Tumor necrosis factor-α (TNF) exerts its transcriptional effects via activation of nuclear transcription factor-κB (NF-κB). NF-κB is sequestered in the cytosol by IκBα and, in most cells, released upon serine phosphorylation of this inhibitory protein which then undergoes rapid, ubiquitin-dependent degradation. In contrast, we find TNF induction of NF-κB in murine bone marrow macrophages (BMMs), is mediated, by c-Src, in a cell, and cytokine specific manner. The non-receptor tyrosine kinase is rapidly mobilized and activated upon TNF exposure. Within the same time frame, TNF induced c-Src associates with IκBα in a long lived complex. The proto-oncogene, when associated with IκBα phosphorylates the inhibitory protein on tyrosine 42. Consistent with the pivotal role played by c-Src in TNF-induced IκBα tyrosine phosphorylation, NF-κB activation by the cytokine, is markedly delayed and reduced in c-src−/−BMMs. Underscoring the physiological significance of c-Src activation of NF-κB, TNF induction of IL-6, which is an NF-κB mediated event, is substantially diminished in c-src−/−BMMs.

The NF-κB family of transcriptional activators regulate genes controlling immune and inflammatory responses, cell proliferation, and apoptosis (1–3). In its inactive state, NF-κB resides in the cytoplasm, bound to the inhibitory protein, IκBα which masks the transcription factor’s nuclear localization sequence (3–5). NF-κB activation by agonists, such as cytokines, involves its dissociation from IκBα, translocation to the nucleus and binding to cognate response elements within target gene promoters (2).

In most cells, mobilization of NF-κB involves phosphorylation of IκBα serine residues 32/36 (3, 4, 6). The phosphorylated inhibitory protein undergoes ubiquitination and is rapidly degraded by proteosomes. A number of kinases which directly serine phosphorylate IκBα prior to proteosomal degradation, are in hand (7–11). Other serine kinases, however, such as casein kinase II, readily phosphorylate IκBα, yet such phosphorylation does not lead to signal-induced degradation of the inhibitory protein (12).

We have demonstrated that pervanadate (pV) activates NF-κB, specifically in Jurkat T cells. This event, however, involves tyrosine rather than serine phosphorylation of IκBα. Similar to serine/threonine phosphorylation induced by casein kinase II, IκBα, when tyrosine phosphorylated by pV, is not rapidly degraded (13). Taken in the context of other studies (14), tyrosine phosphorylation thus appears to prevent degradation of IκBα. On the other hand, NF-κB activation via tyrosine phosphorylation of IκBα has not been shown to be induced by a natural agonist and its physiological relevance, if any, is unknown (13, 14).

The osteoclastogenic cytokine, TNF, exerts much of its transcriptional impact by activating NF-κB (15, 16). In some circumstances, TNF also signals via tyrosine kinases (17–19). Thus, the cytokine presents itself as a candidate physiological activator of NF-κB via tyrosine phosphorylation of IκBα. We find such is the case regarding TNF induction of NF-κB in BMMs. We document c-Src, which stably associates with the inhibitory protein following TNF treatment of BMMs, is the cytokine-induced IκBα tyrosine kinase. c-Src phosphorylates IκBα on tyrosine 42, and such phosphorylation mobilizes NF-κB which translocates to the nucleus. The physiological relevance of c-Src-mediated IκBα tyrosine phosphorylation is underscored by delayed and diminished TNF-induced activation of NF-κB and curtailed expression of the NF-κB dependent cytokine, IL-6, in c-src−/−BMMs.

EXPERIMENTAL PROCEDURES

Reagents—Monoclonal antibody 327 (20), directed against the c-Src protein, full-length c-Src cDNA was a gift of Dr. A. Shaw (Dept. of Pathology, Washington University School of Medicine, St. Louis, MO). Polyclonal anti-IκBα, anti-p50NF-κB, and anti-p65NF-κB antibodies and c-Src specific substrate, p34, were purchased from Santa Cruz (Santa Cruz, CA). Monoclonal anti-phosphotyrosine and polyclonal anti-c-Src antibodies were purchased from UBI (Lake Placid, NY). Recombinant murine TNF (specific activity 1.1 × 108 units/mg) was purchased from Genzyme (Cambridge, MA). In all experiments involving TNF, 10 ng/ml cytokine was used which we have show to be inductive of murine BMM c-Src expression (19). ECL kit was obtained from Amersham Corp. (Arlington Heights, IL). All other chemicals were obtained from Sigma.

Mice—C3H/HeN males (Harlan Industries, Indianapolis, IN) and those in which the c-src gene was deleted (21) were used.

Cell Culture—BMMs were isolated from whole bone marrow of 4–6-week-old mice and incubated in tissue culture plates, at 37 °C in 5% CO2, in the presence of colony stimulating factor-1 (1,000 units/ml) (22). After 24 h in culture, the non-adherent cells were collected and layered on a Ficoll-Hypaque gradient and the cells at the gradient interface were collected and plated in α-minimal essential medium, supple-
conditions were established in our hands as optimal and specific for
To electrophoresis on a SDS-PAGE gel, dried, and exposed to film. These
sample buffer and heating at 65 °C for 5 min. Samples were subjected
to electrophoresis and blotted with anti-NF-κB rabbit pAb. b, BMMs of wild type
(WT) or c-src−/− mice were exposed to TNF (10 ng/ml) with time. EMSA was
performed using a double-stranded oligonucleotide probe containing the sequence
5′-AAGGGGGCTTTCCCTCCT-3′ derived from the B3 (NF-κB) site of the
TNF promoter. NF-κB band shifts and free probe (F) are indicated.

1. TNF promotes nuclear translocation of NF-κB in a c-Src-dependent manner. BMMs of wild type (WT) or c-src−/− mice were exposed to TNF (10 ng/ml) with time. a, the cytosol was immunoprecipitated with anti-IκBα rabbit pAb and the precipitate blotted with anti-NF-κB rabbit pAb. a, the nuclear fraction was lysed and the lysate electrophoresed and blotted with anti-NF-κB rabbit pAb. b, BMMs of wild type (WT) or c-src−/− mice were exposed to TNF (10 ng/ml) with time. EMSA was performed using a double-stranded oligonucleotide probe containing the sequence 5′-AAGGGGGCTTTCCCTCCTCCTC-3′ derived from the B3 (NF-κB) site of the TNF promoter. NF-κB band shifts and free probe (F) are indicated.

2. Immunoprecipitation—Adherent cells were scraped from the dish in the presence of ice-cold lysis buffer (20 mM Tris, pH 7.8, 150 mM NaCl, 1 mM CaCl₂, 0.2% deoxycholic acid, 0.2% Nonidet P-40, 0.02% NaN₃, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM AEBSF, 50 mM sodium fluoride, 0.1 mM NaVO₄), and incubated at 4 °C with gentle rocking for 30 min. The cells were then passed through a 25-gauge needle and spun at 10,000 rpm for 10 min in a microcentrifuge. Lysates were precleared with excess of protein A-Sepharose (Sigma) and protein G-Sepharose (Pharmacia Biotech Inc., Piscataway, NJ). Cleared lysates were incubated with various antibodies or non-immune serum followed with secondary goat anti-mouse or rabbit IgG horseradish peroxidase-conjugated antibody (1 h, room temperature). Membranes were washed with PBS/Tween buffer and exposed to primary antibodies (1 h at room temperature) for 30 min before addition of the labeled probe. After incubation with the labeled probe for an additional 30 min, samples were fractionated on a 4% polyacrylamide gel and visualized by exposing dried gel to film.

3. In Vitro Kinase Assay—The method described by Clark and Brugge (24) was followed, with slight modifications. Beads containing immunoprecipitated c-Src were washed with kinase lysis buffer (0.5 mM Tris-HCl, pH 7.8, 10 mM EDTA, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 mM AEBSF, 5 μg/ml leupeptin, vortexed for 30 s, and rotated for 30 min in 4 °C. The samples were then centrifuged and the nuclear proteins in the supernatant were transferred to fresh tubes and protein content was measured using standard BCA kit (Pierce, Rockford, IL). Nuclear extracts (10 μg) were incubated with an end-labeled double-stranded oligonucleotide probe containing the sequence 5′-AAGGGGGCTTTCCCTCCTCCTC-3′ (27) derived from the B3 site of the TNF promoter. The reaction was performed in a total of 20 μl of binding buffer (20 mM HEPES, pH 7.8, 100 mM NaCl, 0.5 mM dithiothreitol, 1 μg of poly(dI-dC), and 10% glycerol) for 15–20 min at room temperature. For supershift assay, the nuclear extract was incubated with specific antibodies for 30 min before addition of the labeled probe. After incubation with the labeled probe for an additional 30 min, samples were fractionated on a 4% polyacrylamide gel and visualized by exposing dried gel to film.

RESULTS

1. TNF Induces Nuclear Translocation of NF-κB Protein in a c-Src-dependent Manner—TNF provokes nuclear translocation of NF-κB and c-Src expression, events required for osteoclast differentiation (28, 29) and function (30), respectively. These observations prompted us to determine if NF-κB activation and c-Src induction by the cytokine, in osteoclast precursors, are functionally related. As seen in Fig. 1, a and b, TNF treatment (10 ng/ml) of wild type (WT) BMMs promotes rapid shift of the transcription factor from cytoplasm to nucleus. In contrast, NF-κB nuclear translocation, in cytokine-treated BMMs derived from mice deleted of c-src, is markedly reduced and delayed (Fig. 1, c−/−). The fact that TNF activation of an alternative pathway, involving c-Jun N-terminal kinase, is unaltered in c-src−/− cells (not shown) indicates failure to activate NF-κB in these mutants does not represent a global sig-

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*FIG. 1.* TNF promotes nuclear translocation of NF-κB in a c-Src-dependent manner. BMMs of wild type (WT) or c-src−/− mice were exposed to TNF (10 ng/ml) with time. a, the cytosol was immunoprecipitated with anti-IκBα rabbit pAb and the precipitate blotted with anti-NF-κB rabbit pAb. a, the nuclear fraction was lysed and the lysate electrophoresed and blotted with anti-NF-κB rabbit pAb. b, BMMs of wild type (WT) or c-src−/− mice were exposed to TNF (10 ng/ml) with time. EMSA was performed using a double-stranded oligonucleotide probe containing the sequence 5′-AAGGGGGCTTTCCCTCCTCCTC-3′ derived from the B3 (NF-κB) site of the TNF promoter. NF-κB band shifts and free probe (F) are indicated.
naling defect. The functional significance of TNF induced NF-κB nuclear translocation is supported by the appearance, in nuclear extracts, of a moiety recognizing the κB3 sequence of the TNF promoter (TNF-κB3) (Fig. 1c). Consistent with retardation of nuclear translocation of NF-κB in c-src−/− BMMs, appearance of TNF-κB3 binding activity is delayed at least 3-fold in mutant animals. Thus, while alternative mechanisms exist for slower TNF activation of NF-κB, its immediate mobilization requires c-Src.

**TNF-activated NF-κB Contains the p50 and RelA Subunits—**
NF-κB consists of various combinations of Rel family transcription factors, the most common being the p50/RelA dimer. To determine if this complex is induced by TNF treatment of BMMs, nuclear extracts were subjected to electrophoretic mobility shift assay (EMSA), in the presence and absence of anti-p50 and -RelA antibodies, using as probe, the κB3 response element of the TNF promoter (Fig. 2a). While nuclear NF-κB, in untreated BMMs, consists largely of p50 homodimers (compare lanes 1 and 2 versus 1 and 3), inclusion of both antibodies, in assays containing nuclear extract of TNF treated BMMs, markedly reduces the intensity of the band migrating with NF-κB. Thus, the TNF induced DNA binding moiety contains both p50 and RelA. Our data, however, do not exclude the possibility that additional NF-κB/Rel family members form the TNF-induced complex in BMMs. Competition with non-radio-labeled TNF-κB3 and the NF-κB response element derived from the HIV1 promoter (HIV1-κB), establishes specificity of the EMSA probe (Fig. 2b). Similarly, a mutated HIV1-κB (mut.HIV1-κB) oligonucleotide fails to compete with TNF-κB3.

**Absence of c-Src Impairs TNF Induction of IL-6 Secretion—**
BMMs differentiate, in defined circumstances, into bone-resorbing osteoclasts (31). Such commitment involves local secretion of selected cytokines, particularly IL-6 (32). IL-6 is NF-κB-induced (33, 34) and the product of exposure to TNF, itself a potent osteoclastogenic agent (19, 35). Thus, our finding that c-src−/− BMMs inadequately secrete IL-6 in response to TNF (Fig. 3) is consistent with failure of these cells to normally activate NF-κB when exposed to the latter cytokine and underscores the physiological relevance of TNF induced c-Src.

**TNF Induces Rapid Mobilization and Activation of c-Src—**
We have shown that c-Src protein is substantially increased in BMMs following 24 h exposure to TNF (19). The rapidity with which TNF induces NF-κB translocation in a c-Src-dependent manner (10 min) suggests the cytokine may prompt a more immediate appearance of the proto-oncoprotein. In fact, exposure of BMMs, for 10 min, to TNF induces a 60-kDa protein which immunoprecipitates, and is reactive by immunoblot, with anti-c-Src mAb (Fig. 4a). Establishing kinase activity, the 60-kDa protein undergoes autophosphorylation and phosphorylates exogenous substrate when subjected to in vitro kinase assay (Fig. 4b). While c-Src protein is unchanged with 10–60 min exposure to TNF (Fig. 4a), its kinase activity progressively increases (Fig. 4b). Measured as a function of autophosphorylation (Fig. 4b) relative to protein expression (Fig. 4a), densitometric analysis confirms that c-Src specific activity increases 210% between 10 and 30 min and 320% between 10 and 60 min of TNF exposure. The rapidity of c-Src induction and activation is in keeping with the functional role played by the proto-oncogene in TNF mobilization of NF-κB.

**TNF Induces Tyrosine Phosphorylation of IκBa in a c-Src-Dependent Manner—**
Having verified TNF rapidly induces c-Src as an active kinase whose deletion impairs NF-κB activation in BMMs, we turned to the mechanisms by which c-Src activates...
the transcription factor. These experiments focused on the inhibitory protein, IκBα. While TNF fails to alter IκBα expression (Fig. 5a, WT), exposure of BMMs to the cytokine rapidly leads to its tyrosine phosphorylation (Fig. 5b, WT). This observation, taken with the fact TNF induces c-Src expression (Fig. 4) and activates NF-κB (Fig. 1) synchronously with tyrosine phosphorylation of IκBα, raises the possibility the proto-oncogene is, in this circumstance, the IκBα tyrosine kinase. To test this hypothesis, we took advantage of BMMs derived from c-src⁻/⁻ mice. Suggesting a central role of c-Src in IκBα phosphorylation, IκBα in mutant cells, exposed to TNF, fails to undergo enhanced tyrosine phosphorylation (Fig. 5b, src⁻/⁻), while expression of the protein is unaffected (Fig. 5a, src⁻/⁻).

**c-Src Phosphorylates IκBα on Tyrosine 42**—Due to poor efficiency and cell death following lysosomal uptake of double-stranded DNA (36, 37), expression of protein in primary BMMs, using cDNA transfection, is a formidable challenge. Thus, to identify the IκBα tyrosine residue phosphorylated by c-Src, we transfected COS cells with c-Src and various IκBα-mutated cDNAs (Fig. 6) (13). We find c-Src-mediated phosphorylation of IκBα occurs on tyrosine 42. Tyrosine kinase activity was induced by either vanadate (VO4) or pV. VO4 and pV promote IκBα phosphorylation in cells transfected with c-Src and wild type IκBα but not with the Y42F mutant (Fig. 6). The fact that mutation of serines 32 and 36 (S32/36A) (30), the sites of traditional IκBα phosphorylation (6), does not preclude c-Src-induced phosphorylation further confirms the target residue of c-Src is tyrosine 42.

**c-Src Activation of NF-κB in BMMs Is Cytokine and Cell Specific**—TNF-induced NF-κB activation, in BMMs, differs from the classical pathway involving serine phosphorylation of IκBα. This discrepancy prompted us to explore the specificity of the tyrosine kinase-mediated phenomenon. Establishing cytokine and gene specificity, interleukin-4, which activates NF-κB in B lymphocytes (38), fails to do so in BMMs, whether derived from c-Src expressing or deleted mice (Fig. 7a). Furthermore, in contrast to NF-κB, TNF induction of PU.1, a DNA-binding osteoclastogenic transcription factor in BMMs, is independent of c-Src expression. More importantly, in contrast to TNF-treated BMMs in which mobilized IκBα is long lived (Figs. 5 and 8c), exposure of U937 (Fig. 7b), HeLa, and HepG2 cells (39–41) to the cytokine results in rapid degradation of the inhibitory protein.

**TNF Induces c-Src/IκBα Association**—The observation that TNF-induced IκBα phosphorylation is mediated by c-Src raises the likelihood of physical association between the inhibitory protein and tyrosine kinase. In fact, a 60-kDa protein, recognized by anti-c-Src polyclonal antibody (pcAb), immunoprecipitates with IκBα within 10 min of cytokine exposure (Fig. 8a). Similarly, an anti-c-Src mAb immunoprecipitate, derived from TNF-treated cells, immunoblots with anti-IκBα pcAb (Fig. 8b).

To assess stability of the 60-kDa IκBα complex, we exposed BMMs to TNF overnight. The cells were lysed and the lysate immunoprecipitated with anti-IκBα or anti-c-Src antibodies. Mirroring the inductive effect of TNF on the proto-oncogene, the quantity of IκBα-associated protein, which immunoblots with anti-c-Src mAb and migrates with c-Src, is increased with prolonged cytokine exposure (Fig. 8c). Thus, putative c-Src and IκBα remain associated for as long as 15 h in TNF-treated cells. The prolonged expression and activation of c-Src in response to TNF (19) and its extended connection with IκBα, suggest this association prompts prolonged tyrosine phosphorylation and IκBα degradation (Fig. 8c).

**The IκBα-associated Species Is Active c-Src Kinase**—To establish the 60-kDa IκBα-associated moiety is, in fact, c-Src, we again lysed BMMs treated for 15 h, with TNF. Lysates were immunoprecipitated with anti-IκBα or anti-c-Src antibodies and subjected to in vitro kinase assay (Fig. 9a). Anti-IκBα and anti-c-Src antibodies each precipitate only 60-kDa TNF-induced bands, which exhibit similar patterns of progressive
gamma-32P incorporation and migrate with c-Src. As seen in Fig. 9b, the anti-IkBα and anti-c-Src-phosphorylated immunoprecipitates yield identical patterns when subject to partial V8 protease digestion. Thus, TNF induces only one IkBα-associated kinase, namely c-Src. Because we have shown the experiment, as performed, represents c-Src mAb excess (19, 25), 15 h of TNF treatment prompts approximately one-third of total BMM c-Src to associate with IkBα (Fig. 9a, compare lanes 2 and 4). Furthermore, to obtain similar band intensities, equal radioactive counts (derived from lanes 2 and 4; Fig. 9a) were loaded in lanes 1 and 2 (Fig. 9b), respectively.

DISCUSSION

The osteoclast is a macrophage-derived specialized polykaryon and the principal if not exclusive resorptive cell of bone (42, 43). The molecular mechanisms by which mononuclear precursors commit to the osteoclast phenotype are beginning to emerge and involve transcriptional regulators such as c-Fos (44) and PU.1 (45). Interestingly, the importance of these regulatory proteins in osteoclast formation was revealed by the phenotype induced by their molecular deletion, namely osteopetrosis (44, 45). Recently, similar observations have been made following deletion of the p50/p52 heterodimer of the NF-κB/Rel family (28).

The non-receptor tyrosine kinase, c-Src, is also an osteoclast regulatory protein, as absence of its gene prompts osteopetrosis (21, 30). Moreover, c-Src expression, by BMMs, is a marker of commitment to the osteoclast phenotype (19). The fact that TNF exerts many of its effects by activating NF-κB (15, 16), taken with the cytokine's unique capacity to induce c-Src in BMMs (19), suggests the transcription complex and proto-oncogene participate in TNF's potent bone resorptive properties.

This posture is in keeping with the subnormal activation of NF-κB and diminished induction of IL-6, by TNF, in c-src<sup>2</sup>2 BMMs. TNF enhancement of c-Src protein, in BMMs, occurs within 1 day of cytokine exposure (19). In the present study, we find the quantity of the proto-oncogene, in BMM lysates, increases within 10 min of TNF treatment, an event rapidly followed by enhanced specific activity. The brisk appearance of the proto-
c-Src Phosphorylates IkBα and Activates NF-κB

Among others (39–41, 54, 55), eventuates in rapid IkBα degradation. Furthermore, in the face of continuous TNF exposure, c-Src association with IkBα is long lasting. Given the abundance of the proto-oncogene in prolonged TNF-exposed BMMs (19), most of the inhibitory protein, as it is synthesized, may associate with c-Src, thus preventing IkBα binding the transcription factor. In vitro reconstitution experiments utilizing HeLa cell-derived extracts (14) support our contention that tyrosine-phosphorylated IkBα is, probably, protected from inducible degradation. It remains to be determined, however, if protection of IkBα reflects tyrosine phosphorylation, per se, or, alternatively, c-Src association.

Activation of NF-κB by interleukin-1 promotes osteoclast survival (56). More importantly, the osteoclast-deficient osteopetrotic lesion of the p50/p52 deleted mouse (28) is in keeping with our hypothesis that TNF-induced osteoclastogenesis is NF-κB-mediated. The c-src−/− mouse is also osteopetrotic but, in contrast to the NF-κB knockout, contains numerous osteoclasts, incapable, however, of normal bone resorption (30). These disparate phenotypes may, at first glance, seem inconsistent with the posture that NF-κB-mediated osteoclastogenesis requires c-Src. The observations are, however, consonant with our finding that c-Src-independent activation of NF-κB, in BMMs, while extant, is reduced and delayed. This finding is analogous to that of Yeh et al. (57) wherein TNF activation of NF-κB in TRAP2 deficient cells, is retarded. Consistent with the anti-apoptotic properties of NF-κB (58–61), its delayed mobilization is attended by enhanced TNF-induced cytotoxicity. Thus, we propose that expression of the complete osteoclast phenotype may require rapid activation of NF-κB as occurs with TNF induction of c-Src in BMMs. Delayed activation of the transcription factor, as obtained in a c-Src-independent manner may, on the other hand, be sufficient to prompt differentiation of BMMs into osteoclasts which are, however, dysfunctional. Regardless of whether this hypothesis proves true, we have identified a novel, c-Src-dependent mechanism by which TNF activates a transcription factor critical to osteoclastogenesis.

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