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

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 κB (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.

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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 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 βγ integrin-dependent cell adhesion via phosphorylation-independent interaction with the cytoplasmic domain of βγ 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 p110α (α, β, δ) or p110γ, and the other is regulatory subunit p85α (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

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; PFF, phosphatidylinositol phosphate; PMA, phorbol 12-myristate 13-acetate; pV, pervanadate; SSSyk, Syk-specific sense phosphorothioate oligonucleotide(s); SykK-κ, 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). Recent studies have shown that uPA stimulation increases cell motility and uPA secretion in these cells. Taken together, Syk and uPA play 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 (27). The recent data also demonstrated that interleukin-1 (IL-1) stimulates PI 3'-kinase (21). The recent data also demonstrated that interleukin-1 (IL-1) stimulates PI 3'-kinase (21). The recent data also demonstrated that interleukin-1 (IL-1) stimulates PI 3'-kinase (21). The recent data also demonstrated that interleukin-1 (IL-1) stimulates PI 3'-kinase (21). The recent data also demonstrated that interleukin-1 (IL-1) stimulates PI 3'-kinase (21).
kinase assay buffer (50 mM Hepes buffer (pH 8.0) containing 10 mM Na2VO4, 50 mM MgCl2, and 5 mM MnCl2) 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 ASSSyk, 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 MgCl2, and 1 mM EDTA) containing 0.25 mg/ml phosphatidylinositol, 100 mM ATP, and 15 μCi of [γ-32P]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% (v/v) acetic acid. The spots corresponding to the position of radioactive phosphatidylinositol phosphates (PIPs) 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 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 at 37 °C for 0–30 min, and the total proteins in the lysates were measured by Bio-Rad protein assay. Half of the lysates were pretreated with 10 μM ST 638, a tyrosine kinase inhibitor, for 15 min, and the serine-phosphorylated IκBα in 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 cell lysates were collected, and the protein concentration was measured by Bio-Rad protein assay. Half of the lysates were treated with 10 μM ST 638 for 15 min, and the immunoprecipitated samples were resolved by SDS-PAGE, and the tyrosine phosphorylated IκBα in the lysates was detected by Western blot analysis using anti-phospho-IκBα antibody. The blot was reprobed with anti-actin antibody as a 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 cell lysates were subjected to kinase assay. The blots were reprobed with anti-actin antibody as a loading control.

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 PBS, followed by 10% Triton X-100 (PBT) in PBS was used for the permeabilization of cells. 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 then washed, mounted with cetyltrimethylammonium bromide (CTAB) buffer and analyzed by 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 NFκB 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 LY 294002. The luciferase activities were measured by luminometer (Lab Systems) using the dual luciferase assay system according to the manufacturer’s instructions (Promega). The luciferase activities were measured densitometrically and analyzed statistically (Fig. 2, 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 SSSyk or ASSSyk 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. 2A, lane 1) or SSSyk transfected cells (lane 2), but the level of Syk was reduced drastically when cells were transfected with ASSSyk (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. 2B, 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. 2A and B, lower panels). These transfected cells were used for cell migration assays and for the detection of downstream signaling molecules.
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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-32p]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-32p]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-oligoucleotide-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. 3A). 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. 3B). 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. 3C). 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. 3D). The SykK-transfected cells showed 98% migration, whereas the number of cells that migrated using LipofectAMINE Plus-transfected cells were considered as 100% (Fig. 3D). The SykK- transfected cells had no effect on suppression of cell migration in MDA-MB-231 cells. We also stud-
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**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 × 10⁵ 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 dramatic 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.
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. 3A and 4B).

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. 4C, lane 3) compared with LipofectAMINE Plus-transfected cells (lane 1) or cells transfected with SykKK (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. 4D, 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 (Figs. 4E, 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. 4F, 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
MCF-7 and MDA-MB-231 cells were treated individually with 250 μM pV for 0–30 min and immunoprecipitated with anti-IkBα antibody. 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 using anti-phosphotyrosine 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-IkBα 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 half of the 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 SSSyk; 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.
The bands were analyzed by densitometry, and the values of -fold changes are indicated (Fig. 5A).

To confirm further the effects of pV on tyrosine phosphorylation of IkBa and regulation of interaction between phosphorylated IkBa 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 IkBa and p85α subunit of PI 3'-kinase through tyrosine phosphorylation of IkBa in MCF-7 (Fig. 5, upper panel, lanes 1–3) and MDA-MB-231 (Fig. 5C, 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 IkBa and subsequent interaction between tyrosine-phosphorylated IkBa 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 IkBa (Fig. 5D, upper panel, lane 3), but this phosphorylation was not detected in SykK cDNA-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 IkBa (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 IkBa and subsequent interaction between IkBa and PI 3'-kinase in both cell lines.

To determine whether pV induces the colocalization of IkBa 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-IkBα 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 IkBa and PI 3'-kinase in both cell lines (Fig. 5E, 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. 5F).

Syk Inhibits Transcriptional Activity of NFκB—Because Syk suppressed the tyrosine phosphorylation of IkBa, 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. 6A). 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. 6B). 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. 7A, 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 resolved 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. 7C, 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. 7B, 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. 7D) and MDA-MB-231 (Fig. 7E) 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
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. 8A, 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. 8B, lanes 2–4) and decreased when the cells were incubated with increasing doses of wortmannin (Fig. 8C, 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. 8E, 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. 8F, 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. 8D).

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. 8G). 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.
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...
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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 30 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. 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.

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 of UPA (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 IκB 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 transactivation of NFκB-mediated uPA secretion 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 reduced uPA secretion and consequent blocking of invasiveness, migration, and metastatic spread of breast cancer.

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