c-Src interacts with and phosphorylates RelA/p65 to promote thrombin-induced ICAM-1 expression in endothelial cells

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The procoagulant thrombin promotes polymorphonuclear leukocyte (PMN) adhesion to endothelial cells by a mechanism involving expression of intercellular adhesion molecule-1 (ICAM-1) via an NF-κB-dependent pathway. We now provide evidence that activation of c-Src is crucial in signaling thrombin-induced ICAM-1 expression via tyrosine phosphorylation of RelA/p65. Stimulation of human umbilical vein endothelial cells with thrombin resulted in a time-dependent activation of c-Src, with maximal activation occurring at 30 min after thrombin challenge. Inhibition of c-Src by pharmacological and genetic approaches impaired thrombin-induced NF-κB-dependent reporter activity and ICAM-1 expression. Analysis of the NF-κB pathway revealed that the effect of c-Src inhibition occurred independently of IκBα degradation and NF-κB DNA binding function and was not associated with exchange of NF-κB dimers. Phosphorylation of RelA/p65 at Ser32,36, an event mediating the transcriptional activity of DNA-bound RelA/p65, was also insensitive to c-Src inhibition. Interestingly, thrombin induced association of c-Src with RelA/p65, and inhibition of c-Src prevented this response, indicating that this interaction is contingent on activation of c-Src. We also observed that thrombin induced tyrosine phosphorylation of RelA/p65, and this phosphorylation was lost upon inhibition of c-Src, consistent with the requirement of activated c-Src for interaction with RelA/p65. These data implicate an important role of c-Src in phosphorylating RelA/p65 to promote the transcriptional activity of NF-κB and thereby ICAM-1 expression in endothelial cells.

tyrosine kinases; NF-κB; adhesion molecules

THE PROCOAGULANT SERINE PROTEASE thrombin, released during intravascular coagulation initiated by tissue injury or sepsis (6, 11), is an important regulator of polymorphonuclear leukocyte (PMN) adhesion to the endothelium (27, 28, 45). Thrombin promotes endothelial adhesivity by inducing the endothelial cell surface expression of intercellular adhesion molecule-1 (ICAM-1; CD54) (24, 27, 28, 40), a counter receptor for β2-integrins (CD11/CD18), on the surface of leukocytes (33). The interaction of ICAM-1 with β2-integrins ensures a firm and stable adhesion of PMN to the vascular endothelium and thus facilitates their migration across the endothelial barrier (7, 32). We have shown that activation of the transcription factor NF-κB is a requirement for thrombin-induced ICAM-1 expression in endothelial cells (27), and that this response is mediated through activation of the GTP binding protein (G protein)-coupled receptor, protease-activated receptor-1 (PAR-1) (29).

NF-κB is a ubiquitously expressed family of transcription factors controlling the expression of numerous genes involved in inflammation, host defense, cell survival, and proliferation (1, 4, 16). The mammalian NF-κB family consists of five members: RelA (p65), RelB, c-Rel, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100). These proteins share a conserved NH2-terminal 300-amino acid Rel homology domain (RHD) that contains nuclear localization signal (NLS) and is responsible for dimerization, sequence-specific DNA binding, and interaction with inhibitory IκB proteins. A critical feature of RelA, RelB, and c-Rel that distinguishes them from p50 and RelA/p65 subunits, are mostly sequestered in the cytoplasm by IκBα, the prototype of a family of inhibitory protein IκBs that mask the NLS of RelA/p65 (3, 39). Activation of NF-κB is initiated through phosphorylation of IκBα on two specific serine residues (Ser32 and Ser36) by a macromolecular cytoplasmic IκB kinase (IKK) complex composed of the catalytic subunits IKKα and IKKβ and the regulatory subunit NEMO/IKKγ (23, 42). Phosphorylation targets IκBα for polyubiquitination by the E3-SCFβ-TrCP ubiquitin ligase, leading to its degradation by the 26S proteasome (21). The released NF-κB undergoes rapid nuclear translocation and subsequent binding to NF-κB-responsive elements to activate transcription of target genes including ICAM-1. Another important mechanism regulating NF-κB activity is through modulation of its transcriptional function by phosphorylation of RelA/p65 (8, 30, 35, 37, 43). Studies have shown that phosphorylation of RelA/p65 at Ser276, Ser311, Ser529, or Ser536 increases the transcriptional capacity of NF-κB in the nucleus in a cell- and stimulus-specific manner (8, 30, 35, 37, 43). We have shown that thrombin promotes the transcriptional activity of NF-κB by increasing the phosphorylation of RelA/p65 at Ser536 (2, 26).

In addition to serine/threonine kinases, tyrosine kinases, particularly members of the Src family, are implicated in the activation of NF-κB (10, 17, 18, 22, 38). Depending on the cell types and stimulus used, c-Src or other members of the Src

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family are engaged to activate NF-κB through tyrosine phosphorylation of IκBβ or IκBα (10, 17, 18, 22, 38). However, it remains unclear whether tyrosine phosphorylation of RelA/p65 also contributes to NF-κB activity and whether c-Src plays a role in this response. In the present study, we demonstrate that thrombin induces c-Src activation in endothelial cells and that this event is required for its association with RelA/p65. We furthermore show that c-Src-RelA/p65 interaction leads to tyrosine phosphorylation of RelA/p65, which in turn contributes to the mechanism of thrombin-induced ICAM-1 expression by increasing the transactivating potential of NF-κB.

MATERIALS AND METHODS

Reagents. Human thrombin (5,000 NIH U/mg) was purchased from Enzyme Research Laboratories (South Bend, IN). Tyrosine kinase inhibitor genistein and Src kinase inhibitor PP2 were purchased from Calbiochem-Novabiochem (La Jolla, CA). Polyclonal antibodies to IκBα, p65/RelA, c-Src, β-actin, and IgG 2a or mouse monoclonal IgG, and a monoclonal antibody to ICAM-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal antibody to c-Src and an antibody that detects c-Src when phosphorylated at Tyr416 were obtained from Cell Signaling (Beverly, MA). A monoclonal phosphorytrosine antibody was from Upstate Biotechnology or Santa Cruz Biotechnology. In addition, polyvinylidene difluoride (PVDF) membrane was from Millipore (Bradford, MA), plasmid maxi kit was from Qiagen (Valencia, CA), DEAE-dextran was from Sigma Chemical (St. Louis, MO), and protein assay kit and nitrocellulose membrane were from Bio-Rad Laboratories (Hercules, CA). All other materials were from VWR Scientific Products (Gaithersburg, MD).

Endothelial cell culture. Human umbilical vein endothelial cell (HUVEC) cultures were established as described previously (14, 36) by using umbilical cords collected within 48 h of delivery. Cells were cultured as described (25) in gelatin-coated flasks using endothelial basal medium 2 (EBM2) with rabbit kit additives (BioWhittaker, Walkersville, MD). HUVEC used in the experiments were between passages 3 and 6.

Cell lysis, immunoprecipitation, and immunoblotting. After treatment, the cells were lysed in a phosphorylation lysis buffer [50 mM HEPES, 150 mM NaCl, 200 μM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1 mM EDTA, 1.5 mM magnesium chloride, 10% glycerol, 0.5–1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail (Sigma)] or in radioimmune precipitation (RIPA) buffer [50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 0.25 mM EDTA, pH 8.0, 1% deoxycholic acid, 1% Triton X, 5 mM NaF, and 1 mM sodium orthovanadate supplemented with protease inhibitor cocktail (Sigma)]. Cell lysates were analyzed by SDS-PAGE and transferred onto nitrocellulose (Bio-Rad) or PVDF membranes, and the residual binding sites on the filters were blocked by incubation with 5% (wt/vol) nonfat dry milk in TBST [10 mM Tris (pH 8.0), 150 mM NaCl, and 0.05% Tween 20] for 1 h at room temperature or overnight at 4°C. The membranes were subsequently incubated with the indicated antibodies and developed using an enhanced chemiluminescence (ECL) method as previously described (2). For immunoprecipitation, cell lysates were prepared in 400 μl of phosphorylation lysis buffer and then incubated with 15 μl of protein A/G agarose beads (Santa Cruz Biotechnology) and 1 μg of appropriate antibody at 4°C (Santa Cruz Biotechnology) overnight with gentle shaking. The immunoprecipitates were washed four times with the same volume of ice-cold phosphorylation lysis buffer, and the proteins in the immunoprecipitates were extracted by boiling with SDS sample buffer for 5 min. The extracted proteins were then analyzed by immunoblotting as described above.

RT-PCR. Total RNA was isolated using RNasey kit from Qiagen (Valencia, CA), and reverse transcription was performed using oligo (dt) primers and superscript RT (Invitrogen) as described in Ref. 25. Human ICAM-1 and GAPDH were amplified using the following primer set: ICAM-1 (forward 5’-AGCAATTGCAAGAAGATAGGCCAA-3’ and reverse 5’-GGCTCCCTGGGTCTTCCACCC-3’), GAPDH (forward 5’-TATCCTGAAGACTCATGACC-3’ and reverse 5’-TACATGCAACTGTAGGGGG-3’). Reverse transcription product (2 μl) was amplified in a 50-μl volume containing 0.5 μmol of primers and 2.5 units of Taq DNA polymerase. 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 use of 1% agarose gel and identified by ethidium bromide staining. Normalization of ICAM-1 expression was achieved by comparing the expression of GAPDH for the corresponding sample.

Reporter gene constructs, endothelial cell transfection, and luciferase assay. The plasmid pNF-κB-LUC containing five copies of consensus NF-κB sequences linked to a minimal E1B promoter-luciferase gene was purchased from Stratagene (La Jolla, CA). The construct encoding kinase-defective c-Src was prepared as described (19). The pTFKRLUC plasmid (Promega, Madison, WI) containing Renilla luciferase gene driven by the constitutively active thymidylate kinase promoter was used to normalize the transfection efficiencies. Transfections were performed using DEAE-dextran method essentially as described (25). Briefly, 5 μg of DNA were mixed with 50 μg/ml DEAE-dextran in serum-free EB2M, and the mixture was added onto cells that were 60–80% confluent. We used 0.125 μg of pTKRLUC plasmid (Promega) containing Renilla luciferase gene driven by the constitutively active thymidylate kinase promoter to normalize the transfection efficiencies. After 1 h, cells were incubated for 4 min with 10% dimethyl sulfoxide (DMSO) in serum-free EB2M. The cells were then washed 2× with EBM2–10% FBS and grown to confluency. We achieved transfection efficiency of 16 ± 5% (mean ± SD; n = 3) in these cells. Cell extracts were prepared and assayed for Firefly and Renilla luciferase activities using Promega Biotech Dual Luciferase Reporter Assay System. The data were expressed as a ratio of Firefly to Renilla luciferase activity.

Cytoplasmic and nuclear extract preparation. After treatment, cells were washed twice with ice-cold Tris-buffered saline and resuspended in 400 μl of buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF]. After 15 min, NP-40 was added to a final concentration of 0.6%. Samples were centrifuged to collect the supernatants containing cytosolic proteins to determine IκBα degradation by Western blotting. The pellet nuclei were resuspended in 50 μl of buffer B [20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF]. After 30 min at 4°C, lysates were centrifuged, and supernatants containing the nuclear proteins were transferred to new vials. The protein concentration of the extract was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories).

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was performed as described (25). Briefly, 10 μg of nuclear extract were incubated with 1 μg of poly(dI-dC) in a binding buffer [10 mM Tris·HCl (pH 7.5), 50 mM NaCl, 0.5 mM DTT, 10% glycerol (20 μl final volume)] for 15 min at room temperature. Then, end-labeled double-stranded oligonucleotides containing an NF-κB site (30,000 counts/min each) were added, and the reaction mixtures were incubated for 15 min at room temperature. The DNA-protein complexes were resolved in 5% native PAGE in low ionic strength buffer (0.25× Tris-borate-EDTA). The oligonucleotide used for the gel shift analysis was Ig-kB 5’-GTTGAGGCGACCTTCCAGGC-3’. The Ig-kB oligonucleotide contains the consensus NF-κB binding site sequence present in mouse Ig kappa light chain gene (31). The NF-κB sequence motif within the oligonucleotides is underlined.
siRNA transfections, siRNA targeting c-Src (siRNA-c-Src) was synthesized by Custom SMARTPool siRNA Design Service of Dharmacon (Lafayette, CO). The nonspecific, scrambled, control siRNA (siRNA-Scr) was purchased from Dharmacon. HUVEC were transfected with siRNA-c-Src or siRNA-Scr using DharmaFect1 siRNA Transfection Reagent (Dharmacon) according to the manufacturer’s recommendations. Briefly, 50–100 nM siRNA was mixed with the transfection reagent and added to cells that were 50–60% confluent. At 24–36 h after transfection, cells were lysed to determine c-Src expression, IkBα degradation, RelA/p65 phosphorylation, and ICAM-1 expression by Western blotting as described above.

Statistical analysis. Data are expressed as means ± SE. Comparisons between experimental groups were made by Student’s t-test. Differences in mean values were considered significant at P < 0.05.

RESULTS

Inhibition of c-Src impairs thrombin-induced NF-κB activity and ICAM-1 expression. We first determined the ability of thrombin to activate c-Src in endothelial cells by monitoring the phosphorylation of c-Src at Tyr416. Western blot analysis showed that thrombin induced the phosphorylation of c-Src in a time-dependent manner. The phosphorylated form of c-Src was detected as early as 5 min, and the peak phosphorylation occurred 30 min, after thrombin challenge of HUVEC (Fig. 1A). Phosphorylation of c-Src showed a decline at 1 h and returned to baseline 2 h after stimulation (Fig. 1A).

We used general tyrosine kinase (genistein and herbimycin A) and Src family-specific (PP2) inhibitors as well as RNA interference knockdown approach to address the role of c-Src in signaling thrombin-induced ICAM-1 expression. Analysis by RT-PCR showed that thrombin challenge of HUVEC resulted in increased ICAM-1 mRNA expression. Preincubation of cells with genistein or PP2 inhibited thrombin-induced ICAM-1 mRNA expression (Fig. 1B). We also determined the effects of inhibition of c-Src on thrombin-induced ICAM-1 protein expression. Western blot analysis showed that pretreatment of HUVEC monolayers with genistein, herbimycin A, or PP2 inhibited thrombin-induced ICAM-1 protein expression (Fig. 1C), consistent with their effects on ICAM-1 mRNA expression (Fig. 1B). These results suggest a role for the Src family of kinases in the mechanism of thrombin-induced ICAM-1 expression. We next determined whether RNAi knockdown of c-Src reproduces the effect of PP2 on the thrombin response. Results showed that depleting c-Src by this approach inhibited thrombin-induced ICAM-1 protein expression (Fig. 1D), indicating the involvement of c-Src in the response.

Because NF-κB activation is essential for thrombin-induced ICAM-1 gene transcription (27), we addressed the role of c-Src in mediating the transcriptional activity of NF-κB. HUVEC were transfected with pNF-κB-LUC containing five copies of consensus NF-κB sequence linked to a minimal adenovirus E1B promoter-luciferase reporter gene. As shown in Fig. 2A, thrombin-induced NF-κB-dependent reporter activity was markedly reduced in cells pretreated with genistein or PP2. To definitively establish the requirement of c-Src in this response, we determined the effect of expression of the kinase-deficient mutant of c-Src (c-Src<sup>MUT</sup>) on NF-κB-dependent reporter activity. Results showed that coexpression of c-Src<sup>MUT</sup> inhibited thrombin-induced NF-κB-dependent reporter activity (Fig. 2B).

Inhibition of c-Src prevents thrombin-induced NF-κB activity without affecting IkBα degradation and NF-κB DNA binding function. We investigated the possibility that inhibition of c-Src prevents thrombin-induced NF-κB transcriptional ac-

Fig. 1. A: thrombin induces phosphorylation of c-Src in endothelial cells. Confluent human umbilical vein endothelial cell (HUVEC) monolayers were challenged with thrombin (5 U/ml) for the indicated time periods. Total cell lysates were separated by SDS-PAGE and immunoblotted with an antibody to the phosphorylated (Tyr416) form of the Src family kinase as described in MATERIALS AND METHODS. The blots were subsequently stripped and reprobed with an antibody to c-Src. Results are representative of 3 independent experiments. B–D: inhibition or depletion of c-Src reduces thrombin-induced intracellular adhesion molecule-1 (ICAM-1) expression. B: confluent HUVEC monolayers were pretreated with indicated concentrations of genistein and PP2 for 1 h before stimulation with thrombin for 3 h. Total RNA was isolated by TRIzol and analyzed by RT-PCR for ICAM-1 mRNA expression as described in MATERIALS AND METHODS. GAPDH mRNA expression was used as an internal control. Results are representative of 2 independent experiments. C: confluent HUVEC monolayers were pretreated with indicated concentrations of genistein, herbimycin A, and PP2 for 1 h before stimulation with thrombin for 6 h. Total cell lysates were separated by SDS-PAGE and immunoblotted with an antibody to ICAM-1 as described in MATERIALS AND METHODS. Actin levels were used to monitor loading. Results are representative of 3 independent experiments. D: HUVEC were transfected with siRNA-c-Src or siRNA-Scr for 24–36 h as described in MATERIALS AND METHODS. Cells were then challenged with thrombin (5 U/ml) for 6 h. Total cell lysates were immunoblotted with anti-ICAM-1 and anti-c-Src antibodies. RelA/p65 levels were measured to determine the nonspecific effects of siRNA-c-Src. Actin levels were used to monitor loading. Results are representative of 2 to 3 separate experiments.
Inhibition of c-Src prevents thrombin-induced NF-κB activity without altering the composition of NF-κB dimers or Ser536 phosphorylation of RelA/p65. We next asked whether the reduced NF-κB transcriptional activity is associated with a change in the composition of NF-κB dimers binding to DNA after c-Src inhibition. Gel supershift assay showed that the thrombin-induced NF-κB binding complex was completely supershifted when incubated with an antibody to RelA/p65, whereas antibody to p50 produced only a weak supershift (Fig. 5A). These data are consistent with our finding that the thrombin-induced NF-κB binding complex is predominantly composed of RelA/p65 homodimer (27). We essentially obtained similar results in a parallel experiment where cells were pretreated with PP2 (Fig. 5B), indicating that inhibition of c-Src kinase activity does not alter the composition of the NF-κB complex activated by thrombin.
Because phosphorylation of RelA/p65 at Ser\(^{536}\) contributes to the transcriptional competency of NF-κB in the nucleus (26, 30, 41), we determined whether c-Src controls NF-κB transcriptional activity by promoting thrombin-induced phosphorylation of RelA/p65. Results showed that thrombin induced the phosphorylation of RelA/p65 at Ser\(^{536}\) in a time-dependent manner, with maximal induction occurring at 1 h after stimulation (Fig. 6A). In related experiments, we assessed the effect of inhibition by PP2 or depletion by specific siRNA of c-Src on Ser\(^{536}\) phosphorylation of RelA/p65 at this time point. We observed that RelA/p65 phosphorylation by thrombin was refractory to inhibition as well as depletion of c-Src (Fig. 6, B and C).

**Inhibition of c-Src prevents thrombin-induced tyrosine phosphorylation of RelA/p65.** The lack of effect of c-Src inhibition on IkBα degradation and composition and the DNA binding function of the NF-κB complex as well as Ser\(^{536}\) phosphorylation of RelA/p65 prompted us to test the possibility that c-Src regulates the transcriptional function of NF-κB by controlling tyrosine phosphorylation of RelA/p65. Thrombin induced tyrosine phosphorylation of RelA/p65 in a time-dependent manner; phosphorylation of RelA/p65 began at 30 min, peaked at 1 h, and declined to baseline by 2 h after thrombin challenge (Fig. 7A). Unlike RelA/p65, we found that thrombin, under the same experimental conditions, failed to induce tyrosine phosphorylation of IkBα (Fig. 7A). In reciprocal experiments, we analyzed the immunoprecipitates of RelA/p65 from control and thrombin-challenged cells to verify the tyrosine phosphorylation of RelA/p65. Immunoblotting by anti-phosphotyrosine antibody confirmed that thrombin induces tyrosine phosphorylation of RelA/p65 (Fig. 7B). In control IgG immunoprecipitates, we failed to detect the phosphorylation of RelA/p65 (Fig. 7B and data not shown), indicating the specificity of the response. We also determined whether inhibiting or depleting c-Src interferes with the phosphorylation of RelA/p65. Pretreatment of cells with PP2 and siRNA-mediated knockdown of c-Src each prevented the tyrosine phosphorylation of RelA/p65 induced by thrombin (Fig. 7, B and C).

**Inhibition of c-Src prevents its association with RelA/p65 induced by thrombin.** The impaired phosphorylation of RelA/p65 following c-Src inhibition raised the possibility that c-Src associates with RelA/p65 following thrombin challenge of endothelial cells. We assessed this possibility by immunoprecipitating c-Src from control and thrombin-challenged cells and then subjecting these precipitates to immunoblotting for the presence of RelA/p65. We found that thrombin induced the association of c-Src with RelA/p65 within 1 min and that this association was sustained for 1 h after stimulation (Fig. 8A). In control experiments, we failed to detect the presence of RelA/p65 in IgG immunoprecipitates (data not shown), indicating the specificity of the interaction. We subsequently examined whether activation of c-Src is essential for its interaction with RelA/p65. Stimulation of cells with thrombin for 5 min induced the association of c-Src with RelA/p65, and pretreatment of the cells with PP2 prevented this response (Fig. 8B). In view of the sustained c-Src–RelA/p65 association (Fig. 8A), we carried out a similar experiment where the cells were stimulated with thrombin for 1 h. We observed that thrombin induced c-Src–RelA/p65 interaction, as expected, and that this interaction was lost in cells pretreated with PP2 (Fig. 8B). In parallel experiments, analysis of IgG immunoprecipitates showed complete absence of RelA/p65 (Fig. 8B), indicating the specificity of the response. In reciprocal experiments, lysates
from cells challenged with thrombin in the absence and presence of PP2 were immunoprecipitated with RelA/p65. Analysis of these immunoprecipitates by immunoblotting with an antibody to the phosphorylated form (Ser536) of RelA/p65. RelA/p65 levels were used to monitor loading. Results are representative of 3 independent experiments. C: HUVEC were transfected with siRNA-c-Src or siRNA-Scr for 24-36 h as described in MATERIALS AND METHODS. Cells were then challenged with thrombin (5 U/ml) for 1 h. Total cell lysates were immunoblotted with anti-phosphoSer536relA/p65 and anti-c-Src antibodies. RelA/p65 levels were used to monitor loading. Results are representative of 2 separate experiments.

DISCUSSION

The present study provides evidence that c-Src associates with and phosphorylates RelA/p65 in a thrombin-dependent manner and that this event is critical in conferring transcriptional competency to the bound NF-κB and thereby in inducing ICAM-1 expression in endothelial cells. We show that throb-
to the participation of the Src family of kinases in NF-κB signaling of ICAM-1 expression by thrombin. Src kinases known to activate NF-κB include c-Src, Fyn, Lck, and Lyn (10, 12, 17, 18, 22, 38). The present study focused on c-Src, because it is the predominant Src kinase in endothelial cells and is known to be activated by thrombin (9, 34). To this end, we determined the effect of expression of a kinase-deficient c-Src mutant or siRNA-mediated knockdown of c-Src on NF-κB activation and ICAM-1 expression. Interfering with c-Src function by these approaches markedly reduced thrombin-induced NF-κB-dependent reporter activity and ICAM-1 expression, confirming the involvement of c-Src in these responses. These data are consistent with a previous study that implicated a role of c-Src in signaling ICAM-1 expression (17). In that study, it was shown that c-Src is activated by TNFα to mediate ICAM-1 expression in A549 cells in an NF-κB-dependent manner (17). Thus the role of c-Src in inducing NF-κB activation and ICAM-1 expression appears to be intact in other cell types as well. Our results, however, do not exclude the possibility that other members of Src family also play a role in activating NF-κB in endothelial cells by thrombin.

We addressed the mechanisms by which c-Src contributes to thrombin-induced NF-κB activity and thereby ICAM-1 expression. Studies have shown that c-Src can promote NF-κB activation via both IkBα degradation-dependent and -independent mechanisms. Huang et al. (17) have demonstrated that c-Src signals TGFα-induced ICAM-1 expression by a mechanism involving tyrosine phosphorylation-dependent activation of IκKβ in A549 cells. Activated IκKβ in turn phosphorylates IkBα on Ser32 and Ser36 to induce its degradation and, consequently, translocation of NF-κB to the nucleus (17). Other studies have shown that c-Src controls NF-κB activation through tyrosine phosphorylation of IkBα at residue 42 by a pathway that is independent of the IκK complex. Phosphorylation of IkBα at Tyr42 causes it to dissociate from NF-κB and thus facilitates nuclear localization of NF-κB in the absence of ubiquitin-dependent degradation of IkBα (10, 18, 22). Our finding that IkBα degradation by thrombin was insensitive to inhibition/depletion of c-Src suggests that c-Src regulates NF-κB activation downstream of IκKβ. We also noted that thrombin failed to induce tyrosine phosphorylation of IkBα. These results indicate that c-Src signaling of thrombin-induced NF-κB activation occurs independently of IkBα degradation and its dissociation from the NF-κB complex. The absence of these events prompted us to explore the possibility that c-Src induces NF-κB activity by controlling Ser536 phosphorylation of RelA/p65. However, the failure of c-Src inhibition/depletion to prevent thrombin-induced Ser536 phosphorylation of RelA/p65 excluded this possibility.

We considered the possibility that c-Src mediates thrombin-induced NF-κB activity by inducing tyrosine phosphorylation of RelA/p65 in endothelial cells. We determined whether thrombin stimulation of endothelial cells leads to association of c-Src with RelA/p65 and whether this association is critical for tyrosine phosphorylation of RelA/p65. Studies have shown that c-Src can promote NF-κB activation via a mechanism involving tyrosine phosphorylation-dependent activation of IκKβ in A549 cells. Activated IκKβ in turn phosphorylates IkBα on Ser32 and Ser36 to induce its degradation and, consequently, translocation of NF-κB to the nucleus (17). Other studies have shown that c-Src controls NF-κB activation through tyrosine phosphorylation of IkBα at residue 42 by a pathway that is independent of the IκK complex. Phosphorylation of IkBα at Tyr42 causes it to dissociate from NF-κB and thus facilitates nuclear localization of NF-κB in the absence of ubiquitin-dependent degradation of IkBα (10, 18, 22). Our finding that IkBα degradation by thrombin was insensitive to inhibition/depletion of c-Src suggests that c-Src regulates NF-κB activation downstream of IκKβ. We also noted that thrombin failed to induce tyrosine phosphorylation of IkBα. These results indicate that c-Src signaling of thrombin-induced NF-κB activation occurs independently of IkBα degradation and its dissociation from the NF-κB complex. The absence of these events prompted us to explore the possibility that c-Src induces NF-κB activity by controlling Ser536 phosphorylation of RelA/p65. However, the failure of c-Src inhibition/depletion to prevent thrombin-induced Ser536 phosphorylation of RelA/p65 excluded this possibility.

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to note that RelA/p65-IκBα association remained intact at early time points and began to decline at 30 min, and the maximal decrease occurred 1 h after thrombin challenge (data not shown), consistent with the time course of IκBα degradation (Fig. 3A). To account for the delayed RelA/p65 tyrosine phosphorylation despite early onset of c-Src-RelA/p65 association, we proposed a model wherein activated c-Src joins the RelA/p65-IκBα complex by associating with RelA/p65. However, c-Src in this complex is unable to induce concurrent phosphorylation of RelA/p65, possibly because of inaccessibility of the critical tyrosine residue(s) owing to RelA/p65 interaction with IκBα. Two lines of evidence support the possibility that RelA/p65 tyrosine phosphorylation is coupled to the IκBα degradation. These include the following. 1) The time course of tyrosine phosphorylation of RelA/p65 is similar to that of IκBα degradation. 2) Interference with IκBα degradation by proteasome inhibitor MG132 prevents tyrosine phosphorylation of RelA/p65 induced by thrombin (K. M. Bijli, unpublished results). Studies by Zhong et al. (44) have shown a similar activation of NF-κB that involves IκBα degradation-dependent phosphorylation of RelA/p65 at Ser276 by the catalytic subunit of protein kinase A (PKAc). These studies demonstrate that PKAc is maintained in an inactive state through association with IκBα in an NF-κB-IκBα-PKAc complex. Signals that cause IκBα degradation result in activation of PKAc and the subsequent phosphorylation of RelA/p65 at Ser276. It should be noted that Ser276 is located in the RHD of RelA/p65, the domain responsible for interaction of RelA/p65 with IκBα (13); hence, IκBα degradation is required not only for activation of PKAc but also for unmasking the Ser276 for phosphorylation (44).

Analysis by UniProtKB/Swiss-Prot, a protein sequence database, of RelA/p65 has revealed the presence of five potential residues (Tyr20, Tyr150, Tyr237, Tyr306, and Tyr360) for tyrosine phosphorylation. Given that these residues are present within or near the RHD of RelA/p65, it is likely that, in the absence of IκBα degradation, these residues are not accessible for phosphorylation by the bound c-Src. Thus IKKβ-dependent degradation of IκBα by thrombin may result in the unmasking of these residues, rendering them accessible for phosphorylation by c-Src, thereby promoting the transcriptional activity of NF-κB.

Additional studies are required to identify the tyrosine residue(s) phosphorylated by c-Src, as this is crucial not only for verifying the model discussed above but also for elucidating the mechanisms by which tyrosine phosphorylation of RelA/p65 contributes to the transcriptional activity of NF-κB.

In summary, the present study describes an important function of c-Src in signaling thrombin-induced NF-κB activity and ICAM-1 expression in endothelial cells by virtue of interacting with and catalyzing the phosphorylation of RelA/p65. Thus tyrosine phosphorylation, in addition to Ser32 phosphorylation, of RelA/p65 represents another mechanism of controlling the transcriptional capacity of NF-κB, although it remains to be clarified whether these phosphorylation events cooperate or act independently of each other in the thrombin response. Our data are consistent with a model wherein thrombin activation of IKKβ and c-Src acts in concert to promote NF-κB activity and ICAM-1 expression. Activated c-Src associates with RelA/p65 in a c-Src-NF-κB-IκBα complex, and the activated IKKβ promotes the degradation of IκBα in this complex. We postulate that IκBα degradation reveals the tyrosine residue(s), which otherwise lies hidden, for phosphorylation by the bound c-Src, and that this event in turn is critical in promoting the transcriptional capacity of NF-κB, thereby contributing to ICAM-1 expression. Given the crucial role of ICAM-1 in facilitating the recruitment of PMN from blood to the site of inflammation, targeting c-Src may be a useful strategy for dampening the thrombin-activated inflammatory response associated with intravascular coagulation.

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REFERENCES

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