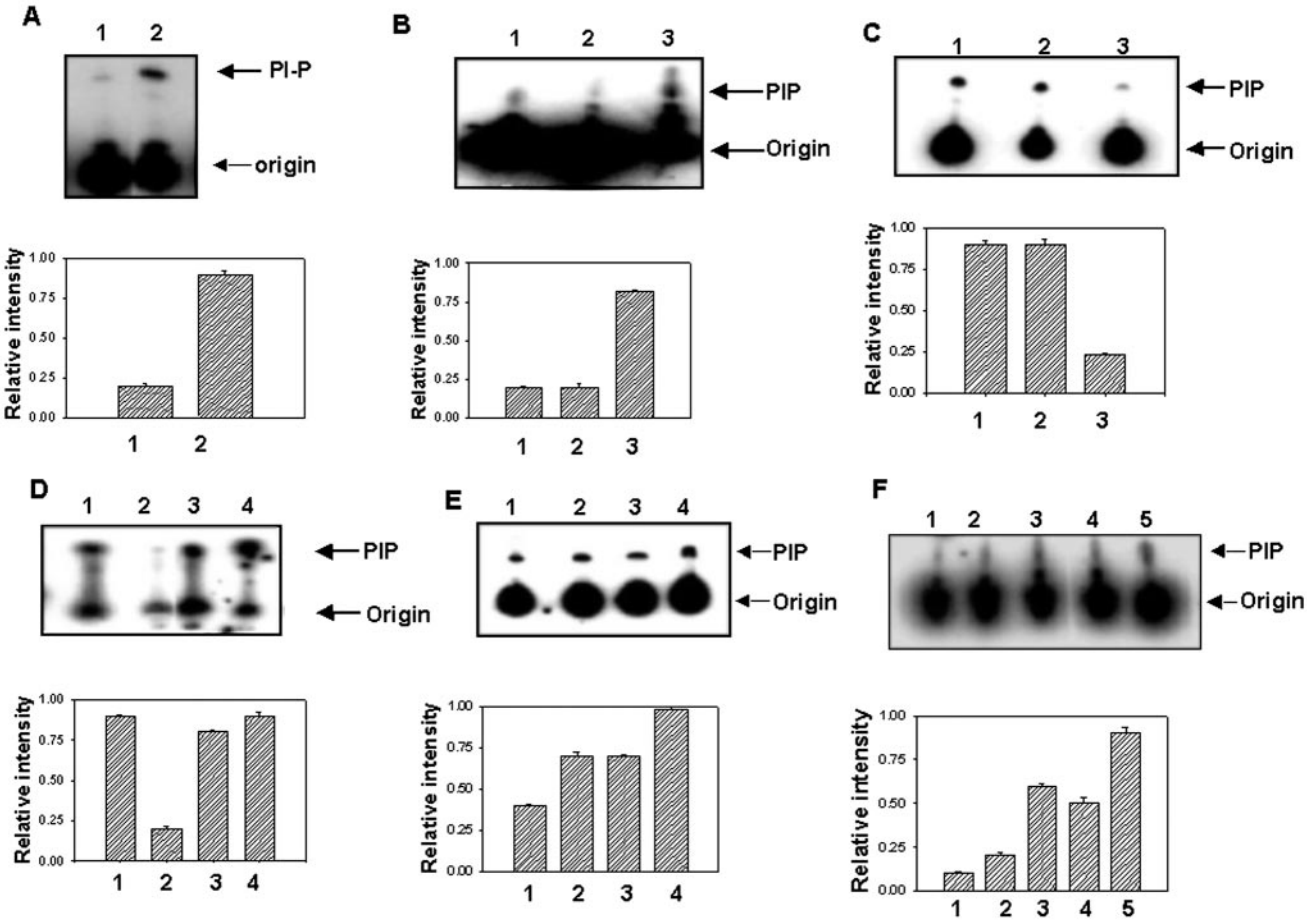

FIG. 4. **PI 3′-kinase activity.** *A*, equal amounts of total proteins from the lysates of MCF-7 and MDA-MB-231 cells were immunoprecipitated with mouse monoclonal anti-p85α antibody, and the immunocomplexes were assayed for their ability to phosphorylate PI to PIP using [γ-^{32}p]ATP at 30 °C for 10 min. The PIP was resolved by TLC and autoradiographed. *Lane 1*, MCF-7 cells; *lane 2*, MDA-MB-231 cells. *B*, the serum-starved MCF-7 cells were transfected with Syk-specific S-oligonucleotides, and then PI 3′-kinase activity was measured as described under "Experimental Procedures." *Lane 1*, with LipofectAMINE Plus; *lane 2*, with SSyk; *lane 3*, with ASSyk. *C*, MDA-MB-231 cells were transfected with Syk cDNA and used for PI 3′-kinase assay. *Lane 1*, with LipofectAMINE Plus; *lane 2*, with SykK$^-$; and *lane 3*, with wild type Syk. *D*, MDA-MB-231 cells were transfected with wild type Syk cDNA followed by treatment with increasing concentrations (0–10 μM) of piceatannol at 37 °C for 30 min and used for PI 3′-kinase assay. *Lane 1*, with LipofectAMINE Plus alone; *lanes 2–4*, cells were transfected with wild type Syk cDNA and then treated with increasing concentrations of piceatannol. *Lane 2*, without piceatannol; *lane 3*, with 5 μM piceatannol; *lane 4*, with 10 μM piceatannol. *E*, MDA-MB-231 cells were treated with 250 μM pV at room temperature for 0–30 min, and the cell lysates were used for the PI 3′-kinase assay. *Lane 1*, untreated cells; *lane 2*, with pV for 5 min; *lane 3*, with pV for 15 min; *lane 4*, with pV for 30 min. *F*, MCF-7 cells were treated with 0–10 μM piceatannol for 30 min or with 250 μM pV for 0–30 min and used for the PI 3′-kinase assay. *Lane 1*, untreated cells; *lane 2*, with 5 μM piceatannol; *lane 3*, with 10 μM piceatannol; *lane 4*, with pV for 15 min; *lane 5*, with pV for 30 min. In all the cases, the *upper arrows* indicate the PIP-specific bands. All of these bands in *A–F* were quantified by densitometric analysis and are represented in the form of a *bar graph*. The mean value of triplicate experiments is indicated.

cell migration compared with SSyk-transfected cells. These data further demonstrated that Syk suppressed the cell migration by inhibiting the PI 3′-kinase activity in MCF-7 cells (Figs. 3*A* and 4*B*).

The MDA-MB-231 cells were transfected with SykK$^-$ or wild type Syk cDNA, cell lysates were immunoprecipitated with anti-p85α antibody, and the kinase assay was performed. The cells transfected with wild type Syk cDNA reduced the PI 3′-kinase activity (Fig. 4*C*, *lane 3*) compared with LipofectAMINE Plus-transfected cells (*lane 1*) or cells transfected with SykK$^-$ (*lane 2*). Because Syk suppressed the PI 3′-kinase activity, we sought to determine whether piceatannol, a Syk inhibitor, reversed the PI 3′-kinase activity. Accordingly, MDA-MB-231 cells were transfected with wild type Syk cDNA, treated with different doses of piceatannol (0–10 μM), and then the kinase assay was conducted. The results demonstrated that piceatannol dose-dependently increased the PI 3′-kinase activity in these cells (Fig. 4*D*, *lanes 3* and *4*) compared with untreated, wild type Syk-transfected cells (*lane 2*). The cells transfected with LipofectAMINE Plus alone showed a higher level of PI 3′-kinase activity (*lane 1*). To check whether pV, a tyrosine phosphatase inhibitor, regulates PI 3′-kinase activity, the MDA-MB-231 cells were treated with 250 μM pV for 0–30 min, and then PI 3′-kinase activity was measured. The results indicated that pV induces the PI 3′-kinase activity in a time-dependent manner in these cells (Fig. 4*E*, *lanes 1–4*). Similarly, MCF-7 cells were also treated with either piceatannol or pV and then used for the kinase assay. The data showed that both piceatannol (Fig. 4*F*, lanes *2* and *3*) and pV (*lanes 4* and *5*) individually enhanced PI 3′-kinase activity compared with untreated (*lane 1*) MCF-7 cells. These data strongly suggested that Syk down-regulates the PI 3′-kinase activity in both MCF-7 and MDA-MB-231 cells. All of these PIP-specific bands were quantified by densitometric analysis and are represented in the form of a *bar graph*. The mean value of triplicate experiments is indicated.

Syk Suppresses pV-induced Tyrosine Phosphorylation of IκBα and Subsequent Interaction between Tyrosine-phosphorylated IκBα and PI 3′-Kinase—To assess the role of pV on tyrosine phosphorylation of IκBα in breast cancer cells, both

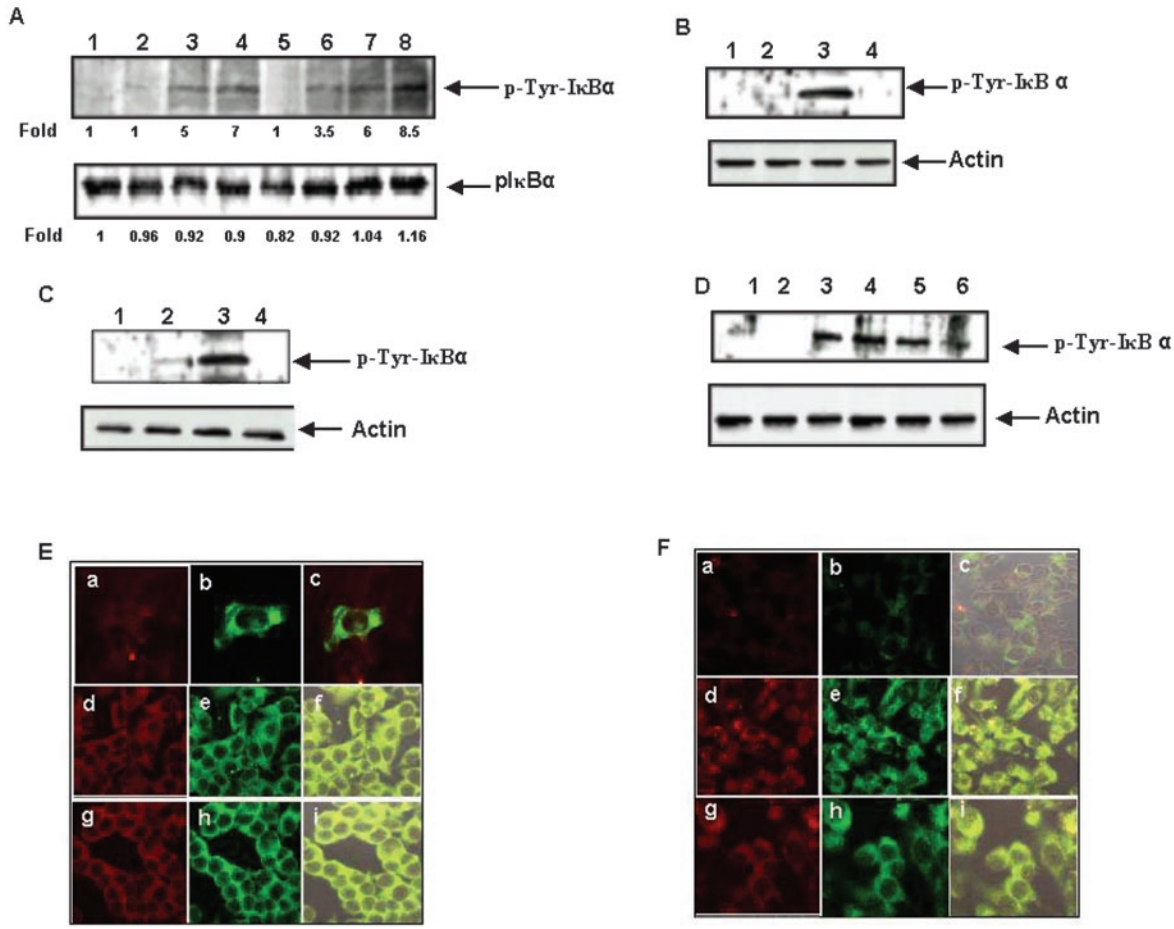

FIG. 5. *A*, role of pV in stimulation of interaction between IκBα and PI 3′-kinase through tyrosine phosphorylation of IκBα. Both MCF-7 and MDA-MB-231 cells were individually treated with pV for 0–30 min, and the cell lysates containing equal amounts of total proteins were immunoprecipitated with nonphosphorylated anti-IκBα antibody. Half of the immunoprecipitated samples were resolved by SDS-PAGE and analyzed by Western blot using anti-phosphotyrosine antibody (*upper panel* in *A*), and the remaining half of the samples were immunoblotted with phosphoserine-specific anti-IκBα antibody (*lower panel* in *A*). *Lanes 1–4*, MCF-7 cells. *Lane 1*, control; *lane 2*, with pV for 5 min; *lane 3*, with pV for 15 min; *lane 4*, with pV for 30 min. *Lanes 5–8*, MDA-MB-231 cells. *Lane 5*, control; *lane 6*, with pV for 5 min; *lane 7*, with pV for 15 min; *lane 8*, with pV for 30 min. The *arrow* in the *upper panel* of *A* indicates a tyrosine-phosphorylated IκBα-specific band, and in the *lower panel* of *A* the *arrow* shows a serine-phosphorylated IκBα-specific band. Note that the level of tyrosine phosphorylation of IκBα is increased in presence of pV (*upper panel* of *A*), but pV has no effect on serine phosphorylation of IκBα (*lower panel* of *A*) in both cell lines, indicating that pV induces tyrosine phosphorylation of IκBα. More tyrosine phosphorylation is observed in MDA-MB-231 cells. All of these bands were quantified by densitometric analysis, and the values of -fold changes are indicated. MCF-7 cells (*B*) and MDA-MB-231 cells (*C*) cells were treated individually with 250 μM pV at room temperature for a period of 0–30 min. In a separate experiment, the cells were also pretreated with ST 638, a protein-tyrosine kinase inhibitor (400 nM) and then treated with 250 μM pV. The cell lysates were immunoprecipitated with monoclonal anti-p85α antibody. The immunocomplexes were resolved by SDS-PAGE and analyzed by Western blot using anti-phosphotyrosine antibody. *Lane 1*, control; *lane 2*, with pV for 15 min; *lane 3*, with pV for 30 min; *lane 4*, with ST 638 and then with pV for 30 min. The *arrows* indicate the tyrosine-phosphorylated IκBα-specific band. As loading controls, both of these blots were reprobed with anti-actin antibody (*lower panels* in *B* and *C*). *D*, effect of Syk-specific S-oligonucleotides on suppression of pV-induced tyrosine phosphorylation of IκBα and subsequent interaction between tyrosine-phosphorylated IκBα and PI 3′-kinase. MCF-7 cells were transfected with Syk-specific S-oligonucleotides, and MDA-MB-231 cells were transfected with Syk cDNA in presence of LipofectAMINE Plus. Both of these transfected cell lines were treated individually with 250 μM pV and lysed in lysis buffer. The lysates were immunoprecipitated with anti-p85α antibody. The immunoprecipitated samples were separated by SDS-PAGE and analyzed by Western blot using anti-phosphotyrosine antibody. *Lane 1*, with LipofectAMINE Plus alone; *lane 2*, with SSyk; *lane 3*, with ASSyk (*upper panel* of *D*, *lanes 1–3*, MCF-7 cells); *lane 4*, with LipofectAMINE Plus alone; *lane 5*, with SykK$^-$; *lane 6*, with wild type Syk (*upper panel* of *D*, *lanes 4–6*, MDA-MB-231 cells). Note that MCF-7 cells transfected with ASSyk induced, but MDA-MB-231 cells transfected with wild type Syk reduced, the tyrosine phosphorylation of IκBα. As loading controls, the same blots were reprobed with anti-actin antibody (*lower panel* of *D*). *E* and *F*, role of pV in regulation of colocalization of IκBα and PI 3′-kinase. MCF-7 (*E*) and MDA-MB-231 (*F*) cells were treated with 250 μM pV at room temperature for 0–30 min, fixed, and incubated with a mixture of monoclonal anti-PI 3′-kinase, p85α, and rabbit polyclonal anti-IκBα antibodies. These cells were incubated further with a mixture of FITC-conjugated anti-rabbit IgG and TRITC-conjugated anti-mouse IgG antibodies and analyzed under confocal microscopy. *a–c*, untreated cells; *d–f*, pV for 15 min and *g–i*, pV for 30 min. *a*, *d*, and *g* are stained with anti-PI 3′-kinase, p85α, and TRITC-conjugated antibodies, respectively; *b*, *e*, and *h* are stained with anti-IκBα and FITC-conjugated antibodies, respectively; and *c*, *f*, and *i* are overlapping forms of TRITC and FITC stained cells. Note that there was more colocalization of IκBα and PI 3′-kinase, p85α in pV-treated cells (*d–i*) compared with untreated cells (*a–c*), but higher levels of colocalization were seen in MDA-MB-231 cells.

MCF-7 and MDA-MB-231 cells were treated individually with 250 μM pV for 0–30 min and immunoprecipitated with anti-IκBα antibody. Half of the immunoprecipitated samples were separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody. The remaining half of the samples was analyzed by Western blot analysis using anti-IκBα (phosphoserine-specific) antibody. The data demonstrated that pV induces the tyrosine phosphorylation of IκBα in both MCF-7 (Fig. 5*A*, *upper panel*, *lanes 1–4*) and MDA-MB-231 (*lanes 5–8*) cells, but the pV-induced tyrosine phosphorylation was higher in MDA-MB-231 cells. In contrast, the serine phosphorylation of IκBα was unchanged in both cell lines (Fig. 5*A*, *lower panel*, *lanes 1–8*), suggesting that pV regulates the tyrosine phosphorylation but not serine phosphorylation of IκBα in these cells.

The bands were analyzed by densitometry, and the values of -fold changes are indicated (Fig. 5*A*).

To confirm further the effects of pV on tyrosine phosphorylation of IκBα and regulation of interaction between phosphorylated IκBα and the p85α subunit of PI 3′-kinase, both cell lines were treated individually with pV under the same conditions described above. The cell lysates were immunoprecipitated with anti-p85α antibody and immunoblotted with anti-phosphotyrosine antibody. In separate experiments, cells were pretreated with ST 638, a protein-tyrosine kinase inhibitor, then treated with pV, immunoprecipitated with anti-p85α antibody, and detected by immunoblotting using anti-phosphotyrosine antibody. The data indicate that pV induces the interaction between IκBα and p85α subunit of PI 3′-kinase through tyrosine phosphorylation of IκBα in MCF-7 (Fig. 5*B*, *upper panel*, *lanes 1–3*) and MDA-MB-231 (Fig. 5*C*, *upper panel*, *lanes 1–3*) cells, whereas ST 638 suppresses pV-induced tyrosine phosphorylation in both cell lines (Fig. 5, *B* and *C*, *upper panel*, *lane 4*). As loading controls, both blots were reprobed with anti-actin antibody (Fig. 5, *B* and *C*, *lower panels*).

To delineate the role of Syk on pV-induced tyrosine phosphorylation of IκBα and subsequent interaction between tyrosine-phosphorylated IκBα and the p85α subunit of PI 3′-kinase, MCF-7 cells were transfected with Syk-specific S-oligonucleotides, and MDA-MB-231 cells were transfected with wild type or SykK$^-$ cDNA. Both cell lines were treated individually with 250 μM pV and lysed in lysis buffer. The cell lysates containing equal amounts of total proteins were immunoprecipitated with anti-p85α antibody and detected by Western blot analysis using anti-phosphotyrosine antibody. MCF-7 cells transfected with ASSyk showed a higher level of tyrosine phosphorylation of IκBα (Fig. 5*D*, *upper panel*, *lane 3*), but this phosphorylation was not detected in SSyk-transfected cells (*lane 2*) or the cells transfected with LipofectAMINE Plus alone (*lane 1*). Similarly, MDA-MB-231 cells transfected with wild type Syk cDNA showed a reduction of tyrosine phosphorylation of IκBα (*lane 6*) compared with SykK$^-$-transfected cells (*lane 5*) or cells transfected with LipofectAMINE Plus alone (*lane 4*). As loading controls, the same blots were reprobed with anti-actin antibody (*lower panel*). These data suggest that Syk suppresses the tyrosine phosphorylation of IκBα and subsequent interaction between IκBα and PI 3′-kinase in both cell lines.

To determine whether pV induces the colocalization of IκBα with the p85 subunit of PI 3′-kinase, both cell lines were treated individually in absence or presence of 250 μM pV, fixed, and incubated with a mixture of anti-IκBα and anti-p85α antibodies. These cells were incubated again with a mixture of anti-rabbit FITC- and anti-mouse TRITC-conjugated IgG. The immunofluorescence-labeled cells were detected by confocal microscopy. The data indicated that pV enhances the colocalization of IκBα and PI 3′-kinase in both cell lines (Fig. 5, *E* and *F*, *d–i*) compared with untreated cells (*a--c*), but a higher level of colocalization was observed in MDA-MB-231 cells (Fig. 5*F*).

Syk Inhibits Transcriptional Activity of NFκB—Because Syk suppressed the tyrosine phosphorylation of IκBα, we sought to determine whether Syk has any role in the transactivation of NFκB in breast cancer cells. Accordingly, MCF-7 cells were transfected with Syk-specific S-oligonucleotides and luciferase reporter construct pNFκB-Luc. Similarly, MDA-MB-231 cells were transfected with wild type or SykK$^-$ cDNA followed by transfection with pNFκB-Luc. The transfection efficiency was normalized by cotransfecting 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. In separate experiments, these transfected cells were also treated individually with increasing concentrations of piceatannol. The data indicated that there was at least an 8-fold increase of NFκB luciferase activity in MCF-7 cells transfected with ASSyk compared with LipofectAMINE Plus or SSyk-transfected cells (Fig. 6*A*). In contrast, MDA-MB-231 cells transfected with wild type Syk showed a 10-fold decrease in NFκB luciferase activity compared with cell transfected with SykK$^-$ or LipofectAMINE Plus alone (Fig. 6*B*). Piceatannol enhanced the NFκB activity in both of these cell lines in a dose-dependent manner (Fig. 6, *A* and *B*). These data clearly demonstrated that Syk suppressed the transcriptional activity of NFκB in both cell lines.

To check the role of PI 3′-kinase inhibitors (wortmannin or LY294002) on pV-induced NFκB activity, both cell lines were transfected individually with pNFκB-Luc and pRL in the presence of LipofectAMINE Plus. These transfected cells were then treated with pV alone, pV with wortmannin, or pV with LY294002. The data indicated that pV up-regulates the NFκB activity, but PI 3′-kinase inhibitors (wortmannin and LY294002) blocked the pV-induced NFκB activity in both cell lines (Fig. 6, *C* and *D*).

PI 3′-Kinase and NFκB Play Crucial Roles in uPA Secretion and Cell Migration—To delineate whether PI 3′-kinase plays any role in uPA secretion, both MCF-7 and MDA-MB-231 cells were treated individually with 250 μM pV alone or pV with 100 nM wortmannin, or pV with 10 μM LY294002. The cells were lysed, and the lysates containing equal amounts of total proteins were resolved by SDS-PAGE and analyzed by Western blot analysis using anti-uPA antibody. The data indicated that MCF-7 cells treated with pV showed a higher level of uPA secretion (Fig. 7*A*, *lane 2*) compared with untreated cells (*lane 1*), whereas wortmannin (*lane 3*) and LY294002 (*lane 4*) separately inhibited the pV-induced uPA secretion in these cells. Similarly, pV also induced uPA secretion (*lane 6*) compared with control (*lane 5*) in MDA-MB-231 cells, whereas both wortmannin (*lane 7*) and LY294002 (*lane 8*) blocked the pV-induced uPA secretion in these cells.

We have also examined the effects of NFκB modulators and other agents on uPA secretion upon treating these cells (MCF-7 and MDA-MB-231) with SN-50, SN-50M, actinomycin-D, curcumin, and PMA. These treated cell lysates containing equal amounts of total proteins were separated by SDS-PAGE and immunoblotted with anti-uPA antibody. The level of uPA expression was reduced significantly when MDA-MB-231 cells were treated individually with SN-50 (NFκB inhibitory peptide) (Fig. 7*C*, *lane 3*), actinomycin-D (protein synthesis inhibitor) (*lane 4*), and curcumin (*lane 5*) compared with untreated cells (*lane 1*). No changes of uPA secretion were observed in cells treated with SN-50M (NFκB control peptide) (*lane 2*). As expected, PMA induces uPA secretion in these cells (*lane 6*). Similar results were obtained in MCF-7 cells (Fig. 7*B*, *lanes 1–6*). The constitutive expression of uPA was much higher in MDA-MB-231 cells compared with MCF-7 cells (Fig. 7, *B* and *C*, *lane 1*). In all of these experiments, the uPA-specific bands were quantified by densitometric analysis, and the values of -fold changes are indicated.

Because the NFκB-responsive element is present in the promoter region of uPA, we sought to determine whether NFκB-regulated uPA expression has any role in the migration of breast cancer cells. Accordingly, both cell lines were pretreated individually with SN-50, SN-50M, actinomycin-D, curcumin, and PMA and used for the migration assay. The results indicated that SN-50, actinomycin-D, and curcumin separately inhibited the cell migration in both MCF-7 (Fig. 7*D*) and MDA-MB-231 (Fig. 7*E*) cells compared with untreated cells. The cells treated with SN-50M had no effect on suppression of cell migration. As expected, PMA induced cell migration in both cell

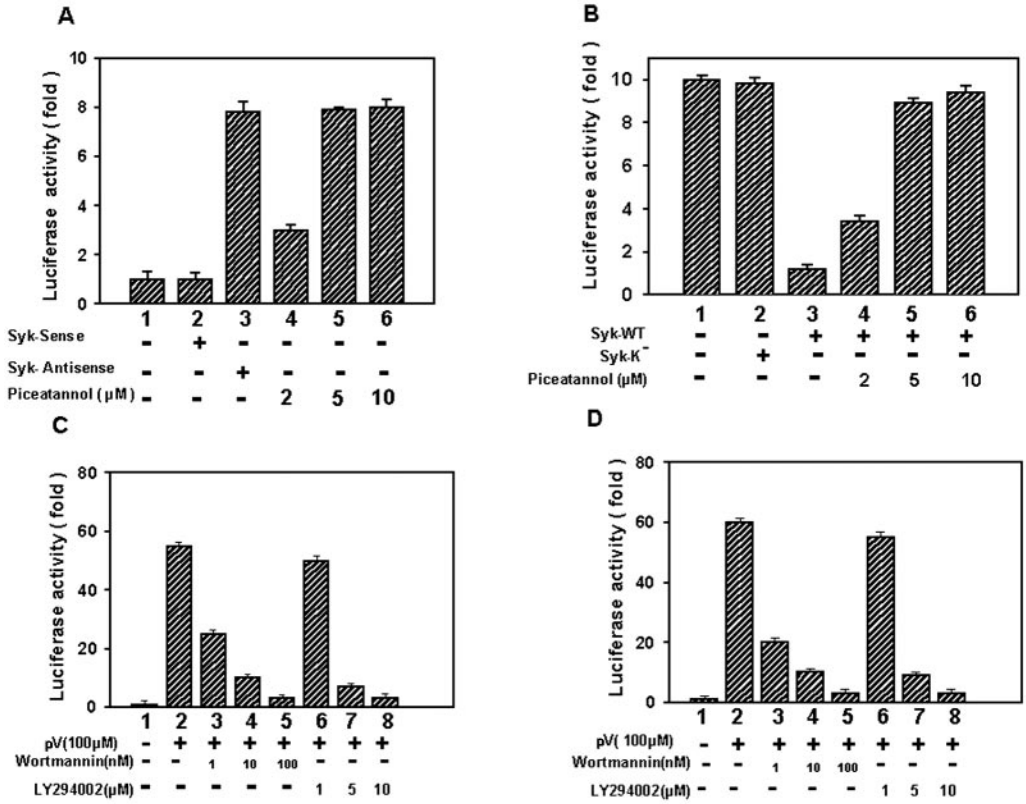

FIG. 6. *A* and *B*, effect of Syk on NFκB transactivation in MCF-7 (*A*) and MDA-MB-231 (*B*) cells. *A*, MCF-7 cells were transiently transfected with Syk-specific S-oligonucleotides and luciferase reporter construct (pNFκB-Luc) in the presence of LipofectAMINE Plus as described under "Experimental Procedures." The transfection efficiency was normalized by cotransfecting the cells with pRL vector. The non-S-oligonucleotide-transfected cells were also treated with varying concentrations (0–10 μM) of piceatannol. These cells were harvested, and luciferase activity was measured. The cells transfected with ASSyk showed a dramatic increase of NFκB luciferase activity compared with SSyk-transfected cells or cells transfected with LipofectAMINE Plus alone. Piceatannol enhanced the luciferase activity in a dose-dependent manner. *B*, MDA-MB-231 cells were transfected with Syk cDNA and pNFκB-Luc using LipofectAMINE Plus as described under "Experimental Procedures." The wild type Syk cDNA-transfected cells were also treated with varying concentrations of piceatannol. MDA-MB-231 cells transfected with wild type Syk drastically suppressed the luciferase activity compared with SykK⁻-transfected cells or cells transfected with LipofectAMINE Plus alone. Piceatannol enhanced the luciferase activity in wild type Syk-transfected cells. *C* and *D*, effects of pV and PI 3′-kinase inhibitors on NFκB transactivation in MCF-7 (*C*) and MDA-MB-231 (*D*) cells. Both MCF-7 and MDA-MB-231 cells were transfected individually with pNFκB-Luc. These cells were then treated with pV alone, pV with 1–100 nM wortmannin or 1–10 μM LY294002, and luciferase activity was measured. In both of these cell lines, pV enhanced the luciferase activity, but pV-induced luciferase activity was blocked by wortmannin or LY294002 in a dose-dependent manner. The values were normalized to *Renilla* luciferase activity. The -fold changes were calculated, and the results are expressed as the means ± S.E. of three determination.

lines (Fig. 7, *D* and *E*). These and previous data suggested that uPA secretion and cell migration are regulated by PI 3′-kinase and NFκB (30).

Syk Suppresses PI 3′-Kinase-mediated uPA Secretion and Cell Migration—To ascertain whether Syk regulates PI 3′-kinase-dependent uPA secretion, MCF-7 cells were either transfected with Syk-specific S-oligonucleotides or treated with piceatannol. These cells were used for the detection of uPA by Western blot analysis using anti-uPA antibody. The cells transfected with ASSyk showed a higher level of uPA expression (Fig. 8*A*, *lane 3*) compared with Syk-transfected cells (*lane 2*) or cells transfected with LipofectAMINE Plus alone (*lane 1*). The level of uPA secretion was increased dose-dependently when the cells were treated with increasing concentrations of piceatannol (Fig. 8*B*, *lanes 2–4*) and decreased when the cells were incubated with increasing doses of wortmannin (Fig. 8*C*, *lanes 2–4*) or LY294002 (data not shown). The very low level of constitutive uPA expression was observed in untreated cells (Fig. 8, *B* and *C*, *lane 1*). The MDA-MB-231 cells transfected with wild type Syk showed significant reduction of uPA secretion (Fig. 8*E*, *lane 2*) compared with SykK⁻-transfected cells (*lane 3*) or cells transfected with LipofectAMINE Plus alone (*lane 1*). Wortmannin inhibited the expression of uPA in these cells in a dose-dependent manner (Fig. 8*F*, *lanes 1–4*). The constitutive expression of uPA was much higher in highly invasive MDA-MB-231 cells rather than low invasive MCF-7 cells (Fig. 8).

To delineate the effect of Syk on uPA-mediated cell migration, the MCF-7 cells were either treated with anti-uPA antibody or transfected with Syk-specific S-oligonucleotides and then treated with anti-uPA antibody. The data showed that the cell migration was reduced drastically when the cells were treated with anti-uPA antibody (47%). The cells transfected with ASSyk showed enhancement of cell migration (252%) compared with SSyk-transfected cells or cells transfected with LipofectAMINE Plus alone, whereas the ASSyk-transfected cells, when treated with anti-uPA antibody, showed moderate cell migration (153%) (Fig. 8*D*).

Similarly, MDA-MB-231 cells transfected with wild type Syk cDNA followed by treatment with anti-uPA antibody reduced the migration dramatically (22%) compared with wild type Syk-transfected cells (24%) or cells treated with anti-uPA antibody (47%) (Fig. 8*G*). The cells transfected with LipofectAMINE Plus alone or SykK⁻ showed maximum migration (100%). These data demonstrated that Syk suppresses the PI 3′-kinase-dependent uPA secretion and uPA-mediated cell migration in both MCF-7 and MDA-MB-231 cells.

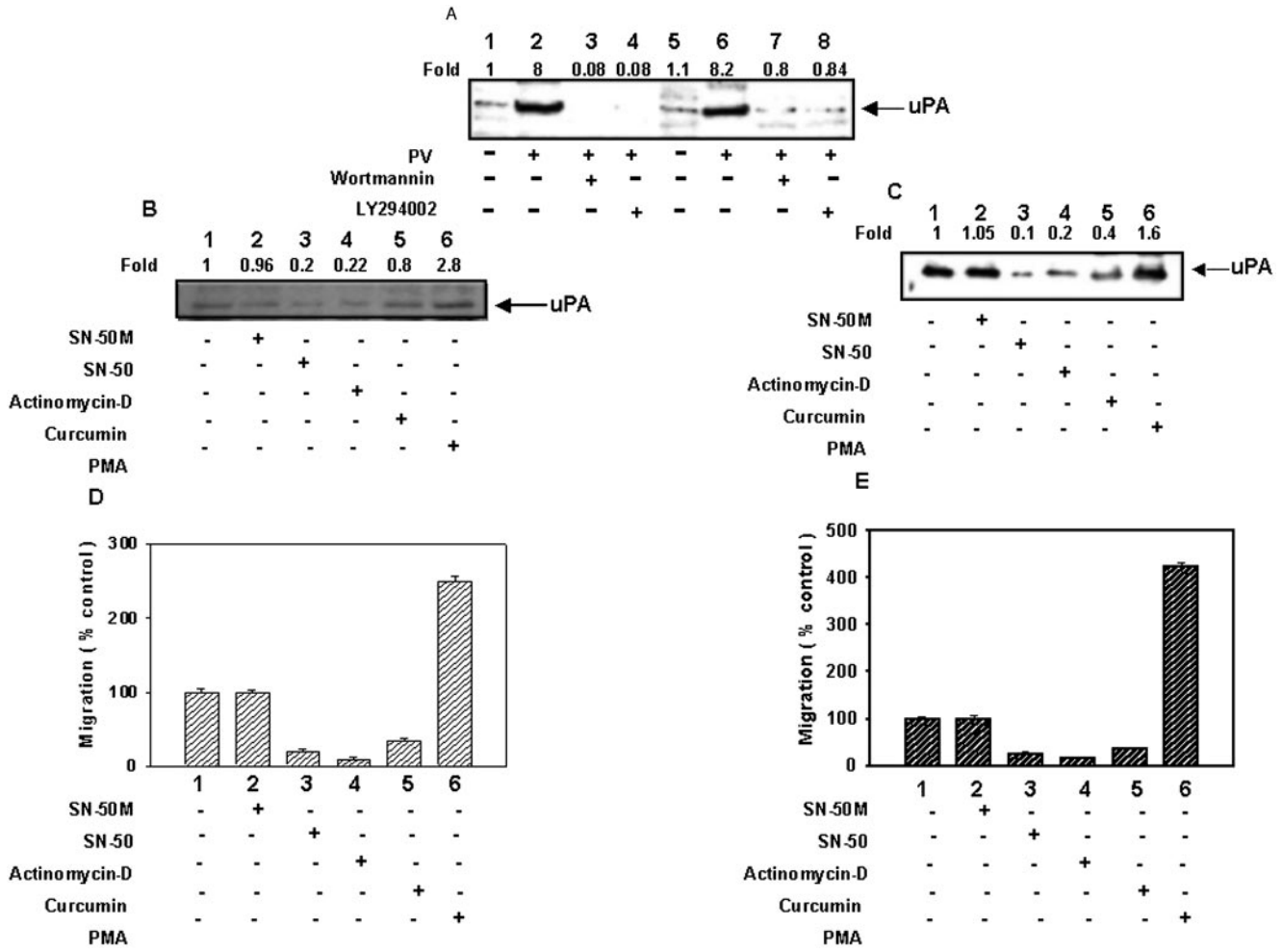

FIG. 7. *A*, effects of pV and PI 3′-kinase inhibitors on uPA secretion. MCF-7 and MDA-MB-231 cells were either treated with 250 μM pV alone, pV with 100 nM wortmannin or 10 μM LY294002, and lysed. The level of uPA in the lysates was detected by Western blot analysis using anti-uPA antibody. *Lanes 1–4*, MCF-7 cells. *Lane 1*, untreated cells; *lane 2*, with pV; *lane 3*, with wortmannin and pV; *lane 4*, with LY294002 and pV. *Lanes 5–8*, MDA-MB-231 cells. *Lane 5*, untreated cells; *lane 6*, with pV; *lane 7*, with wortmannin and pV; and *lane 8*, with LY294002 and pV. The *arrow* indicates the uPA-specific band. *B* and *C*, effects of NFκB modulators and other agents on uPA secretion. *B*, MCF-7 cells were treated individually with 100 μg/ml SN-50, 100 μg/ml SN-50M, 10 μg/ml actinomycin-D, 50 μM curcumin, and 5 ng/ml PMA. The cells were lysed, and uPA expression in the lysates was detected by Western blot analysis. *Lane 1*, untreated cells; *lane 2*, with SN-50M; *lane 3*, with SN-50; *lane 4*, with actinomycin-D; *lane 5*, with curcumin; *lane 6*, with PMA. *C*, MDA-MB-231 cells were treated under the same conditions as those described above. *Lane 1*, untreated cells; *lane 2*, with SN-50M; *lane 3*, with SN-50; *lane 4*, with actinomycin-D; *lane 5*, with curcumin; *lane 6*, with PMA. The level of uPA was decreased significantly when both of these cell lines were pretreated with SN-50, actinomycin-D, or curcumin. As expected, PMA induced uPA expression. Note that the constitutive expression of uPA was much higher in MDA-MB-231 cells compared with MCF-7 cells. The level of uPA was quantified by densitometric analysis and is represented as -fold changes (*A–C*). *D* and *E*, effects of NFκB modulators and other agents on cell migration. Both MCF-7 (*D*) and MDA-MB-231 (*E*) cells were pretreated individually with SN-50M, SN-50, actinomycin-D, PMA, and curcumin under the same conditions as described above. These treated cells were used for the migration assay as described under "Experimental Procedures." The number of untreated cells migrated was considered as 100%. The cells pretreated with SN-50, actinomycin-D, or curcumin showed a dramatic reduction of cell migration compared with untreated cells or cells treated with SN-50M. As expected, PMA induced cell migration. The results are expressed as the means ± S.E. of three determinations.

DISCUSSION

In this study, we have demonstrated that Syk, a nonreceptor protein-tyrosine kinase, inhibited PI 3′-kinase activity and subsequently suppressed cell motility in both highly invasive (MDA-MB-231) and low invasive (MCF-7) breast cancer cells. Syk is expressed in MCF-7 cells, but its expression is not detectable in highly invasive MDA-MB-231 cells. The wild type Syk cDNA was transfected in MDA-MB-231 cells, and its expression and autophosphorylation activity in these cells were comparable with the endogenous Syk activity in MCF-7 cells (data not shown). Furthermore, we have demonstrated that Syk suppresses the pV-induced interaction of p85 subunit of PI 3′-kinase and tyrosine-phosphorylated IκBα. Syk reduced the NFκB luciferase activity and piceatannol, a Syk inhibitor, enhanced the NFκB activity in both MCF-7 and MDA-MB-231 cells. The inhibition of PI 3′-kinase activity down-regulates the NFκB transactivation as well as cell motility in these cells. Moreover, inhibition of PI 3′-kinase and NFκB activities by their specific inhibitors reduced uPA secretion and cell motility in these cells. Syk also inhibits the uPA secretion in both MCF-7 and MDA-MB-231 cells. These data demonstrated that Syk suppresses cell motility and down-regulates NFκB activity by inhibiting the PI 3′-kinase activity and uPA secretion in both MCF-7 and MDA-MB-231 cells.

ZAP-70, a nonreceptor protein-tyrosine kinase that shares the same tandem SH2 domains with Syk at the amino terminus, was not detected in the breast cancer cells (data not shown). Previous reports have indicated that pV, a phosphotyrosine phosphatase inhibitor, induces autophosphorylation of Syk in MCF-7 cells (13). However, our data revealed that pV had no effect on activation of Syk in MCF-7 cells. Earlier reports have shown that PI 3′-kinase signaling is required for

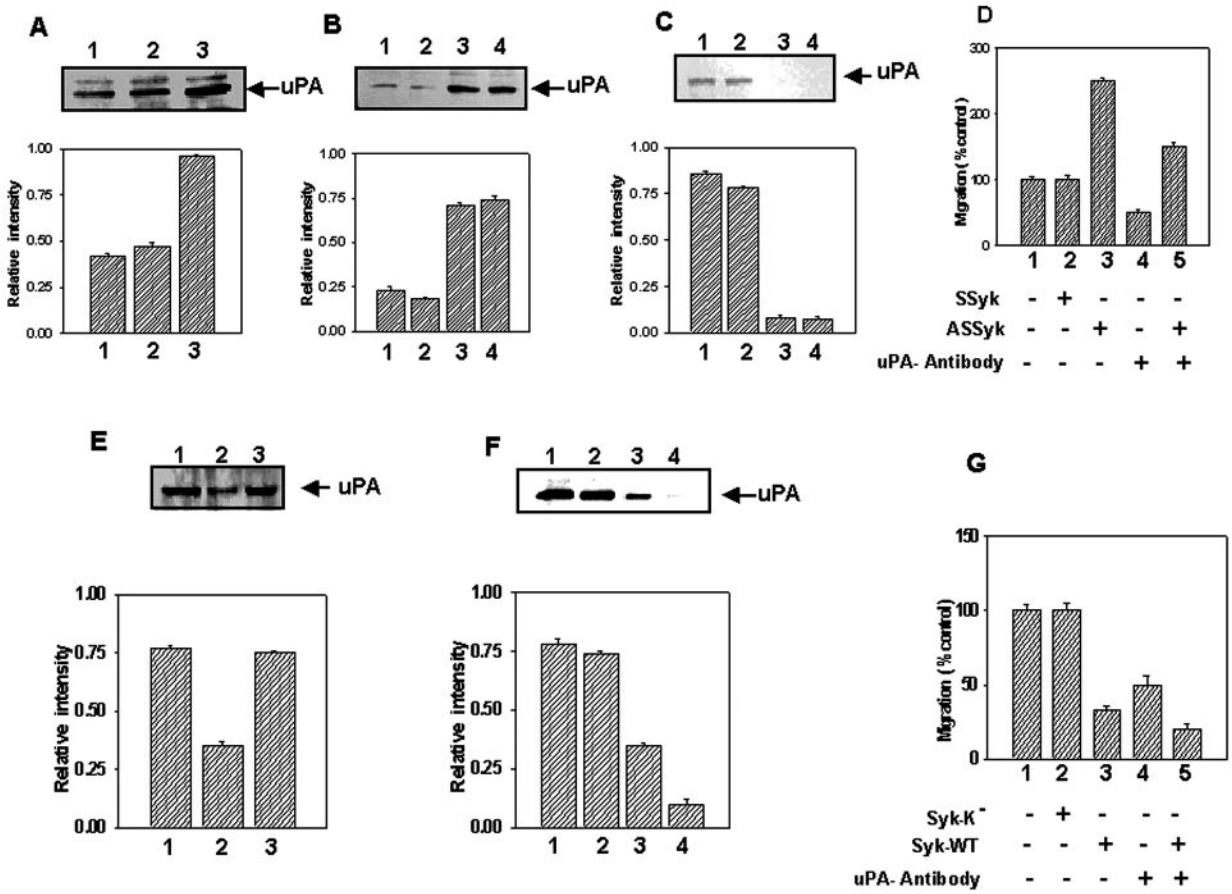

FIG. 8. **effects of Syk and PI 3′-kinase inhibitor on uPA secretion and cell migration.** *A*, MCF-7 cells were transfected with Syk-specific S-oligonucleotides, lysed, and the level of uPA was detected by Western blot analysis. *Lane 1*, with LipofectAMINE Plus alone; *lane 2*, with SSyk; *lane 3*, with ASSyk. *B*, MCF-7 cells were treated with 0–20 μM piceatannol, and uPA was detected by Western blot analysis. *Lane 1*, untreated cells; *lane 2*, with 1 μM piceatannol; *lane 3*: with 10 μM piceatannol; *lane 4*, with 20 μM piceatannol. *C*, MCF-7 cells were treated with 0–100 nM wortmannin, a PI 3′-kinase inhibitor, and the level of uPA was analyzed. *Lane 1*, untreated cells; *lane 2*, with 10 nM wortmannin; *lane 3*, with 50 nM wortmannin; *lane 4*, with 100 nM wortmannin. Note that ASSyk and piceatannol enhanced the uPA secretion, whereas wortmannin reduced the uPA secretion in these cells (*A–C*). *D*, MCF-7 cells were transfected with Syk-specific S-oligonucleotides as described above, treated with or without 10 μg/ml anti-uPA antibody, and used for migration assay. The cells transfected with ASSyk showed a dramatic increase in cell migration compared with cells transfected with SSyk or LipofectAMINE Plus alone. Pretreatment of nontransfected cells with anti-uPA antibody showed a drastic reduction of cell migration, whereas ASSyk-transfected cells, when treated with anti-uPA antibody, showed moderate inhibition of cell migration. The number of cells migrated in LipofectAMINE Plus-transfected cells was considered as 100%. The results are expressed as the means ± S.E. of three determinations. *E*, MDA-MB-231 cells were transfected with Syk cDNA, and expression of uPA was detected by Western blot analysis. *Lane 1*, with LipofectAMINE Plus; *lane 2*, with wild type Syk; *lane 3*, with SykK⁻. *F*, MDA-MB-231 cells were treated with increasing concentrations of wortmannin (0–100 nM), and the level of uPA was detected. *Lane 1*, untreated cells; *lane 2*, with 10 nM wortmannin; *lane 3*, with 50 nM wortmannin; *lane 4*, with 100 nM wortmannin. Wild type Syk and wortmannin suppressed the uPA secretion in these cells (*E* and *F*). Note that the constitutive expression of uPA was higher in MDA-MB-231 cells compared with MCF-7 cells. *G*, MDA-MB-231 cells were transfected with wild type or SykK⁻ cDNA, treated in the absence or presence of 10 μg/ml anti-uPA antibody, and used for the cell migration assay. The wild type Syk-transfected cells showed a dramatic reduction of cell migration compared with cells transfected with LipofectAMINE Plus alone or SykK⁻. The cells transfected with wild type Syk followed by treatment with anti-uPA antibody showed maximum inhibition of cell migration, whereas the nontransfected cells treated with anti-uPA antibody showed moderate inhibition of cell migration. The number of cells migrated in LipofectAMINE Plus-transfected cells was used as 100%. The results are expressed as the means ± S.E. of three determinations. In all Western blot experiments, the *arrows* indicate the uPA-specific band. The bands were quantified by densitometry and are represented in the form of a *bar graph*. The mean values of triplicate experiments are indicated in the *bar graph*.

depolarization and cell migration of MCF-7 cells by insulin-like growth factor I (31). The data also suggested that increased PI 3′-kinase activity is correlated with the migratory potential and metastatic activity of highly invasive breast cancer (MDA-MB-231) cells (30). Using genetic (wild type Syk and SykK⁻) and pharmacological (piceatannol) inhibitors of Syk, we have demonstrated that Syk is involved in the suppression of cell motility, PI 3′-kinase activity, and NFκB-mediated uPA secretion in both MCF-7 and MDA-MB-231 cells. The 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 (32, 33). Recently, Sliva *et al.* (30) 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.

It has been documented recently that PI 3′-kinase plays a significant role in NFκB activation in different cell types (34, 35). Tumor necrosis factor α-induced NFκB activation is not affected by PI 3′-kinase inhibitors (wortmannin and LY294002). Similarly, a PI 3′-kinase inhibitor has no effect on interleukin-1-dependent IκBα degradation, nuclear translocation of NFκB, and NFκB-DNA binding (22). Our results demonstrated that Syk down-regulates NFκB transactivation by inhibiting the interaction between the tyrosine-phosphorylated IκBα and the p85 subunit of PI 3′-kinase. Earlier reports have shown that pV and tumor necrosis factor α induced NFκB

activation in Jurkat cells, and only pV-induced activation of NFκB is inhibited by wortmannin (21). Both wortmannin and LY294002 abrogated the transactivation of NFκB but had no effect on NFκB-DNA binding in MDA-MB-231 cells (30). Our data also revealed that Syk and PI 3′-kinase inhibitors down-regulate the transactivation of NFκB but not NFκB-DNA binding (data not shown) in both MCF-7 and MDA-MB-231 cells. Thus we conclude that Syk-regulated transactivation of NFκB is independent of NFκB-DNA binding in breast cancer cells.

We have shown that MDA-MB-231 cells transfected with wild type Syk but not with SykK$^-$ suppressed the cellular migration and treatment of wild type Syk-transfected cells with increasing concentrations of piceatannol enhanced the cell migration in these cells. Both wortmannin and LY294002 inhibited, but pV induced the migration of MCF-7 and MDA-MB-231 cells. The pV-induced migration is blocked by wortmannin or LY294002 in these cells. Similarly, the NFκB inhibitory peptide SN-50, curcumin, and protein synthesis inhibitor (actinomycin-D) reduced cell migration. Pretreatment of non-transfected or Syk-transfected cells with anti-uPA antibody reduced the migration of these cells. These data suggested that Syk suppressed the cell migration by down-regulating the constitutively active NFκB activation and uPA secretion by inhibiting the PI 3′-kinase activity in breast cancer cells.

Our data also revealed that pV induces tyrosine phosphorylation of IκBα and subsequently enhances the interaction between tyrosine-phosphorylated IκBα with the p85α domain of PI 3′-kinase in a time-dependent manner in both cell lines. ST 638, a tyrosine kinase inhibitor, blocked the pV-induced tyrosine phosphorylation of IκBα. However, pV had no effect on serine phosphorylation of IκBα in these cells, suggesting that pV-induced transactivation of NFκB occurs through tyrosine phosphorylation of IκBα. Syk suppressed the tyrosine phosphorylation of IκBα and reduced the interaction between tyrosine-phosphorylated IκBα and p85 subunit of PI 3′-kinase. These data demonstrated that Syk regulates the transactivation of NFκB by inhibiting the direct interaction of tyrosine-phosphorylated IκBα and the p85 domain of PI 3′-kinase and suggested an alternative pathway not involving the phosphorylation and degradation of IκBα pathways.

Matrix metalloproteinases play a major role in the regulation of cancer cell migration, extracellular matrix invasion, and metastasis by degrading the extracellular matrix proteins (3, 4, 36). We and others have shown recently that NFκB plays significant roles in the activation of matrix metalloproteinases-1, -2, -3, and -9 (27, 37, 38). uPA is also responsible for the migration and regulation of matrix metalloproteinases activation through NFκB-mediated pathways (39). In this study, we have detected the level of uPA in both highly invasive (MDA-MB-231) and low invasive (MCF-7) breast cancer cells. The constitutive secretion of uPA is significantly higher in MDA-MB-231 cells; however, a low level of uPA expression is observed in MCF-7 cells. The data also indicate that Syk down-regulates whereas pV up-regulates the uPA production in these cells. The PI 3′-kinase inhibitors wortmannin and LY294002 or the NFκB inhibitor SN-50 reduced uPA secretion, especially in MDA-MB-231 cells.

In summary, we have demonstrated for the first time that overexpression of wild type Syk kinase but not the SykK$^-$ suppresses cell motility and reduces the activation of PI 3′-kinase in MDA-MB-231 cells. In contrast, in ASSyk but not SSyk, when transfected to the MCF-7 cells, the level of PI 3′-kinase activity as well as cell motility were increased. In the wild type Syk cDNA-transfected MDA-MB-231 cells, when treated with piceatannol, a Syk inhibitor, PI 3′-kinase activity increased. pV, a tyrosine phosphatase inhibitor, enhances the activity of PI 3′-kinase and induces the interaction between p85α, the regulatory subunit of PI 3′-kinase, and IκBα through tyrosine phosphorylation of IκBα. Syk suppresses the transactivation of NFκB by inhibiting the tyrosine phosphorylation of IκBα. PI 3′-kinase inhibitor reduces the pV-induced transactivation of NFκB. Both PI 3′-kinase inhibitors wortmannin and LY294002 and NFκB inhibitory peptide SN-50 suppress cell motility, indicating that PI 3′-kinase and NFκB play significant roles in this process. ASSyk-transfected MCF-7 cells enhanced uPA secretion, whereas wild type Syk cDNA-transfected MDA-MB-231 cells reduced uPA production, indicating that Syk down-regulates uPA secretion. These data suggested that Syk suppresses the NFκB transactivation by inhibiting the direct interaction of tyrosine-phosphorylated IκBα with the p85α domain of PI 3′-kinase in breast cancer cells. Finally, these data demonstrated that Syk down-regulates PI 3′-kinase activity and suppresses the constitutive NFκB activity and uPA secretion that ultimately lead to the suppression of cell motility of breast cancer cells (Fig. 9). These findings may be useful in designing novel therapeutic interventions using Syk as a target molecule that will disrupt the PI 3′-kinase and NFκB signaling pathways, resulting in reduction of uPA secretion and consequent blocking of invasiveness, migration, and metastatic spread of breast cancer.

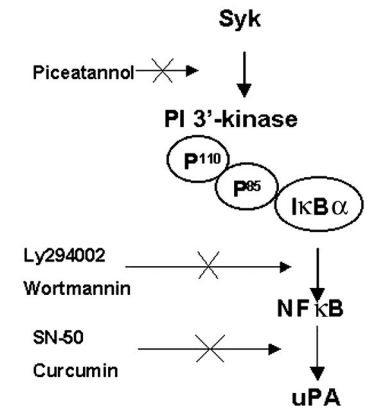

FIG. 9. **Molecular mechanism of Syk-regulated NFκB activation and uPA secretion through activation of PI 3′-kinase in breast cancer cells.** Syk suppresses cell motility and PI 3′-kinase activity in breast cancer cells. Syk also inhibits NFκB activation by blocking the interaction between p85α subunits of PI 3′-kinase and tyrosine-phosphorylated IκBα and subsequently reduces the uPA secretion in these cells. Wortmannin, LY294002, SN-50, and curcumin specifically disrupt these signaling pathways.

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