Activation of Syk by Protein Kinase C-δ Regulates Thrombin-induced Intercellular Adhesion Molecule-1 Expression in Endothelial Cells via Tyrosine Phosphorylation of RelA/p65

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Protein kinase C-δ (PKC-δ) plays a pivotal role in mediating thrombin-induced NF-κB activation and ICAM-1 expression in endothelial cells. However, the downstream mechanisms mediating its function are unclear. In this study, we show that PKC-δ-mediated activation of protein-tyrosine kinase Syk plays an important role in thrombin signaling of NF-κB activation and intercellular adhesion molecule-1 (ICAM-1) expression in endothelial cells. Stimulation of human umbilical vein endothelial cells with thrombin resulted in a time-dependent phosphorylation of Syk on tyrosine 525 and 526, an indication of Syk activation. Inhibition of PKC-δ by pharmacological and genetic approaches prevented Syk activation by thrombin. These results place Syk downstream of PKC-δ in transmitting thrombin-activated signaling in endothelial cells. Consistent with this, thrombin-induced NF-κB activity and ICAM-1 expression were prevented by the expression of a kinase-defective mutant or RNA interference knockdown of Syk. Similarly, inhibiting Syk also prevented NF-κB activity of ICAM-1 expression induced by a constitutively active mutant of PKC-δ. Analysis of the NF-κB pathway showed that Syk contributes to thrombin-induced NF-κB activation by controlling its transactivation potential and that this response is associated with tyrosine phosphorylation of RelA/p65. Thus, these data unveil a novel pathway in which Syk signals downstream of PKC-δ to mediate thrombin-induced ICAM-1 expression in endothelial cells by increasing transcriptional capacity of NF-κB via a mechanism that relies on tyrosine phosphorylation of RelA/p65.

The procoagulant serine protease thrombin, released during intravascular coagulation initiated by tissue injury or sepsis (1, 2), induces the expression of the intercellular adhesion molecule-1 (ICAM-1) in endothelial cells (3–6). ICAM-1, the ligand for the leukocyte β2 integrins (CD11/CD18) (7), enables polymorphonuclear leukocytes to adhere firmly to the vascular endothelium and thereby migrate across the microvascular barrier (8, 9). We have shown that the transcription factor NF-κB (RelA/p65) is an essential regulator of ICAM-1 expression following thrombin stimulation of protease-activated receptor-1 (PAR-1) in challenge of endothelial cells (3, 10). A key signal mediating RelA/p65 activation by thrombin involves activation of protein kinase C-δ (PKC-δ) (4). Activated PKC-δ leads to activation of IκB kinase (IKKβ), which in turn phosphorylates IκBα, the prototype of a family of inhibitory proteins that sequester NF-κB as an inactive complex in the cytoplasm (4, 10). Phosphorylation of IκBα triggers the rapid ubiquitination and subsequent degradation of this inhibitor in proteasome complex (11–14). The liberated NF-κB migrates to the nucleus where it binds to cognate κB enhancer elements and activates target genes such as ICAM-1 (3, 4, 10). In addition, NF-κB activity can be regulated through modulation of its transcriptional function by phosphorylation of RelA/p65. Phosphorylation of RelA/p65 at serines 276, 311, 529, or 536 has been shown to increase the transcriptional capacity of NF-κB in a cell- and stimulus-specific manner (15–19). We have shown that thrombin promotes the transcriptional capacity of NF-κB by increasing the phosphorylation of RelA/p65 at serine 536 (20, 21).

Besides serine/threonine kinases, tyrosine kinases, particularly members of the Src family, are implicated in the mechanism of NF-κB activation (22–27). Depending upon the cell types and stimulus used, c-Src or other members of the Src family are engaged to activate NF-κB through tyrosine phosphorylation of IKKβ or IκBα (22–28). We have recently demonstrated that c-Src plays an important role in signaling thrombin-induced NF-κB activation in endothelial cells (29). Activation of c-Src by thrombin leads to tyrosine phosphorylation of RelA/p65, which in turn contributes to the mechanism

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2 The abbreviations used are: ICAM-1, intercellular adhesion molecule-1; HUVEC, human, umbilical vein endothelial cells; NF-κB, nuclear factor-κB; IKK, IκB kinase; PKC, protein kinase C; TNF-α, tumor necrosis factor-α; IL-8, interleukin-8; EMSA, electrophoretic mobility shift assay; PMSF, phenylmethylsulfonyl fluoride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DTT, dithiothreitol; FBS, fetal bovine serum; RT, reverse transcription.
of NF-κB activation and thereby ICAM-1 expression in endothelial cells (29).

Spleen tyrosine kinase (Syk), a 72-kDa nonreceptor protein-tyrosine kinase, is largely confined to cells of hematopoietic origin (30, 31). It plays a pivotal role in the signaling machinery of various receptors of the adaptive immune system (32, 33). Recent studies indicate that Syk is also critically involved in signaling mechanisms of innate immunity (34–36). It is an essential component of integrin signaling in neutrophils and is required for the β₂ integrin-mediated respiratory burst, spreading, and site-directed migration (34–38). Syk also plays a central role in E-selectin-induced rolling of neutrophils on ICAM-1 (39). Additionally, Syk is implicated in airway and pulmonary inflammation, and depletion of Syk by aerosolized antisense oligonucleotide suppresses these responses (40, 41). Studies also demonstrate the presence of Syk in endothelial cells (42, 43), but its function remains largely unclear.

In this study, we addressed the possible role of Syk in the mechanism of thrombin-induced NF-κB activation and ICAM-1 expression in endothelial cells. Our results show that thrombin induces Syk activation via a PKC-δ-dependent pathway, and its activation is crucial in mediating thrombin-induced ICAM-1 expression by a mechanism involving tyrosine phosphorylation-dependent induction of RelA/p65 transcriptional function.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Human thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). Piceatannol, PP2, chelerythrin, LY 294002, rottlerin, and Go6976 were all purchased from Calbiochem-Novabiochem. Polyclonal antibodies to PKC-δ, β-actin, IκBα, and a monoclonal antibody to ICAM-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal antibody to Syk was obtained from Labvision (Fremont, CA), and antibodies detecting Syk when phosphorylated at Tyr525/526 and c-Src when phosphorylated at Tyr416 were obtained from Cell Signaling (Beverly, MA). In addition, polyvinylidene difluoride membrane was from Millipore Corp. (Bradford, MA); plasmid maxi kit was from Qiagen (Hilden, Germany); Lipofectamine 2000 transfection reagent was purchased from Life Technologies (Valencia, CA); DEAE-dextran was from Sigma; protein assay kit and nitrocellulose membrane were from Bio-Rad; Lipofectamine 2000 transfection reagent was purchased from Invitrogen. Nucleofector HUVEC kit and electroporation system were from Amaxa (Gaithersburg, MD). All other materials were from VWR Scientific Products Corp. (Gaithersburg, MD).

**cDNA and siRNA Constructs**—The construct pNF-κB-LUC containing five copies of consensus NF-κB sequences linked to a minimal E1B promoter-luciferase gene was obtained from Stratagene (La Jolla, CA). The GeneSuppressor plasmid encoding control or Syk siRNA was obtained from Imgenex (San Diego, CA). Human Syk cDNA (a gift from B. Mueller-Hilke, University of Rostock, Germany) and the construct encoding kinase-deficient mutant (48) were kindly provided by K. Sada (Kobe University Graduate School of Medicine, Japan). The kinase-deficient mutant of Syk (Syk-KD) was created by replacing Lys³⁴² with Arg (K³⁴²R) (48). Expression vectors encoding kinase-defective and constitutively active forms of mouse PKC-δ were gifts from I. B. Weinstein (Columbia University, New York) (49). The constitutively active form of PKC-δ (PKC-δ-CAT) contains only the catalytic domain and was generated by deletion of the regulatory domain (49). The kinase-deficient mutant of PKC-δ (PKC-δ-KD) was created by replacing Lys³⁷⁶ with Arg (K³⁷⁶R) (49). The constructs Gal4-p65 and Gal4-LUC were gifts from M. W. Mayo (University of Virginia, Charlottesville).

**Endothelial Cell Culture**—Human umbilical vein endothelial cell (HUVEC) cultures were established as described previously (44, 45) using umbilical cords collected within 48 h of delivery. Cells were cultured as described (45) in 0.1% gelatin-coated flasks using endothelial basal medium 2 (EBM2) with bullet kit™ additives (BioWhittaker, Walkersville, MD). In all experiments, unless otherwise indicated, cells were washed twice with serum-free MCDB-131 medium and incubated in the same serum-free medium for 0.5–1 h prior to thrombin challenge. Cells used in the experiments were between 3 and 6 passages.

**Cell Lysis, Immunoprecipitation, and Immunoblotting**—Cells were lysed in a phosphorylation lysis buffer (50 mM HEPES, 150 mM NaCl, 200 μM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM EDTA, 1.5 mM magnesium chloride, 100 mM sodium fluoride, 10% glycerol, 0.5–1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor mixture (Sigma)) or in radioimmune precipitation buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM NaF, 0.25 mM EDTA (pH 8.0), 1% deoxycholic acid, 1% Triton X-100, 1 mM sodium orthovanadate supplemented with protease inhibitor mixture (Sigma)). Cell lysates were resolved on SDS–PAGE and transferred onto nitrocellulose or polyvinylidene difluoride membranes. The residual binding sites on the membranes were blocked by incubation with 5% (w/v) nonfat dry milk in TBS (10 mM Tris (pH 8.0), 150 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature. The membranes were then incubated with appropriate antibodies and developed using an ECL method as described previously (46). For immunoprecipitation, cell lysates were prepared in 300 μl of phosphorylation lysis buffer and then subjected to preclarifying with 10 μl of protein A/G-agarose beads (Santa Cruz Biotechnology) for 4 h at 4 °C. The preclarified lysate was subjected to immunoprecipitation by incubating with 0.6–1 μg of appropriate antibody and 10 μl of the protein A/G-agarose beads at 4 °C overnight with gentle shaking as described (29). The immunoprecipitates were washed four times with the same volume of ice-cold phosphorylation lysis buffer. The proteins in the immunoprecipitates were extracted by boiling with SDS sample buffer for 5 min. The extracted proteins were subsequently analyzed by immunoblotting as described above.

**Reverse Transcription (RT)-PCR**—Total RNA was isolated using RNaseasy kit from Qiagen (Valencia, CA). RT was performed using oligo(dT) primers and superscript reverse transcriptase (Invitrogen) as described (47). Human ICAM-1 and GAPDH were amplified using the following primer set: ICAM-1 (forward, 5’-AGCAATGTGCAAGAAGATAGC-3’; reverse, 5’-GGTCCCTGGCGTGTCCACC-3’); GAPDH (forward, 5’-TATCGTGGAAGGACTC-ATGACC-3’; reverse, 5’-TACATGGCAACT- GTGAAGGG-3’). RT product (2 μl) was amplified in a 50 μl volume containing 2.5
units of TaqDNA polymerase and 0.5 μmol of primers. The reaction conditions were as follows: 95°C for 30 s, 67°C for 30 s, and 72°C for 30 s for 25 cycles for ICAM-1 amplification, and 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min for 30 cycles for GAPDH amplification. PCR products were resolved by 1.2% agarose gel and visualized by ethidium bromide staining. Normalization of ICAM-1 expression was achieved by comparing the expression of GAPDH for the corresponding sample.

Endothelial Cell Transfection—The cDNA and siRNA constructs were purified using the Endo-free Qiagen kit and transfected into cells by electroporation or using Lipofectamine 2000. For electroporation, 0.5 × 10⁶ cells were suspended in 100 μl of HUVEC nucleofector solution, mixed with 2.5–3 μg of cDNA, and rapidly electroporated by Amaxa nucleofector device using the manufacturer’s recommended program (U-01). Electroporated cells were immediately transferred into wells containing 37°C prewarmed EBM2, 10% FBS in 6-well plates and allowed to grow for 36–48 h before using them for various analyses. In some experiments, HUVEC were transfected using Lipofectamine 2000 essentially as per manufacturer’s recommendations. Briefly, cDNA (1.5 μg) was mixed with 1.5 μl of Lipofectamine 2000 in 100 μl of serum-free EBM2. After a 15–20-min incubation at room temperature, 0.9 ml of the mixture was gently added onto cells that were 60–80% confluent. The protein concentration of the extract was measured using a Bio-Rad protein determination kit (Bio-Rad).

**RESULTS**

Thrombin Activates Syk to Promote ICAM-1 Expression in Endothelial Cells—We determined the ability of thrombin to activate Syk in endothelial cells by monitoring the phosphorylation of Syk at Tyr⁵²⁵/⁵²⁶. Western blot analysis showed that thrombin induced a time-dependent phosphorylation of Syk. The phosphorylated form of Syk was detected within 30 min, and the peak phosphorylation occurred between 1 and 2 h after thrombin challenge (Fig. 1A). We next asked if activation of Syk contributes in the mechanism of ICAM-1 expression induced by thrombin. To this end, we used piceatannol, a relatively specific inhibitor of Syk, to determine whether inhibition of Syk influences thrombin-induced ICAM-1 expression. Analyses by RT-PCR showed a dose-dependent decrease in ICAM-1 mRNA expression in cells pretreated with piceatannol (Fig. 1B). Inhibiting Syk by piceatannol also caused a marked inhibition of ICAM-1 protein expression (Fig. 1C), consistent with its effect on ICAM-1 mRNA expression (Fig. 1B). To verify these results, we employed genetic approaches involving transfection of the construct pcDNA3-Syk-KD encoding kinase-defective Syk mutant (Syk-KD) to inhibit Syk activity or the GeneSuppressor plasmid encoding specific siRNA (siRNA-Syk) to deplete Syk. Inhibiting or depleting Syk by the above approaches prevented ICAM-1 expression by thrombin (Fig. 1D and data in supplemental Fig. S1). These results demonstrate an important role of Syk in signaling ICAM-1 expression following thrombin challenge of endothelial cells.

Syk Signals Thrombin-induced ICAM-1 Expression by Inducing NF-κB Activity—Given the essential role of NF-κB in mediating thrombin-induced ICAM-1 transcription (3), we addressed the possibility that Syk regulates ICAM-1 expression by controlling the activity of NF-κB. HUVEC were co-transfected with pNF-κB-LUC and pcDNA3-Syk-KD. Expression of Syk-KD significantly impaired thrombin-induced NF-κB-dependent reporter activity (Fig. 2). We essentially obtained similar results when the above experiment was carried out in human lung microvascular endothelial cells (data not shown).
Syk Mediates Thrombin-induced NF-κB Activity Independently of IkBα Degradation, RelA/p65 Nuclear Translocation, and DNA Binding—We previously showed that thrombin-induced NF-κB complexes are predominantly composed of RelA/p65 homodimer (3, 46). Therefore, we addressed the possibility that Syk mediates thrombin-induced NF-κB activity and thereby ICAM-1 expression by facilitating IkBα degradation-dependent nuclear translocation and, subsequently, DNA binding of RelA/p65. For this purpose, HUVEC were transfected with pcDNA3-Syk-KD to inhibit the endogenous Syk activity, and the nuclear and cytoplasmic extracts were prepared after stimulation of these cells with thrombin. Analysis of cytoplasmic extracts by immunoblotting showed that expression of Syk-KD failed to prevent thrombin-induced IkBα degradation (Fig. 3A). Similarly, analyses of nuclear extracts by immunoblotting and EMSA revealed that thrombin-induced nuclear localization and DNA binding function of RelA/p65 were both insensitive to expression of Syk-KD (Fig. 3, B and C). These results indicate that Syk contributes to thrombin-induced transcriptional activity of NF-κB through a mechanism that is independent of IkBα degradation, nuclear translocation, and DNA binding of RelA/p65.

Syk Mediates Thrombin-induced NF-κB Activity by Increasing the Transactivation Potential of RelA/p65—The lack of effect of Syk inhibition on IkBα degradation, nuclear translocation, and DNA binding of RelA/p65 prompted us to investigate whether Syk regulates NF-κB activity by controlling the transactivation function of RelA/p65. To determine this possibility, we used a construct Gal4-p65 encoding a fusion protein in which the DNA binding domain of the yeast Gal4 transcription factor is fused with the transactivation domain of Gal4-LUC, which contains four copies of Gal4 DNA-binding consensus sites, derived from yeast Gal4 gene promoter (52)) and then stimulated with thrombin. Results showed a marked increase in constitutive transactivation of Gal4-LUC by the nuclear fusion proteins Gal4-p65 as compared with the cells expressing Gal4 alone (Fig. 4), as expected. The transactivation by Gal4-p65 showed further increase upon stimulation with thrombin (Fig. 4). Expression of Syk-KD was effective in preventing both the constitutive and thrombin-stimulated transactivation by Gal4-p65 (Fig. 4). These results are consistent with a role of Syk in mediating NF-κB activity by virtue of increasing the transactivation potential of RelA/p65.

Syk Mediates Thrombin-induced Tyrosine Phosphorylation of RelA/p65—Because phosphorylation of RelA/p65 at Ser^536 is implicated in enhancing transcriptional capacity of NF-κB (20, 21, 53), we determined whether Syk controls NF-κB transcriptional activity by promoting Ser^536 phosphorylation of RelA/p65. We found that expression of Syk-KD failed to inhibit the phosphorylation of RelA/p65 at Ser^536 induced by thrombin (supplemental Fig. S2). We next addressed the possibility that Syk increases the transcriptional function of NF-κB by inducing
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Tyrosine phosphorylation of RelA/p65. Immunoblotting of RelA/p65 immunoprecipitates by anti-phosphotyrosine antibody (G410) showed that thrombin induced the tyrosine phosphorylation of RelA/p65 and that this response was inhibited in cells expressing Syk-KD (Fig. 5A). In control IgG immunoprecipitates, we failed to detect the presence and, consequently, phosphorylation of RelA/p65 (data not shown), indicating the specificity of the response.

The involvement of Syk in mediating tyrosine phosphorylation of RelA/p65 raised the possibility that Syk associates with RelA/p65 following thrombin challenge of endothelial cells. We assessed this possibility by immunoprecipitating RelA/p65 from control and thrombin-challenged cells and then analyzing these precipitates by immunoblotting for the presence of Syk. Because Syk is present in low levels in endothelial cells, we carried out this experiment using cells transfected with the construct pApuro-Syk-WT encoding wild type Syk (Syk-WT). Overexpression of Syk in cells transduced with pApuro-Syk-WT was confirmed by immunoblotting (Fig. 5B). Results showed that thrombin induced the association of RelA/p65 with endogenous as well as overexpressed Syk (Fig. 5C, 2nd and 3rd lanes). We also observed a constitutive association, albeit to a lesser extent, between Syk and RelA/p65 in cells overexpressing Syk (Fig. 5C, 4th lane); however, such an association of RelA/p65 was not detected with endogenous Syk (Fig. 5C, 1st lane). In control experiments, we failed to detect the presence of RelA/p65 and Syk in IgG immunoprecipitates (Fig. 5C, 5th to 7th lanes), indicating the specificity of the interaction. Together, these results suggest a role of Syk in signaling thrombin-induced ICAM-1 expression by increasing the transcriptional activity of NF-κB by virtue of associating and phosphorylating RelA/p65.

Requirement of PKCδ in Thrombin-induced Syk Activation—

As PKC-δ plays a crucial role in mediating NF-κB activation in endothelial cells (4), we determined whether Syk signals downstream of PKC-δ in the mechanism of thrombin-induced NF-κB activity. We used general and isoform-specific
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PKC inhibitors to address the role of PKC-δ in signaling Syk activation. Because Syk phosphorylation peaked at 1 h after thrombin challenge (Fig. 1A), we used this time point to examine the role of PKC-δ in the response. Pretreatment of cells with chelerythrin, a general PKC inhibitor, or rottlerin, a relatively specific inhibitor of PKC-δ, each prevented thrombin-induced Syk phosphorylation (Fig. 6, A and B). In parallel experiments, pretreatment of cells with Gö6976, a relatively specific inhibitor of PKC-α, failed to inhibit Syk phosphorylation/activation by thrombin (data not shown). These data suggest a role of PKC-δ in mediating Syk activation by thrombin. To further verify the requirement of PKC-δ in Syk activation, cells were transfected with the construct pCDNA3-PKC-δ-KD encoding kinase-defective mutant of PKC-δ (PKC-δ-KD) to inhibit the activity of endogenous PKC-δ. Expression of PKC-δ-KD was confirmed by the increased levels of PKC-δ in cells transfected with pCDNA3-PKC-δ-KD (Fig. 6C, 3rd and 4th lanes). Results showed that thrombin-induced Syk phosphorylation was inhibited in cells expressing PKC-δ-KD (Fig. 6C), confirming the involvement of PKC-δ in the response.

We also examined whether phosphorylation of Syk by thrombin requires its association with PKC-δ. Cells were challenged with thrombin, and the lysates were immunoprecipitated with an anti-PKC-δ antibody. Immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotted with an anti-Syk antibody. Syk was not detectable in anti-PKC-δ immunoprecipitates after thrombin challenge (supplemental Fig. S3A). To exclude the possibility that Syk is not detected in PKC-δ immunoprecipitates because of its low levels in endothelial cells, we carried out a similar experiment in which cells were transfected with a construct encoding wild type Syk (Syk-WT) to overexpress Syk. Cells were then challenged with thrombin, and the lysates were immunoprecipitated with an anti-Syk or anti-PKC-δ antibody. We failed to detect PKC-δ in Syk immunoprecipitates (supplemental Fig. S3B) or Syk in PKC-δ immunoprecipitates (supplemental Fig. S3C). These data indicate that PKC-δ-Syk association is not required for thrombin-induced phosphorylation of Syk, and point to the participation of an intermediate tyrosine kinase that may be engaged in a PKC-δ-dependent manner to phosphorylate Syk in intact cells.

PKC-δ Controls Syk Activation through c-Src—Because c-Src plays an important role in mediating ICAM-1 expression by promoting the transcriptional activity of NF-κB (29), we explored the possibility that PKC-δ activates Syk through c-Src. For this purpose, we first studied the effect of PKC-δ inhibition on c-Src activation. Preincubation of cells with rottlerin or expression of PKC-δ-KD each caused a significant inhibition of thrombin-induced Tyr416 phosphorylation of c-Src, an indicator of its activation (Fig. 7A). We next asked if activation of c-Src in turn is required for Syk activation. Inhibition of c-Src by PP2 markedly reduced Syk phosphorylation (Fig. 7B), indicating a role of c-Src in Syk activation by thrombin. Taken together, these data provide the evidence for the existence of PKC-δ→c-Src→Syk pathway that is activated by thrombin to promote the transcriptional activity of NF-κB and expression of ICAM-1 in endothelial cells.

Syk Mediates PKC-δ-induced NF-κB Activity and ICAM-1 Expression—The involvement of PKC-δ as an upstream component of thrombin-induced Syk activation led us to investigate whether inhibition of Syk influences NF-κB activity induced by PKC-δ. Cells were transfected with pNF-κB-LUC in combination with the construct pCDNA3-PKC-δ-CAT encoding the constitutively active mutant of PKC-δ (PKC-δ-CAT). We observed that the expression of PKC-δ-CAT induced NF-κB-dependent luciferase activity in the absence of thrombin challenge (Fig. 8A). Co-expression of Syk-KD inhibited the PKC-δ-CAT-induced NF-κB activity (Fig. 8A). Similarly, PKC-δ-CAT-induced NF-κB activity was also sensitive to expression of the kinase-defective c-Src (c-Src-KD) mutant (supplemental Fig. S4), consistent with the role of c-Src in linking PKC-δ to Syk activation. We also assessed the effect of Syk inhibition on PKC-δ-induced ICAM-1 expression. Results showed that expression of PKC-δ-CAT was sufficient to induce ICAM-1 expression and that this response was inhibited in cells co-ex-
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![Figure 6](image)

**FIGURE 6.** A and B, inhibition of PKC-δ prevents thrombin-induced phosphorylation of Syk. Confluent HUVEC monolayers were pretreated with chelerythrine (2 μM) (A), rottlerin (10 μM) (B), or transfected with the construct pcDNA3-PKCδ-KD encoding kinase-defective mutant of PKC-δ (PKCδ-KD) (C) and then challenged with thrombin for 1 h. Total lysates were separated by SDS-PAGE and immunoblotted with an anti-phospho-Syk (Y525/526) antibody. The blots were subsequently stripped and reprobed with an anti-Syk antibody to monitor loading. Results in A are representative of two independent experiments. The bar graph in B represents the effect of rottlerin on thrombin-induced Syk phosphorylation. The phosphorylated Syk level normalized to the total Syk level is expressed relative to the untreated control set at 1. Data are mean ± S.E. (n = 3–4 for each condition). †, p < 0.05 compared with untreated control; ‡, p < 0.05 compared with thrombin-stimulated control. C, effect of kinase-defective mutant of PKC-δ on thrombin-induced phosphorylation of Syk. HUVEC were transfected with the construct pcDNA3-PKCδ-KD encoding kinase-deficient mutant of PKC-δ (PKCδ-KD) or vector alone by electroporation as described under "Experimental Procedures." After 24 h, cells were challenged with thrombin (5 units/ml) for 1 h. Total lysates were separated by SDS-PAGE and immunoblotted with an anti-phospho-Syk (Y525/526) or anti-PKCδ antibody. The blots were subsequently stripped and reprobed with an anti-Syk antibody to monitor loading. The bar graph represents the effect of PKCδ-KD on thrombin-induced Syk phosphorylation. The phosphorylated Syk level normalized to the total Syk level is expressed relative to the untreated control set at 1. Data are mean ± S.E. (n = 3–4 for each condition). †, p < 0.05 compared with untreated control; ‡, p < 0.05 compared with thrombin-stimulated control.

pressing Syk-KD (Fig. 8B), consistent with the effect of Syk inhibition on NF-κB activity (Fig. 8A). Collectively, these results indicate that Syk signals downstream of PKC-δ to mediate thrombin-induced ICAM-1 expression by promoting NF-κB activity in endothelial cells.

**DISCUSSION**

The Syk tyrosine kinase, predominantly expressed in cells of hematopoietic origin, plays a crucial role in the signaling mechanisms of adaptive and innate immunity (32–36). In neutrophils, its activation is essential for the β2 integrin-mediated respiratory burst, spreading, and site-directed migration (34–37). Syk is also present in low levels in endothelial cells (42, 43); however, its role in contributing to the inflammatory response remains unknown. We have previously shown that thrombin promotes endothelial adhesiveness toward neutrophils by a mechanism involving expression of ICAM-1 via PKC-δ-dependent activation of NF-κB (4). In this study, we provide evidence that Syk lies downstream of PKC-δ in the endothelium, and its activation is crucial in signaling thrombin-induced ICAM-1 expression by promoting tyrosine phosphorylation-dependent induction of RelA/p65 transcriptional function. Thus, this study links two apparently different signaling pathways, the PKC-δ and the Syk pathways, and reveals the importance of this linkage in mediating NF-κB activation and in thereby promoting ICAM-1 expression in endothelial cells.

We used a combination of pharmacological and genetic approaches to address the contribution of Syk in the mechanism of NF-κB activation and ICAM-1 expression following thrombin challenge of endothelial cells. RNA interference knockdown of Syk inhibited thrombin-induced ICAM-1 expression, suggesting the involvement of Syk in the response. To ascertain whether kinase activity of Syk is required for NF-κB activation and ICAM-1 expression, we exposed the cells to piceatannol or transfected them with Syk-KD prior to thrombin challenge. Inhibiting Syk activity by these approaches mimicked the effect of Syk depletion on thrombin responses. These data define an important role of Syk in signaling thrombin-induced NF-κB activation and ICAM-1 expression in endothelial cells. Our findings are consistent with the described role of Syk in regulating NF-κB-dependent responses in immune cells. For example, treatment of fibronectin-adhered neutrophils with TNF-α, IL-8, and granulocyte-macrophage colony-stimulating factor results in NF-κB activation in a Syk-dependent manner (35). Syk also plays a role in mediating H2O2- and TNF-α-induced NF-κB activation in Jurkat cells and thereby protecting them from undergoing apoptosis (54, 55). In addition to immune cells, activation of Syk is implicated in β2 integrin signaling and TNF-α-induced expression of NF-κB-dependent genes, ICAM-1 and IL-6, in airway epithelial cells (56). It should, however, be noted that whereas the above studies demonstrate a role of Syk in activating IKK to facilitate IκBα phosphorylation/degradation-dependent activation of NF-κB, our results show that thrombin engages Syk to induce the transcriptional activation of NF-κB through a RelA/p65 tyrosine phosphorylation-dependent mechanism. Considered together, these findings indicate that activation of NF-κB by Syk is not restricted to cells of hematopoietic origin and appears to be functional in other cell types as well;
The identification of Syk as a downstream effector of PKC-δ provides further insight into the mechanisms by which PKC-δ regulates NF-κB activity and ICAM-1 expression in endothelial cells. Moreover, these findings are also of general significance as PKC-δ is implicated in controlling NF-κB activation in a variety of cell types. For example, TNF-α-induced NF-κB activation in human neutrophils is mediated by PKC-δ (58). Similarly, activation of PKCδ/NF-κB is critical for interleukin-8 (IL-8) production in human bronchial epithelial cells by lysophosphatidic acid (59) and in human airway epithelial cells by TNF-α (60). Studies by Wang et al. (61) showed the requirement of PKC-δ/NF-κB-dependent pathway in the regulation of phorbol 12-myristate 13-acetate-induced cIAP-2 expression in human colon cancer cells. In another study, Xu et al. (62) demonstrated that PKC-δ plays an important role in regulating IL-13-induced 15-lipoxygenase expression in human monocytes, and subsequently modulates the inflammatory responses mediated by 15-lipoxygenase products. PKC-δ is also implicated in the regulation of lipopolysaccharide-induced inducible nitric-oxide synthase expression in RAW 264.7 macrophages (63). Given that Syk is expressed in all the cell types described above, it is likely that PKC-δ/Syk/NF-κB signaling pathway is conserved in these cells as well and is activated by a host of stimuli to promote NF-κB-dependent responses.

Because PKC isoforms do not directly phosphorylate proteins at tyrosine residues, and we do not observe any association between PKC-δ and Syk after thrombin challenge, our data point to the participation of an intermediate tyrosine kinase(s) that may link PKC-δ signaling to Syk activation. Importantly, we have recently shown a role of c-Src in signaling thrombin-induced NF-κB activity and ICAM-1 expression in endothelial cells by mediating tyrosine phosphorylation of RelA/p65 (29). It should also be noted that in many cases Src family and Syk kinases function together, with activation of Src family kinases generally preceding the activation of Syk kinase (64). Indeed, we found that inhibiting c-Src prevented thrombin-induced Syk activation in endothelial cells. Moreover, we found that Syk associates with RelA/p65 and that inhibition of Syk impairs tyrosine phosphorylation of RelA/p65, consistent with a role of c-Src → Syk signaling in thrombin-induced NF-κB activation. Additionally, we observed that inhibiting PKC-δ impaired c-Src activation, indicating the requirement of PKC-δ in c-Src activation by thrombin. Consistent with this, PKC-δ-CAT-induced NF-κB activity was impaired in cells expressing c-Src-KD mutant. However, the precise mechanism by which PKC-δ controls thrombin-induced c-Src activation may depend on the cell type and the stimulus used (35, 54, 56, 57).

The blots were subsequently stripped and reprobed with an anti-Syk antibody to monitor loading. The bar graph represents the effect of PP2 on thrombin-induced Syk phosphorylation. The phosphorylated Syk level normalized to the total Syk level is expressed relative to the untreated control set at 1. Data are mean ± S.E. (n = 4 for each condition). **, p < 0.01 compared with untreated control; #, p < 0.01 compared with thrombin-stimulated control. B, inhibition of c-Src prevents thrombin-induced phosphorylation of Syk. Confluent HUVEC monolayers were pretreated with Src family kinase inhibitor PP2 (10 μM) and then challenged with thrombin for 1 h. Total lysates were separated by SDS-PAGE and immunoblotted with an anti-phospho-Syk (Tyr525/526) antibody.
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viation in endothelial cells remains to be clarified. Given that serine phosphorylation is implicated in the activation of c-Src (65–67) and that PKC-δ can mediate serine phosphorylation of c-Src (68), it is likely that PKC-δ mediates c-Src activation through its phosphorylation on specific serine residue(s). Collectively, these findings are consistent with the notion that thrombin engages PKC-δ → c-Src → Syk cascade to signal NF-κB activation by promoting the tyrosine phosphorylation of RelA/p65.

We previously showed that thrombin activation of the PKC-δ pathway leads to activation of IKKβ, which in turn mediates the release of RelA/p65 for its nuclear uptake and binding to the ICAM-1 promoter, secondary to phosphorylation and degradation of IκBα (4). Taken together, our findings are consistent with a model wherein PKC-δ regulates thrombin-induced NF-κB activation and ICAM-1 expression by a dual mechanism involving IKK-dependent release of RelA/p65 secondary to IκBα phosphorylation/degradation and c-Src/Syk-dependent tyrosine phosphorylation of liberated RelA/p65. Our results that inhibition of IκBα degradation by proteasome inhibitor MG132 prevents thrombin-induced tyrosine phosphorylation of RelA/p65 is consistent with the possibility that RelA/p65 tyrosine phosphorylation is coupled to the IκBα degradation.

In summary, this study defines an important role of endothelial Syk in signaling downstream of PKC-δ to mediate thrombin-induced ICAM-1 expression by increasing transcriptional capacity of NF-κB via a mechanism that appears to rely on tyrosine phosphorylation of RelA/p65. The mechanisms by which tyrosine phosphorylation of RelA/p65 contribute to the transcriptional activity of NF-κB are unclear. As serine phosphorylation of RelA/p65 (Ser^{376} or Ser^{311}) enhances the transcriptional capacity of NF-κB by increasing the association of RelA/p65 with its co-activator CBP/p300 (17, 19), it is likely that tyrosine phosphorylation of RelA/p65 contributes to the transcriptional activity of NF-κB by a similar mechanism; however, this possibility remains to be addressed. Given that RelA/p65 has five potential residues (Tyr^{250}, Tyr^{150}, Tyr^{257}, Tyr^{306}, and Tyr^{360}) for tyrosine phosphorylation (29), additional studies are required to (i) identify the tyrosine residue(s) phosphorylated by Syk and (ii) define the precise mechanisms by which tyrosine phosphorylation of RelA/p65 promotes the transcriptional activity of NF-κB. The crucial role of Syk in medi-

pcDNA3-PKCδ-CAT and pcDNA3-Syk-KD using Lipofectamine 2000 as described under "Experimental Procedures." After 24–36 h, total lysates were immunoblotted with an antibody to ICAM-1, Syk, or PKC-δ. Low exposure of the Syk blot was taken to allow the visualization of overexpressed Syk-KD but not the endogenous Syk protein. PKCδ-CAT corresponds to ~47 kDa as it contains only the catalytic domain and therefore can be distinguished from the endogenous PKC-δ (76 kDa). Actin levels were used to monitor loading. The bar graph represents the effect of Syk-KD on PKC-δ-CAT-induced ICAM-1 protein expression. ICAM-1 protein expression normalized to actin level is expressed relative to vector (pcDNA3) alone control set at 1. Data are mean ± S.E. (n = 3 for each condition). **, p < 0.01 compared with vector (pcDNA3)-transfected control; #, p < 0.05 compared with PKCδ-CAT-transfected control.

3 K. M. Bijil, unpublished observation.

FIGURE 8. Effect of kinase-defective mutant of Syk on PKC-δ-induced NF-κB activity and ICAM-1 expression. A, HUVEC were co-transfected with the constructs NF-κB-LUC and pcDNA3-PKCδ-CAT encoding constitutively active PKC-δ (PKCδ-CAT) in combination with pcDNA3-Syk-KD using DEAE-dextran method as described under "Experimental Procedures." After 12 h, cells were challenged with thrombin for 10 h. Cell extracts were prepared and assayed for Firefly and Renilla luciferase activities. Data are mean ± S.E. (n = 6 to 12 for each condition). **, p < 0.01 compared with vector (pcDNA3)-transfected control; ##, p < 0.01 compared with PKCδ-CAT-transfected control. B, HUVEC were co-transfected with the constructs NF-κB/Renilla luciferase construct reporter plasmid (pκB-Luc) and pcDNA3-PKCδ-CAT in combination with pcDNA3-Syk-KD using Lipofectamine 2000 as described under "Experimental Procedures." After 24–36 h, total lysates were immunoblotted with an antibody to ICAM-1, Syk, or PKC-δ. Low exposure of the Syk blot was taken to allow the visualization of overexpressed Syk-KD but not the endogenous Syk protein. PKCδ-CAT corresponds to ~47 kDa as it contains only the catalytic domain and therefore can be distinguished from the endogenous PKC-δ (76 kDa). Actin levels were used to monitor loading. The bar graph represents the effect of Syk-KD on PKC-δ-CAT-induced ICAM-1 protein expression. ICAM-1 protein expression normalized to actin level is expressed relative to vector (pcDNA3) alone control set at 1. Data are mean ± S.E. (n = 3 for each condition). **, p < 0.01 compared with vector (pcDNA3)-transfected control; #, p < 0.05 compared with PKCδ-CAT-transfected control.
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atting ICAM-1 expression in endothelial cells and also in promoting β₃ integrin-mediated respiratory burst and site-directed migration of neutrophils (37, 38) suggests a pivotal role of Syk in controlling inflammatory responses. Thus, targeting Syk may be a useful strategy for dampening the thrombin-activated inflammatory response associated with intravascular coagulation.

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