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Transcription-Independent Turnover of IκBα during Monocyte Adherence: Implications for a Translational Component Regulating IκBα/MAD-3 mRNA Levels

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We identified IκBα/MAD-3 as an immediate-early gene in human monocytes that is expressed in response to a variety of signals, including adhesion, lipopolysaccharide, and phorbol myristate acetate. Within 5 min of monocyte adhesion, the level of the IκBα protein is markedly diminished but is rapidly replaced in a cycloheximide-sensitive manner within 20 min. Accompanying the rapid turnover of the IκBα protein is simultaneous translocation of NF-κB-related transcription factors to nuclei of adhered monocytes. The demonstration that NF-κB can regulate IκBα/MAD-3 gene transcription in other cell types suggested that the rapid increase in steady-state IκBα/MAD-3 mRNA levels we observed within 30 min of monocyte adherence would result from NF-κB-dependent transcriptional stimulation of the IκBα/MAD-3 gene. Nuclear run-on analyses indicated that, instead, while several immediate-early cytokine genes, such as the interleukin 1β (IL-1β) gene, were transcriptionally activated during monocyte adhesion, the rate of IκBα/MAD-3 gene transcription remained constant. The adherence-dependent increase in IκBα/MAD-3 mRNA levels was also not a consequence of mRNA stabilization events. Interestingly, while increases in both IL-1β and IκBα/MAD-3 mRNA levels were detected in nuclei of adherent monocytes, cytoplasmic levels of IL-1β mRNA increased during adherence whereas those of IκBα/MAD-3 mRNA did not. Taken together, our data suggest that two interactive mechanisms regulate monocytic IκBα/MAD-3 mRNA levels. We propose that adherent monocytes regulate nuclear processing (or decay) of IκBα/MAD-3 mRNA, thereby increasing mRNA levels without stimulating IκBα/MAD-3 gene transcription. Moreover, since inhibition of protein synthesis leads to accumulation of IκBα/MAD-3 mRNA without stimulating IκBα/MAD-3 gene transcription, we suggest that low cytoplasmic levels of IκBα/MAD-3 mRNA are maintained by a translation-dependent degradation mechanism.

Transcription factor NF-κB plays a critical role in the regulation of many immediate-early response genes associated with the host response to infection and tissue damage (reviewed in references 25 and 42). Rel/NF-κB activity is modulated, in part, by a growing family of regulatory molecules, including IκBα, IκBγ, the C-terminal domains of precursors for the p50 and p52 components of NF-κB (p105 and p100, respectively), and Bcl-3 (for a recent review, see reference 8). The IκB inhibitors function by sequestering Rel family members in the cytoplasm, effectively interfering with their transcription activation potential (reviewed in references 8 and 27). Recently, several groups have reported that cytokine, phorbol myristate acetate, or lipopolysaccharide (LPS) stimulation of a variety of malignant cell lines results in rapid degradation of the IκBα protein (9, 11, 17, 45, 47, 60). In each case, loss of IκBα was associated with simultaneous translocation of p50/65 to the nucleus. This indicates that dissociation of NF-κB from IκBα, a prerequisite for κB-dependent transcriptional activation, requires proteolytic processing of the IκBα inhibitor prior to nuclear translocation of NF-κB. Induced proteolysis of IκBα is coincident with changes in its phosphorylation state (9, 17, 23, 61), an observation which couples κB-dependent transcriptional activation to various signal transduction pathways.

IκBα/MAD-3 was first cloned from a cDNA library derived from human monocytes adhered to plastic for 30 min (29). Numerous immediate-early genes associated with inflammation and wound repair are expressed following monocyte adherence (22, 30, 31, 59). In monocytes, several cytokine genes are transcriptionally activated following various stimuli (3, 46). The high levels of cytokine mRNA produced within 30 min of adhesion (up to 0.1 to 0.2% of the total mRNA for interleukin 1β [IL-1β] and IL-8 [59]) suggested this was also a result of transcriptional activation, although we did not formally demonstrate this until recently (43). Steady-state mRNA levels for IκBα/MAD-3 and c-Fos also rapidly accumulate in adherence-stimulated monocytes (22, 29). Since these gene products are intimately associated with transcriptional control, we proposed that modulation of monocytic IκBα activity may have an important homeostasis function. Specifically, rapid up-regulation of IκBα and other regulatory proteins following exposure to inflammatory stimuli may help monocytes suppress responses to their own cytokine secretions.

The promoter of the IκBα/MAD-3 gene contains multiple NF-κB-binding sites (14, 20, 41), and overexpression of the p65 component of NF-κB can transcriptionally activate IκBα/MAD-3 (55). We assumed that transcriptional up-regulation of the IκBα/MAD-3 gene would also be the primary mechanism by which IκBα protein levels are up-regulated during monocyte adherence. In this report, however, we detail a series of studies that suggest an alternative pathway of regulation. We present data which led us to propose a mechanism by which adherent monocytes satisfy the requirement for translation of replacement IκBα protein by regulating the nuclear processing of IκBα/MAD-3 mRNA. Our evidence for this model is threefold. (i) The transcriptional rate of the IκBα/MAD-3 gene does

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not increase during monocyte adherence. (ii) Increased IκBα/MAD-3 mRNA levels following monocyte adherence are not a consequence of mRNA stabilization events. (iii) Mature IκBα/MAD-3 mRNA accumulates in the nuclei of adherent monocytes. Moreover, since inhibition of protein synthesis in monocytes leads to accumulation of IκBα/MAD-3 mRNA without stimulating IκBα/MAD-3 gene transcription, we also propose that low cytoplasmic levels of IκBα/MAD-3 mRNA are maintained by a translation-dependent degradation mechanism.

MATERIALS AND METHODS

Isolation of monocytes. Human monocytes were isolated from randomly selected healthy donors as previously described (22, 66). Whole blood (240 ml) was diluted 4:1 with RPMI 1640 medium (Gibco) and layered over Ficoll/Histopaque 1077 (Sigma). Following centrifugation at 400 × *g* (10), buffy coat cells were collected and washed repeatedly in a sterile 0.9% saline solution (Baxter) to remove platelets. Monocytes were then separated from the remaining mononuclear cells by centrifugation through Percoll (Pharmacia) density gradients (62). The purified monocytes were washed twice more with saline, suspended in cold RPMI 1640, counted, and used as described below.

Culture conditions. Monocytes isolated as described above were cultured in RPMI 1640 medium without serum at 37°C under 5% CO_2. When cultured adherently, 5×10^6 to 10×10^6 monocytes were plated on polystyrene tissue culture dishes (Corning). Nonadherent cultures were incubated in polypropylene tubes (Falcon) at cell concentrations of not more than 10^6/ml. When included, 10 μg of either LPS (Sigma), cycloheximide (Sigma), or puromycin (Sigma) per ml was added to the cultures. To minimize cell-cell interactions, all nonadherent monocyte cultures were continually mixed on a tube rotator.

Integrin engagement. Monocytes isolated as described above were suspended in ice-cold RPMI 1640 medium at 10^7/ml. Anti-β1 integrin monoclonal antibody (MAb) TS2/16, a gift from Francisco Sánchez-Madrid (Universidad Autónoma de Madrid) (12, 32), was added to the suspension at a final concentration of 1 μg/ml and incubated on ice for 40 min. The antibody-treated cells were then collected by centrifugation, washed with ice-cold medium, suspended at 10^6/ml in prewarmed (37°C) RPMI 1640 medium, and incubated for the time(s) indicated in the figure legends. In these experiments, nonadherent control cells were subjected to all of the manipulations applied to the antibody-treated cells but received no antibody.

Electrophoretic mobility shift assay (EMSA). For EMSAs, monocyte nuclear and cytoplasmic extracts were made by using a modification of the procedure described by Schütze and coworkers (53). Each treatment group utilized 5×10^6 to 10×10^6 monocytes. Following nonadherent incubation, monocytes were collected by centrifugation, washed with cold phosphate-buffered saline, and suspended in ice-cold cytoplasmic extraction buffer (CEB; 10 mM Tris-HCl [pH 7.9], 60 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol). After equilibration in CEB for 5 min, the cells were collected by centrifugation and lysed on ice in 50 times the packed cell volume of NP-40/CEB/PI (CEB containing 0.4% Nonidet P-40 [NP-40], 1 mM phenylmethylsulfonyl fluoride, 50 μg of antipain per ml, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml, 40 μg of bestatin per ml, 3 μg of E64 per ml, 1 mM 1,10-phenanthrolene, and 100 μg of chymostatin per ml). Adherent cells were equilibrated with 2 ml of ice-cold CEB buffer and subsequently recovered from the tissue culture dish by being gently scraped into 500 μl of NP-40/CEB/PI. Nuclei were pelleted, and the supernatant (cytoplasmic extract) was collected and frozen. The isolated nuclei were washed in cold CEB containing protease inhibitors but no detergent, suspended, and then mixed in 25 μl of nuclear extraction buffer (20 mM Tris-HCl [pH 8.0], 0.4 M NaCl, 1.5 mM $MgCl_2$, 1.5 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 50 μg of antipain per ml, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml, 40 μg of bestatin per ml, 3 μg of E64 per ml, 1 mM 1,10-phenanthrolene, 100 μg of chymostatin per ml, and 25% glycerol). After 10 min of incubation on ice, the samples were clarified by centrifugation and the supernatants (nuclear extracts) were collected and snap frozen on dry ice before storage at −70°C. Protein concentrations were determined by the bicinchoninic acid method (Pierce).

EMSAs (5) were performed by a slight modification of the method we previously reported (29). Briefly, DNA-protein-binding reactions were performed in 10 mM Tris-HCl (pH 7.9)–50 mM NaCl–0.5 mM EDTA–1 mM dithiothreitol, 5 μg of bovine serum albumin–0.1 μg of poly(dI-dC)–4% Ficoll in a final volume of 20 μl. Each reaction contained 1 μg of monocyte nuclear extract and 10,000 to 20,000 cpm of major histocompatibility complex enhancer probe. In parallel EMSAs, specific NF-κB subunits were identified in shifted complexes by using supershifting antibodies (38, 49) against the p50, p65, and Rel components of human NF-κB (data not shown).

Western immunoblot analysis. Whole monocyte extracts were prepared from 4×10^5 cells by standard methods (51), resolved by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis, and analyzed by immunoblotting with polyclonal rabbit anti-IκBα/MAD-3 serum. Immunoreactive proteins were visualized with either an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Western Blue; Promega) or an alkaline phosphatase-conjugated donkey anti-rabbit secondary antibody (ECL; Amersham). Specific immunoreactivity of the primary antibody against IκBα was demonstrated by subjecting the primary antibody to competition with synthetic IκBα/MAD-3 peptides (data not shown).

Northern (RNA) analysis. Steady-state RNA levels for individual RNA species were determined by hybridization of gene-specific cDNA probes to Northern blots as previously described (59). RNA was purified from monocytes (5×10^6 cells per sample point) by the guanidinium isothiocyanate-CsCl method (15). For this study, 3 to 5 μg of purified RNA was loaded into each lane of denaturing agarose gels (51). Following autoradiography, the Northern blots were exposed to a Phosphorimager screen (Molecular Dynamics) for quantitation of steady-state mRNA levels (Molecular Dynamics Imagequant 3.3 software).

Nuclear run-on analysis. Following culture, nuclei were extracted from monocytes (1.5×10^7 cells per treatment group) and run-on transcriptions were performed by using slight modifications of previously reported procedures (3, 46). Following nonadherent incubation, monocytes were collected by centrifugation, washed with cold phosphate-buffered saline, and lysed for 10 min on ice with 1.5 ml of NP-40 lysis buffer (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$, and 0.4% [vol/vol] NP-40) containing 100 U of RNasin per ml. Adherent cells were lysed directly on the tissue culture plates with 2 ml of NP-40 lysis buffer. Nuclei were collected by centrifugation at 500 × *g* for 5 min and subsequently washed twice with 1 ml of the lysis buffer containing 20 U of RNasin per ml. Run-on transcriptions were initiated by suspending the washed nuclei in 100 μl of a reaction mixture containing 10 mM Tris-HCl (pH 8.0); 5 mM $MgCl_2$; 100 mM KCl; 1 mM dithiothreitol; 40 U of RNasin per ml; 500 μM each GTP, CTP, and ATP; and 100 μCi of [α-^{32}P]UTP (3,000 Ci/mmol) and incubating the suspension at 30°C for 30 min with mixing every few minutes. Reactions were terminated by the addition of 500 μl of a solution containing 4 M guanidinium isothiocyanate, 25 mM sodium citrate, 100 mM 2-mercaptoethanol, and 0.5% (wt/vol) Sarkosyl. The ^{32}P-labeled RNA was subsequently purified by phenol-$CHCl_3$ extraction and precipitated with 2-propanol. Typically, each reaction yielded approximately 1×10^6 to 1.5×10^6 cpm of ^{32}P-labeled RNA which was denatured for 30 min at 60°C in a solution consisting of 6× SSPE (51), 1× Denhardt's solution, 0.5% (wt/vol) SDS, and 50% (vol/vol) formamide. Control plasmid pEMSV (pEMSVscribeα2; Hal Weintraub, Fred Hutchinson Cancer Research Center, Seattle, Wash.) and plasmids encoding cDNAs for IL-1β and IκBα/MAD-3 (29, 59) were denatured and slot blotted onto Nytran membranes in accordance with the directions of the manufacturer (Schleicher & Schuell). To test transcriptional attenuation of IκBα, 5′ and 3′ portions of the IκBα/MAD-3 cDNA were separated following restriction with *Xmn*I (which cleaves the MAD-3 cDNA into 921- and 629-bp 5′ and 3′ fragments, respectively). The purified fragments (1 μg of each) were denatured and transferred to Nytran filters as described above.

Total counts per minute were equilibrated for all sample points in each experiment (one experiment per donor) and hybridized to slot-blotted cDNAs for 60 h at 42°C in 6× SSPE–1× Denhardt's solution–0.5% SDS–50 μg of denatured salmon sperm DNA per ml–50% (vol/vol) formamide. Following hybridization, the filters were washed twice for 30 min each time in 2× SSPE–0.1% SDS at 42°C, twice for 30 min each time in 0.2× SSPE–0.1% SDS at 42°C, and once for 20 min in 0.2× SSPE–0.1% SDS at 56°C before exposure to Kodak XAR film. Subsequent to autoradiography, the filters were exposed to a Phosphorimager screen for quantitation of run-on activity.

PCR analysis. RNA was isolated either from whole cells or from nuclear and cytoplasmic fractions (obtained as described above) by the guanidinium isothiocyanate-CsCl method (15). Total, nuclear, and cytoplasmic RNAs (50 ng of each) were subsequently reverse transcribed (with random hexamers as primers) and PCR amplified as previously reported (35, 64). To amplify cDNA products arising from both mature and unprocessed RNAs, exon-specific PCR primer pairs were designed for both IL-1β and IκBα/MAD-3. For IL-1β, PCR primers corresponding to exons 4 (5′-TCAGTTGTTGTGGCCATGGACAAGCT GAGG) and 5 (5′-CGTGCAGTTCAGTGATCGTACAGGTGCAT) were synthesized (16). With these primers, the expected PCR amplification product sizes for IL-1β are 181 (mature) and 728 (unprocessed) bp. For IκBα/MAD-3, PCR primers corresponding to exons 5 (5′-TATTCTCCCTACCAGCTCAC) and 6 (5′-GCTTAACACTCCTGGCTGTT) were synthesized (36). With these primers, the expected PCR amplification product sizes for IκBα/MAD-3 are 569 (mature) and 921 (unprocessed) bp. The sequences of PCR primers used for amplification of β-actin cDNA have previously been reported (35). As controls, independent PCRs with the above-described primer pairs were performed on purified human genomic DNA and on dilution curves of IL-1β-, IκBα/MAD-3-, and β-actin-encoding plasmid DNAs.

RESULTS

IκBα/MAD-3 protein is rapidly lost and replaced following monocyte adherence. In a variety of cell lines, IκBα is rapidly degraded in response to stimulation by cytokines, phorbol myristate acetate, or LPS (9, 11, 17, 45, 47, 60). Degradation of IκBα is one of the initial steps in a pathway which ultimately leads to NF-κB-dependent transcriptional induction of genes

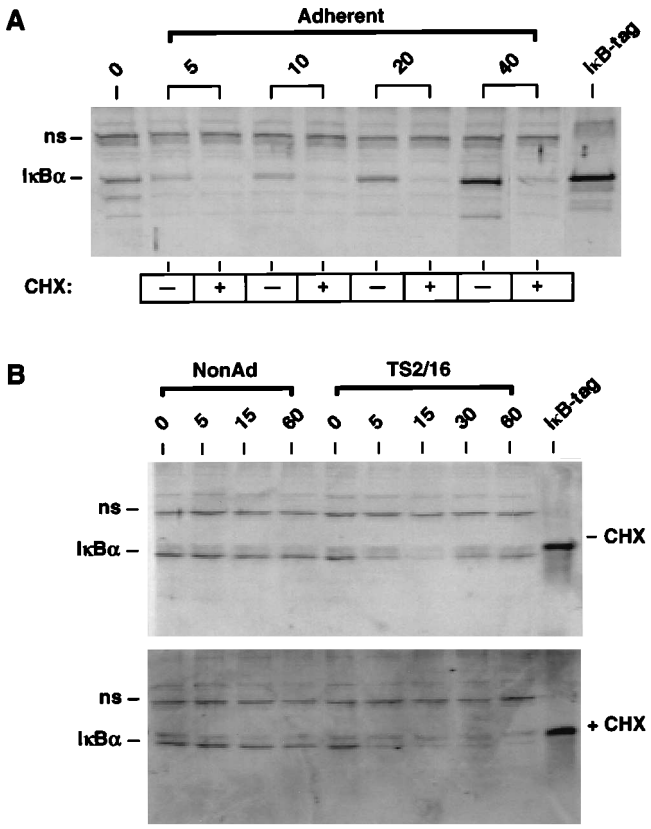

FIG. 1. IκBα/MAD-3 protein is rapidly lost and replaced following monocyte adherence and β1 integrin engagement. Human monocytes were stimulated either by adherence to plastic (A) or by nonadherent (NonAd) incubation with anti-β1 integrin MAb TS2/16 (B) for the times indicated (in minutes). Where indicated, cells were stimulated in the presence of 10 μg of cycloheximide (CHX) per ml. Following stimulation, whole-cell extracts were prepared, and for each sample point, the protein equivalent of 4×10^5 cells was resolved by SDS–10% polyacrylamide gel electrophoresis and analyzed by immunoblotting with polyclonal rabbit anti-IκBα/MAD-3 serum. The IκBα control standard, IκB-tag, is a baculovirus-synthesized recombinant IκBα protein containing an additional 1-kDa tag. Specific immunoreactive IκBα bands were determined by subjecting the primary antibody to competition with synthetic IκBα/MAD-3 peptides (28a). Nonspecific (ns) immunoreactive bands are indicated.

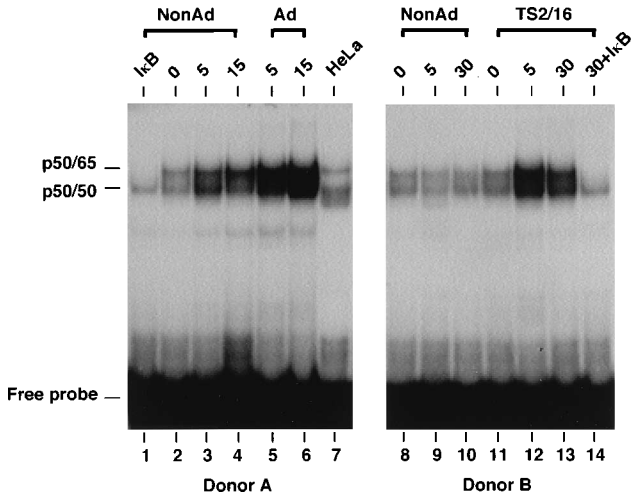

FIG. 2. Monocyte adherence and β1 integrin engagement result in immediate translocation of NF-κB activity. Freshly isolated human monocytes were stimulated either by adherence (Ad) to plastic (lanes 5 and 6) or by nonadherent (NonAd) incubation with anti-β1 integrin MAb TS2/16 (lanes 11 to 13) for the times indicated (in minutes). Nuclear extracts were prepared and EMSAs were performed as described in Materials and Methods. The mobility of the class I major histocompatibility complex enhancer probe in these extracts suggested the presence of both p50/65 and p50/50 NF-κB–DNA complexes. Specific NF-κB subunits were identified in parallel EMSAs with supershifting antibodies raised against components of NF-κB (data not shown). To further confirm the identity of the p50/65 complex, exogenous IκBα was added to nuclear extract from nonadherent time zero cells (lane 1) and to nuclear extract from cells MAb treated for 30 min (lane 14). The mobility of the major histocompatibility complex probe in nuclear extract prepared from PMA-stimulated HeLa cells was used as a control (lane 7).

containing κB enhancer elements in their promoter sequences. To determine whether primary human monocytes degrade IκBα in response to adhesion, we performed immunoblots of whole-cell extracts prepared from monocytes cultured nonadherently and from monocytes cultured adherently for various times. In the absence of any stimuli, monocytes cultured nonadherently maintained relatively constant levels of IκBα protein. Stimulation of monocytes either by adhesion (Fig. 1A) or by nonadherent incubation with LPS (data not shown) induced rapid (5 min) loss of detectable IκBα. Within 20 min of the stimulation, the amount of IκBα protein had been restored to the initial, unstimulated level. Cycloheximide completely inhibited the replacement of IκBα (Fig. 1A), suggesting that accumulation of IκBα protein following monocyte adherence was dependent upon new protein synthesis rather than upon transient modification(s) of preexisting protein.

Monocyte adhesion to components of the extracellular matrix is mediated via the integrin family of cell surface receptors (reviewed in reference 7). We previously reported that the gene induction profiles stimulated by adherence could be reproduced by stimulating integrin adhesion receptors nonadherently with MAbs raised against integrin β1 subunits (66). To investigate whether degradation and subsequent resynthesis of IκBα during adhesion stimulation of monocytes occurs as a consequence of direct integrin engagement, we performed immunoblots on extracts prepared from monocytes incubated nonadherently with anti-β1 MAb TS2/16 (12). Nonadherent stimulation of β1 integrins with MAb TS2/16 caused loss of detectable IκBα (Fig. 1B). The loss, which occurred more slowly than adhesion, was immediately compensated for by synthesis of new IκBα protein, which also was slightly delayed in comparison with that following adhesion. As during the adhesion response, replacement of IκBα protein following β1 integrin stimulation was sensitive to cycloheximide (Fig. 1B).

Monocyte adherence results in translocation of NF-κB activity. It is well established that nuclear translocation of transcription factor NF-κB parallels cytokine-induced degradation of IκBα (9, 11, 45, 60). To examine whether degradation of IκBα following monocyte adherence led to translocation of NF-κB, we performed EMSAs on extracts prepared from unstimulated and adherence-stimulated monocytes (Fig. 2). While unstimulated monocytes had low levels of p50/50 or p50/65 in their nuclei (lanes 2 and 8), monocytes stimulated by adhesion or stimulated nonadherently with LPS (data not shown) rapidly accumulated additional nuclear NF-κB-binding activity (lanes 5 and 6). Similar results were obtained when monocytes were stimulated nonadherently through their β1 integrins with MAb TS2/16 (Fig. 2). In our study, nuclear κB-binding activity reached a maximum within 15 min of adherence (lane 6) and within 5 min of β1 integrin stimulation (lane 12). The observed rapid accumulation of nuclear κB-binding activity demonstrated that monocytes can actively

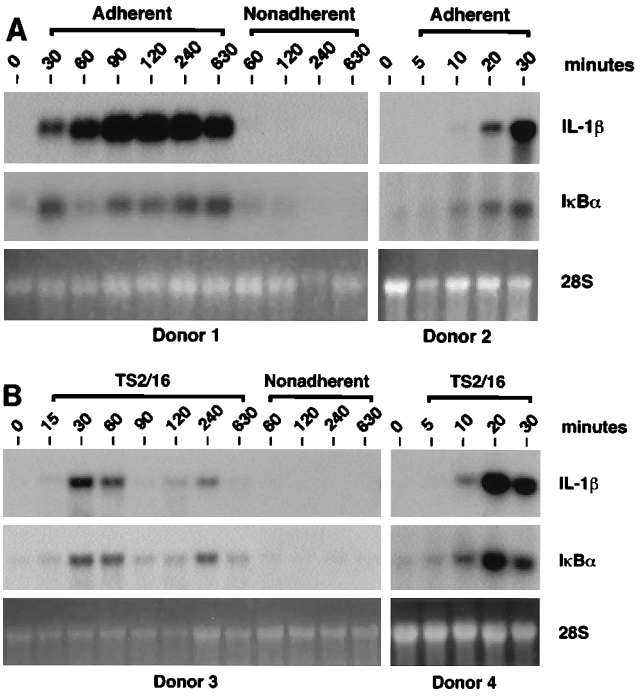

FIG. 3. IκBα/MAD-3 mRNA rapidly accumulates following monocyte adherence and β1 integrin engagement. Freshly isolated human monocytes were stimulated by adherence to plastic (A) or by nonadherent incubation with anti-β1 integrin MAb TS2/16 (B). RNA for Northern analysis was then isolated from cells stimulated for the times indicated (in minutes). Kinetics of IL-1β and IκBα mRNA synthesis are presented and represent the results obtained with material from four independent donors. The ethidium-stained 28S rRNA bands are presented as loading indicators for each Northern blot.

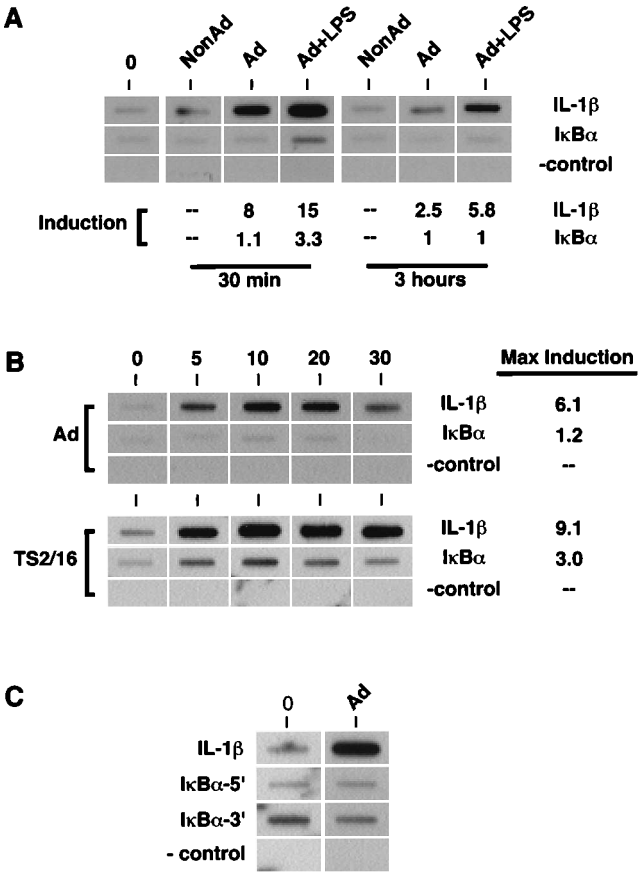

FIG. 4. Transcription of the IκBα/MAD-3 gene in monocytes is not increased during adherence but is increased by direct engagement of β1 integrins. (A) Nuclei were prepared from monocytes immediately following their isolation (0 min), following 30 min and 3 h of nonadherent culture (NonAd), following 30 min and 3 h of adherent culture on tissue culture plastic (Ad), or following 30 min and 3 h of adherent culture in the presence of 10 μg of LPS per ml (Ad+LPS). ^{32}P-labeled nuclear run-on RNA was prepared from each sample and hybridized to slot blots of cDNAs for IL-1β and IκBα/MAD-3 as indicated. Hybridization of run-on RNA to the pEMSV cloning vector was used as a control, (−control). (B) Nuclei were prepared from monocytes stimulated either by adherence to plastic or by nonadherent incubation with anti-β1 integrin MAb TS2/16 for the times indicated (in minutes). ^{32}P-labeled run-on RNA from each sample was hybridized to slot blots of cDNAs for IL-1β, IκBα/MAD-3, and pEMSV (−control) as described in Materials and Methods. (C) Nuclei were prepared from monocytes immediately following their isolation (0 min) and following 20 min of adherent culture on tissue culture plastic (Ad). ^{32}P-labeled nuclear run-on RNA was prepared from each sample and hybridized to slot blots of IL-1β cDNA, restriction fragments specific for either the 5′ (IκBα-5′) or 3′ (IκBα-3′) end of the IκBα/MAD-3 cDNA, and the pEMSV cloning vector (−control) as indicated.

modulate those transcriptional components which are commonly associated with immediate-early genes expressed in response to integrin-mediated adhesion (37).

IκBα/MAD-3 mRNA rapidly accumulates following monocyte adherence and β1 integrin engagement. We previously reported the cloning of IκBα/MAD-3 from a cDNA library constructed from monocytes adhered for 30 min (29). A more extensive kinetic analysis (Fig. 3A) indicated that steady-state levels of IκBα/MAD-3 transcripts increased approximately sevenfold within 30 min of adherence. We also observed that this rapid, adherence-induced accumulation of IκBα/MAD-3 mRNA was followed by a transient reduction in the steady-state level of the mRNA, which ultimately was followed by additional accumulation of IκBα/MAD-3 mRNA. The biphasic response of IκBα/MAD-3 mRNA was not observed for other adhesion-induced immediate-early genes, such as IL-1β (Fig. 3A).

mRNA levels for IκBα/MAD-3, like those for other adherence-inducible immediate-early genes, also increase in nonadherent monocytes stimulated with anti-β1 integrin MAb TS2/16 (Fig. 3B). While the kinetics of β1 integrin-mediated accumulation of IκBα/MAD-3 mRNA closely approximated those of adherence, the duration of the increased steady-state IκBα/MAD-3 mRNA level was more transient. This transient nature of mRNA accumulation was also evident for IL-1β mRNA in TS2/16-stimulated monocytes (Fig. 3B). As during adhesion, integrin-mediated accumulation of IκBα/MAD-3 mRNA was also biphsic. Unlike adhesion, however, the second peak of IκBα/MAD-3 mRNA occurred later (240 min poststimulation; Fig. 3B) and was accompanied by a second wave of IL-1β mRNA (Fig. 3B).

The transcriptional rate of the IκBα/MAD-3 gene is not increased during monocyte adherence. We have demonstrated here that adherence stimulates monocytes to rapidly accumulate both IκBα/MAD-3 mRNA and protein. Our coincidental observation that adherent monocytes also rapidly translocate NF-κB to the nucleus, in conjunction with the demonstration of NF-κB utilization by the IκBα/MAD-3 promoter (41), led us to examine whether transcription of the IκBα/MAD-3 gene was increased during adherent stimulation of monocytes. To address this issue, we performed nuclear run-on analyses on monocytes cultured either nonadherently or adherently for 30 min and 3 h. Surprisingly, adherence failed to elevate the level of transcription for the IκBα/MAD-3 gene at either time point (Fig. 4A). Addition of LPS to adherent monocytes increased

the transcriptional rate of IκBα/MAD-3 at least threefold within 30 min. The LPS-mediated effect on IκBα/MAD-3 transcription was transient; transcription of the IκBα/MAD-3 gene returned to basal, unstimulated levels within 3 h (Fig. 4A). In marked contrast to IκBα/MAD-3, the transcriptional rate of the IL-1β gene was dramatically increased during adherence (six- to eightfold within 30 min; Fig. 4A), as were the transcriptional rates of several other cytokine genes, including IL-8 and tumor necrosis factor alpha (43).

The rapid accumulation of IκBα mRNA (30 min) made us question whether we had missed a potential window of increased IκBα/MAD-3 transcriptional activity. To address this issue, we cultured monocytes adherently for 5, 10, 20, and 30 min. Nuclear run-on analyses of these cells again demonstrated the rapid transcriptional up-regulation of IL-1β (Fig. 4B). During the same time frame, however, we observed no changes in the transcription of the IκBα/MAD-3 gene (Fig. 4B), suggesting that accumulation of IκBα/MAD-3 mRNA immediately following adhesion of monocytes is not dependent upon increased transcription of the IκBα/MAD-3 gene. Although transcriptional activity of the IκBα/MAD-3 gene was unaltered at 1 h (data not shown) and 3 h (Fig. 4A) postadherence, we did not perform detailed run-on analyses between these time points and therefore cannot exclude the possibility that increased transcription may account for the second wave of IκBα/MAD-3 mRNA seen in Fig. 3.

To directly determine whether monocytes respond transcriptionally to signals generated from their adhesion receptors, we stimulated monocytes nonadherently with MAb TS2/16 and determined their transcriptional response by nuclear run-on analysis. Direct engagement of the β subunit of β1 integrins with this antibody increased transcriptional rates for several immediate-early cytokine genes, including IL-1β, transcription of which was induced approximately ninefold over that in unstimulated cells (Fig. 4B). The kinetics of the observed increase were similar to those of the increase generated by adhesion. Unlike adhesion, however, direct integrin engagement with the anti-β1 integrin MAb increased the transcriptional rate of the IκBα/MAD-3 gene up to threefold in 10 min (Fig. 4B). This rather surprising observation, that transcription of the IκBα/MAD-3 gene is stimulated by engagement of monocyte integrin receptors but not by monocyte adherence, may indicate that different signalling pathways are triggered by the two stimuli.

The absence of a detectable transcriptional response for the IκBα/MAD-3 gene during adhesion prompted us to examine whether adherent monocytes control IκBα/MAD-3 mRNA levels by regulating transcriptional elongation through the IκBα/MAD-3 gene. We restricted the MAD-3 cDNA clone to generate 5′ and 3′ IκBα/MAD-3 probes for use in our run-on analyses. Comparison of ^{32}P-labeled nuclear RNAs from monocytes cultured either nonadherently or adherently for 20 min revealed no detectable change in transcription across either end of the IκBα/MAD-3 gene (Fig. 4C). Similar results were obtained with genomic clones spanning the IκBα/MAD-3 gene (generously provided by C. Ito and A. S. Baldwin; data not shown). It appears, therefore, that a 3′ block in IκBα/MAD-3 transcription elongation does not account for the apparent discrepancy between our run-on and Northern data.

Increased IκBα/MAD-3 mRNA levels following monocyte adherence are not a consequence of mRNA stabilization events. Many immediate-early mRNAs associated with transcription and inflammation are short-lived molecules which are targeted for rapid degradation by repetitive AU-rich sequence elements (AREs) in their 3′ noncoding regions (18, 26, 56). The 3′ untranslated region of the IκBα/MAD-3 RNA contains at least three ARE-like repeats (29). To directly test whether monocytes regulate the stability of IκBα/MAD-3 mRNA in response to adhesion, we compared mRNA half-lives in monocytes cultured nonadherently and those cultured adherently or cultured nonadherently in the presence of the anti-β1 integrin MAb TS2/16. After 30 min of stimulation, the cells were treated with 5 μg of actinomycin D per ml to inhibit RNA synthesis and the decay rates of IL-1β, IκBα/MAD-3, and β-actin mRNAs were assessed by Northern analysis (Fig. 5). In nonadherent monocytes, both IL-1β and IκBα/MAD-3 mRNAs displayed half-lives of approximately 20 min, while that of β-actin mRNA was approximately 3 h (lanes 1 to 5). The decay rates for IκBα/MAD-3 and β-actin mRNAs were unaltered by adhesion or by β1 integrin engagement, indicating that their stability is not regulated by these stimuli (lanes 6 to 15). Nonadherent stimulation of monocytes with MAb TS2/16 also had no effect on the decay rate of IL-1β mRNA (lanes 11 to 15). In contrast, the stability of IL-1β mRNA was increased nearly 20-fold in adherent monocytes, which displayed a half-life of 6 to 8 h (lanes 6 to 10). The stability of IL-1β mRNA in monocytes adhered for 30 min was more than twice that reported by Arend and coworkers for monocytes cultured in the presence of LPS (3). These data indicate that while mRNA stabilization mechanisms are induced during monocyte adherence, the observed accumulation of IκBα/MAD-3 transcripts following adhesion is not a consequence of stabilized IκBα/MAD-3 mRNA.

Inhibition of protein synthesis leads to accumulation of IκBα/MAD-3 mRNA without stimulation of IκBα/MAD-3 gene transcription. Recent investigations have established that 3′ AREs can mediate their effect through a cotranslational mechanism. Namely, those ARE-containing RNAs which are efficiently translated are rapidly degraded (1, 52). This observation, coupled with the reported rapid turnover of IκBα protein (48), suggests a potential pathway for IκBα/MAD-3 regulation by mRNA destabilization. When we treated nonadherent monocytes with cycloheximide, we observed a dramatic increase in the steady-state level of IκBα/MAD-3 mRNA (Fig. 6A) which could not be accounted for by a cycloheximide-dependent increase in transcription of the IκBα/MAD-3 gene (Fig. 6B). Cycloheximide also stimulated the accumulation of various immediate-early cytokine mRNAs, including IL-1β mRNA (Fig. 6A), but unlike that of the IκBα/MAD-3 gene, transcription of the IL-1β gene was markedly enhanced by cycloheximide (Fig. 6B). Like cycloheximide, puromycin treatment of nonadherent monocytes also led to an increase in steady-state levels of IκBα/MAD-3 mRNA (Fig. 6A), further suggesting a link between translation and steady-state mRNA levels. Interestingly, puromycin treatment did not affect IL-1β mRNA levels. The transcription-independent accumulation of IκBα/MAD-3 mRNA in the absence of protein synthesis suggested that one aspect of adhesion signalling in monocytes could involve transient interruption of a translationally linked RNA degradation process.

Mature IκBα/MAD-3 mRNA accumulates in the nuclei of adherent monocytes. Since well-established regulatory mechanisms did not adequately explain the accumulation of IκBα/MAD-3 mRNA following monocyte adhesion, we initiated an investigation into whether IκBα/MAD-3 mRNA levels can be regulated by a nuclear posttranscriptional mechanism. Precedence for this line of investigation has previously been established in other studies which investigated the regulation of fibronectin gene expression induced by either fibroblast cell adhesion (21) or Ha-*ras* expression in human osteosarcoma cells (13). To determine whether monocytes alter their splicing patterns or subcellular distribution of IκBα/MAD-3 mRNA

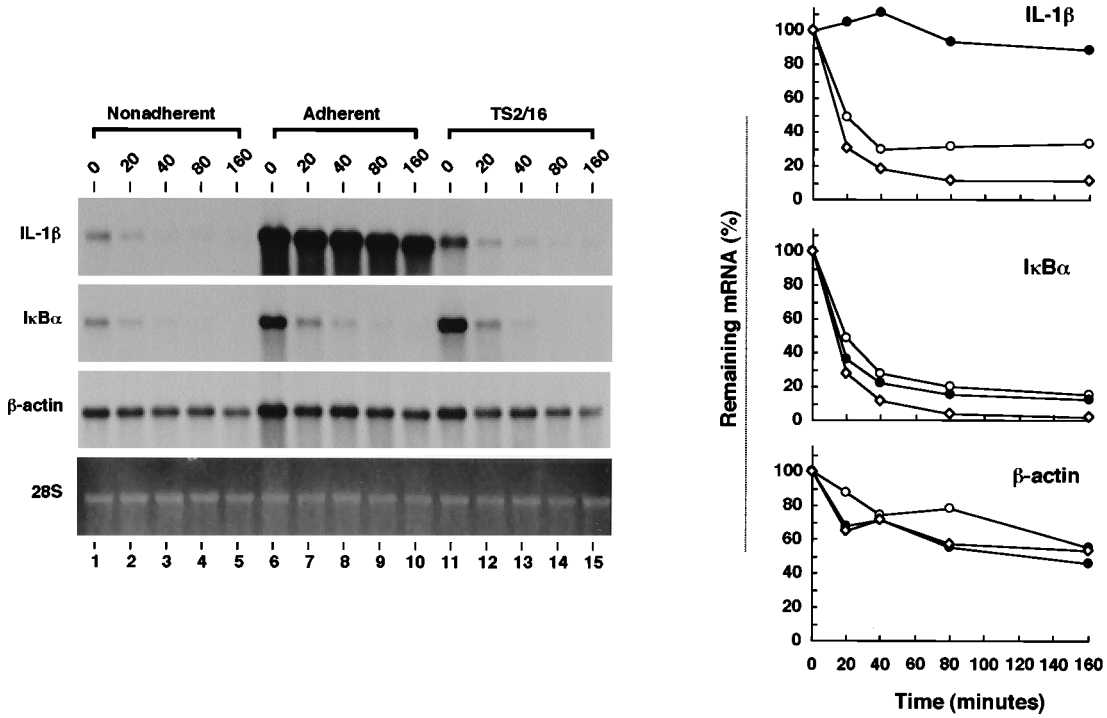

FIG. 5. Increased IκBα/MAD-3 mRNA levels following monocyte adherence are not a consequence of mRNA stabilization events. Freshly isolated human monocytes were cultured nonadherently with no stimulus (lanes 1 to 5), adherently on plastic (lanes 6 to 10), or nonadherently with anti-β1 integrin MAb TS2/16 (lanes 11 to 15). After 30 min, the monocyte cultures were treated with 5 μg of actinomycin D per ml for the times indicated prior to collection of the cells and isolation of the RNA for Northern analysis. The kinetics of degradation for IL-1β, IκBα, and β-actin mRNAs are presented for one representative donor. The decay for each mRNA was measured by Phosphorimager analysis, and the results are presented graphically to the right of each blot. Symbols: ○, unstimulated nonadherent monocytes; ●, adherence-stimulated monocytes; ◇, TS2/16-stimulated monocytes.

during adhesion, we purified nuclear and cytoplasmic RNAs from unstimulated monocytes and from monocytes stimulated by adherence for 20 min. Since only a small percentage (approximately 6%) of the total monocyte RNA isolated was nuclear, we analyzed the fractionated RNAs by PCR. Exonic PCR primers failed to amplify products corresponding to unspliced IL-1β and IκBα/MAD-3 mRNAs in either unstimulated or adherence-stimulated monocytes (Fig. 7). The same PCR primers amplified products from genomic DNA of the appropriate intron-containing size. We concluded that splicing of both IL-1β and IκBα/MAD-3 mRNAs is efficient in monocytes and that alternative splicing is not an important mechanism regulating adherence-dependent synthesis of monocytic IL-1β and IκBα/MAD-3 mRNAs. In contrast, our PCR analysis revealed that while increases in both IL-1β and IκBα/MAD-3 mRNA levels can be detected in the nuclei of adherent monocytes, cytoplasmic levels of IL-1β mRNA increase during adherence whereas those of IκBα/MAD-3 mRNA do not (Fig. 7). These data suggest that the subcellular distribution of IκBα/MAD-3 mRNA is tightly regulated in adherent monocytes, presumably because of adhesion-dependent changes in the rate(s) of splicing and/or nuclear decay of IκBα/MAD-3 mRNA.

DISCUSSION

Monocytes play a key role in various aspects of the host defense system, including the release of preformed mediators, as well as rapid induction of important cytokines and growth factors (for a recent review, see reference 6). An important aspect of this activity is the ability of monocytes to suppress their own response to these effectors before causing damage beyond that required to eliminate the foreign organism or repair the damaged tissue. For example, monocytes rapidly secrete IL-1β in response to bacterial products and rapidly follow this with far stronger production of a specific IL-1 inhibitor, the IL-1 receptor antagonist (4). Such specific responses serve to attenuate the inflammatory reaction. Since a number of immediate-early cytokine genes are transcriptionally regulated by Rel/NF-κB-like transcription factors (40, 42), a more general mechanism for down-regulating cytokine responses may be mediated by the IκB-like inhibitors of Rel family members. We have previously reported that IκBα/MAD-3 mRNA levels rapidly increase following monocyte adhesion (29). Since adhesive interaction with the extracellular matrix is one of the primary events during monocyte extravasation and is likely to be involved in the initiation of intracellular signalling cascades (34, 37, 54), we expect that regulation of IκBα activity during adherent events is crucial for regulation of cytokine production in monocytes.

In the present study, we demonstrated that primary human monocytes rapidly degrade IκBα in response to stimulation by adherence. Coincident with IκBα degradation was immediate translocation of transcription factor NF-κB to the nucleus. Transcriptional activation of IL-1β and other immediate-early cytokine genes closely paralleled the adherence-mediated activation of κB-binding activity. While many of the effects caused by adhesion could be reproduced nonadherently by incubating monocytes with a MAb raised against the β1 family of integrin adhesion receptors, there were a few notable exceptions. Most striking of these was our observation that signalling through β1 integrins readily increased the transcriptional rate of the IκBα/MAD-3 gene whereas bona fide

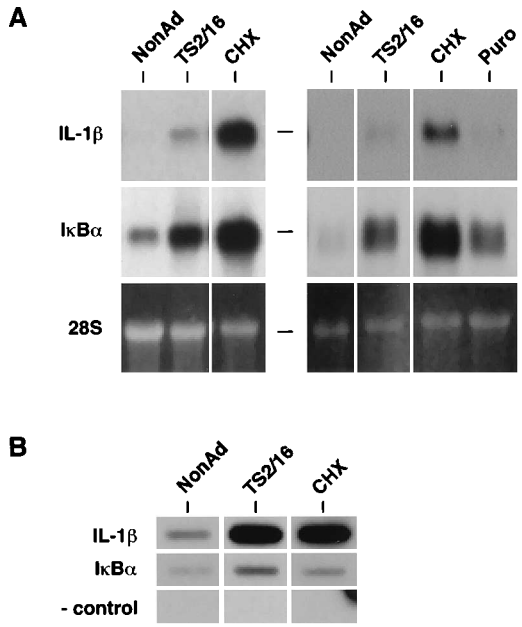

FIG. 6. Inhibition of protein synthesis leads to accumulation of IκBα/MAD-3 mRNA without stimulation of IκBα/MAD-3 gene transcription. (A) Freshly isolated human monocytes were cultured for 60 min nonadherently with no stimulus (NonAd), with an anti-β1 integrin MAb (TS2/16), and with cycloheximide (CHX) or puromycin (Puro) to inhibit protein synthesis. RNA was then isolated for Northern analysis of steady-state mRNA levels for both IL-1β and IκBα/MAD3. The ethidium-stained 28S rRNA bands were used as a loading indicator for each Northern blot. Results obtained with material from two independent donors are presented. (B) ^{32}P-labeled nuclear run-on RNA was prepared from each of the cultures described for the left half of panel A and hybridized to slot blots of cDNAs for IL-1β and IκBα/MAD-3 as indicated. As a hybridization control, the empty cloning vector pEMSV was also included on the blot (−control).

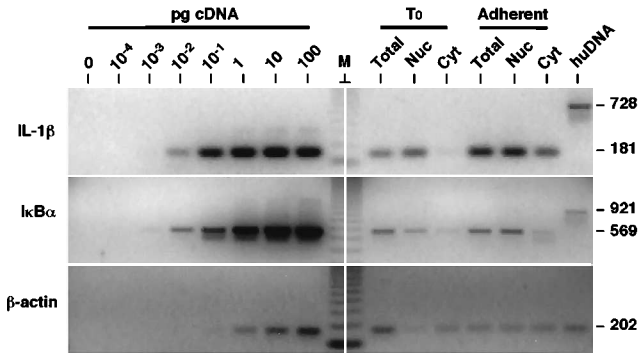

FIG. 7. Mature IκBα/MAD-3 mRNA accumulates in the nuclei of adherent monocytes. Total, nuclear (Nuc), and cytoplasmic (Cyt) RNAs were isolated from freshly isolated (0 min) monocytes or from monocytes stimulated by adherence for 20 min (Adherent) as described in Materials and Methods. Purified RNAs were reverse transcribed and subsequently analyzed by PCR for IL-1β and IκBα/MAD-3 cDNAs as indicated. To detect any intron-containing cDNAs, we utilized primers capable of amplifying across intron 4 of the IL-1β gene (16) and across intron 5 of the IκBα/MAD-3 gene (36) (see Materials and Methods). Presented here are negatives of the ethidium-stained agarose gels used to resolve the PCR-amplified products. The PCR-amplified products of cDNA dilution curves and of human genomic DNA (huDNA) were used as controls (as were the PCR-amplified products for β-actin). DNA size standards are shown (123-bp ladder [lane M]; note that for the IκBα gel, the smallest visible size standard is that corresponding to 369 bp). To the right are the sizes of PCR products corresponding to the precursor and mature forms of mRNAs for IL-1β (728 and 181 bp, respectively), IκBα (921 and 569 bp, respectively), and β-actin (202 bp).

adhesion did not. In this study, we used adherence to tissue culture plastic as a model for adherence to components of the extracellular matrix. Although adherence to plastic may involve occupancy of multiple adhesion receptors (24, 63), we have recently obtained data for monocytes adhered to immobilized fibronectin, collagen, and laminin (major ligands for β1 integrins [2]) indicating that the results will be similar to those obtained with plastic. Namely, adherence to immobilized extracellular matrix components increased the transcriptional rates of several immediate-early cytokine genes but did not increase transcription of the IκBα/MAD-3 gene (data not shown). Taken together, our results suggest that regulation of IκBα protein levels during monocyte adherence requires neither transcriptional activation of the IκBα/MAD-3 gene nor stabilization of IκBα/MAD-3 mRNA, although both mechanisms are clearly implicated in the expression of other immediate-early genes, such as IL-1β.

Degradation and subsequent resynthesis of IκBα protein are commonly observed as a consequence of cytokine, phorbol myristate acetate, and LPS stimulation of myeloid, epithelial, and fibroblast cells (9, 11, 33, 48). The rate at which IκBα protein was degraded varied among different cell lines, but in each of these studies translocation of the RelA/p50 species of NF-κB paralleled the loss of IκBα protein. In U937 cells, replacement of IκBα was fairly rapid, requiring 20 to 40 min, while in HeLa, Jurkat, and THP-1 monocytic cells, replacement required 1 to 2 h (9, 11, 17, 60). Here we show that adherence stimulates primary human monocytes to rapidly (within 5 min) degrade IκBα. As in other studies with other stimuli and cell types, loss of monocytic IκBα protein was quickly compensated for by new protein synthesis, but in monocytes, resynthesis of IκBα seemed to occur more rapidly than in other cell types (10 to 20 min; Fig. 8 contains a summary of the IκBα data). The kinetics of IκBα replacement indicate that synthesis of new IκBα protein was initiated immediately following adherence. Indeed, the line representing the rate of replacement of IκBα extrapolates through the origin, suggesting that in monocytes, IκBα is continually synthesized at a constant rate, even in unstimulated cells.

In unstimulated cells of lymphoid and epithelial lineages, IκBα protein has a relatively short half-life of approximately 30 min (48). The continual degradation and replacement of IκBα may indicate a requirement for low basal levels of nuclear NF-κB activity. Our present data support this hypothesis, since we detected nuclear κB-binding activity in unstimulated monocytes. Moreover, our results indicate that unstimulated monocytes maintain low basal levels of transcription for several NF-κB-dependent cytokine genes, including IL-1β. Adhesion induced rapid translocation of additional κB-binding activity to the nuclei of adherent monocytes. This translocation paralleled the loss of IκBα protein and was accompanied by a striking increase in the transcriptional rates of numerous immediate-early cytokine genes. We suggest, therefore, that degradation of IκBα in monocytes is an important prerequisite for the adhesion-dependent transcriptional activation of many immediate-early genes associated with inflammation (37).

Analysis of the IκBα/MAD-3 promoter has revealed several potential NF-κB-binding sequences required for activation of IκBα transcription in other cell types (14, 20, 36, 41). Consequently, we expected that transcriptional activation was likely to be responsible for the rapid accumulation of IκBα/MAD-3 transcripts seen following monocyte adherence. In the present study, however, we were unable to demonstrate that monocytes transcriptionally regulate the IκBα/MAD-3 gene during adherence (Fig. 4). Nevertheless, the gene is transcribed, albeit at low levels, in both unstimulated and adherent monocytes. In

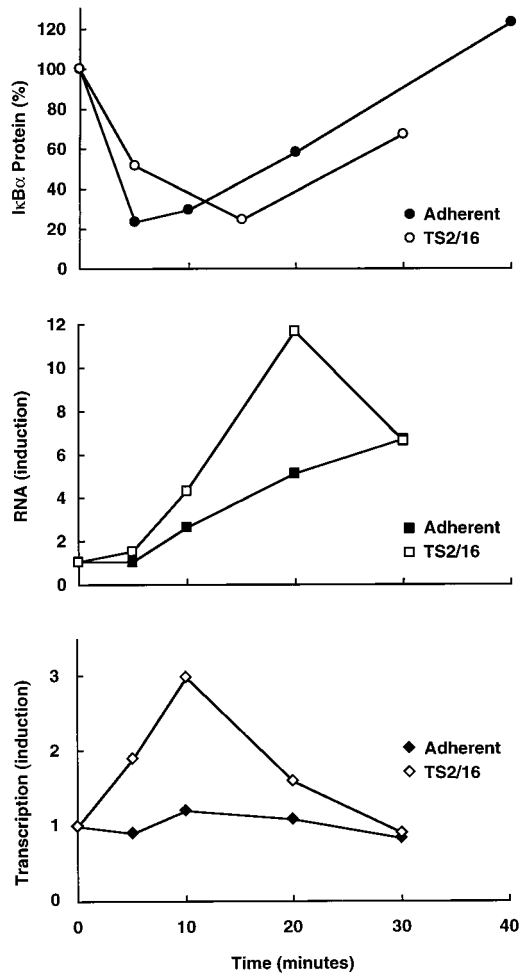

FIG. 8. Summary of the effects on IκBα/MAD-3 expression in monocytes stimulated by adherence (closed symbols) and by nonadherent engagement of the β chain of β1 integrins with MAb TS2/16 (open symbols). The early (0 to 40 min) effects of adherence and integrin engagement are presented for IκBα protein levels (circles), steady-state mRNA levels (squares), and relative levels of transcription induction (diamonds).

marked contrast to adhesion, increased transcription of the IκBα/MAD-3 gene was readily detected following either LPS treatment or nonadherent incubation with an anti-β1 integrin MAb (Fig. 4 and 6). The latter is particularly intriguing since adherence of monocytes to the extracellular matrix components of the basement membrane is mediated primarily through their β1 integrin receptors. These observations appear to indicate that transcriptional regulation of the IκBα/MAD-3 gene is signal specific in monocytes. It has now been well documented that several eukaryotic and viral genes are regulated at the level of transcriptional elongation (for a review, see reference 65). Since we detected no change in monocytic IκBα/MAD-3 gene transcription following adherence, we considered the possibility that any integrin-mediated transcriptional response of the IκBα gene is attenuated during bona fide adherent interactions. Our run-on analyses with 5′ and 3′ IκBα/MAD-3 cDNA (Fig. 4C) and genomic probes, however, indicated that adherent monocytes do not control IκBα/MAD-3 mRNA levels by regulating transcriptional elongation through the IκBα/MAD-3 gene.

Since transcription rates for several cytokine genes are considerably increased during monocyte adherence, the lack of a transcriptional response for the IκBα/MAD-3 gene during adherence was unexpected. Because of this, we considered the possibility that monocytes regulate the stability of IκBα/MAD-3 mRNA in response to adhesion, a particularly attractive model in view of our observation that IκBα/MAD-3 mRNA contains multiple AREs. Functional ARE repeats confer instability on the transcript on which they occur (reviewed in references 19 and 50). The recent demonstration that degradation of granulocyte-macrophage colony-stimulating factor mRNA can be blocked by inhibiting ribosome binding indicates that translational efficiency is closely associated with the decay of short-lived mRNAs containing AREs (1). When we treated nonadherent monocytes with the translational inhibitors cycloheximide and puromycin, we observed increased steady-state levels of IκBα/MAD-3 mRNA approximating those seen following adherence. For cycloheximide, the increase in IκBα/MAD-3 mRNA occurred without increasing the rate of IκBα/MAD-3 gene transcription. Rapid accumulation of various immediate-early cytokine mRNAs also occurred following inhibition of protein synthesis with cycloheximide but not after inhibition with puromycin. Accumulation of IκBα mRNA in the absence of protein synthesis suggested that one aspect of adhesion signalling in monocytes may involve transient interruption of a cotranslational RNA degradation process similar to that which regulates granulocyte-macrophage colony-stimulating factor mRNA levels. We found, however, that the rapid increase in both IκBα mRNA and protein levels following adherence could not be explained by mRNA stabilization mechanisms since half-life of IκBα/MAD-3 mRNA was unaltered by adhesion. Interestingly, the stability of IL-1β mRNA was enhanced nearly 20-fold by adhesion, indicating that monocytes can selectively alter the stability of specific mRNAs during adherent interactions.

Taken together, our current data suggest that two interactive mechanisms may regulate monocytic IκBα/MAD-3 mRNA levels. First, regulation of IκBα/MAD-3 mRNA levels may have a nuclear component which acts subsequent to transcription. Several independent investigations have documented posttranscriptional nuclear events regulating gene expression by differential splicing (reviewed in references 28 and 57) and by differentially affecting the stability of precursor RNAs (39, 58). For example, in describing an investigation of the Rev transactivator of human immunodeficiency virus type 1, Malim and Cullen reported that Rev not only affects the transport of viral mRNAs to the cytoplasm but also prevents nuclear degradation of precursor viral mRNAs (44). In the absence of Rev protein, unspliced viral mRNAs are retained in the nucleus, presumably by splicing factors, and subsequently degraded. In a study investigating Ha-*ras* effects on fibronectin gene expression in human osteosarcoma cells, Chandler and coworkers demonstrated that *ras*-mediated down-regulation of fibronectin gene expression occurred postranscriptionally by an unidentified mechanism which affects accumulation of processed nuclear fibronectin RNAs (13). Such observations raise the interesting possibility that normal human monocytes control mature IκBα/MAD-3 mRNA levels by regulating the rate(s) of splicing and/or decay of nuclear IκBα/MAD-3 RNA. Our current data do not rule out such a mechanism, since adherent monocytes increase steady-state levels of mature IκBα/MAD-3 mRNA without increasing transcription of the IκBα gene. In further support of this idea, our PCR analysis indicates that the early (20 min) increase in IκBα/MAD-3 mRNA levels is primarily nuclear; only low levels of mRNA were detected in the cytoplasm.

We argued above that accumulation of IκBα/MAD-3 mRNA following adhesion could not be explained by mRNA

stabilization mechanisms. In view of this, our observation that translational inhibitors increase steady-state levels of IκBα/MAD-3 mRNA led us to propose an alternative mechanism to explain our current results. Unlike many cytokines, which require secondary signals (like bacterial endotoxin) to initiate translation of adhesion-induced mRNAs (30), translation of IκBα/MAD-3 mRNA seems constitutive in monocytes (Fig. 1 and 8), as it is in murine B cells (48). Therefore, a mechanism involving cotranslational degradation of cytoplasmic IκBα/MAD-3 mRNA similar to that regulating granulocyte-macrophage colony-stimulating factor mRNA levels (1) could explain the observed low levels of cytoplasmic IκBα/MAD-3 mRNA.

Although neither of the above-described mechanisms (regulation of nuclear processing and translation-dependent degradation) can, by itself, completely explain all of the data presented, an adherence-dependent change in the rate(s) of splicing and/or nuclear decay of IκBα/MAD-3 mRNA coupled with cotranslational degradation of cytoplasmic IκBα/MAD-3 mRNA would have the effect of (i) increasing steady-state IκBα/MAD-3 mRNA levels (independently of transcription), (ii) provide for constitutive synthesis of IκBα protein, and (iii) account for low levels of cytoplasmic IκBα/MAD-3 mRNA. The question of whether human monocytes utilize both nuclear and cytoplasmic mechanisms to regulate IκBα/MAD-3 mRNA levels during adhesive interactions is the focus of our current investigations.

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REFERENCES

1. **Aharon, T., and R. J. Schneider.** 1993. Selective destabilization of short-lived mRNAs with the granulocyte-macrophage colony-stimulating factor AU-rich 3′ noncoding region is mediated by a cotranslational mechanism. Mol. Cell. Biol. **13:**1971–1980.
2. **Albelda, S. M., and C. B. Buck.** 1990. Integrins and other cell adhesion molecules. FASEB J. **4:**2868–2880.
3. **Arend, W. P., D. F. Gordon, W. M. Wood, R. W. Janson, F. G. Joslin, and S. Jameel.** 1989. IL-1β production in cultured human monocytes is regulated at multiple levels. J. Immunol. **143:**118–126.
4. **Arend, W. P., F. G. Joslin, R. C. Thompson, and C. H. Hannum.** 1989. An IL-1 inhibitor from human monocytes. Production and characterization of biologic properties. J. Immunol. **143:**1851–1858.
5. **Baldwin, A. S.** 1990. Analysis of sequence-specific DNA-binding proteins by the gel mobility shift assay. DNA Protein Eng. Tech. **2:**73–76.
6. **Baumann, H., and J. Gauldie.** 1994. The acute phase response. Immunol. Today **15:**74–80.
7. **Beekhuizen, H., and R. van Furth.** 1993. Monocyte adherence to vascular endothelium. J. Leukocyte Biol. **54:**363–378.
8. **Beg, A. A., and A. S. Baldwin.** 1993. The IκB proteins: multifunctional regulators of Rel/NF-κB transcription factors. Genes Dev. **7:**2064–2070.
9. **Beg, A. A., T. S. Finco, P. V. Nantermet, and A. S. Baldwin.** 1993. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NF-κB activation. Mol. Cell. Biol. **13:**3301–3310.
10. **Böyum, A.** 1968. Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest. **21**(Suppl. 97)**:**77–89.
11. **Brown, K., S. Park, T. Kanno, G. Franzoso, and U. Siebenlist.** 1993. Mutual regulation of the transcriptional activator NF-κB and its inhibitor, IκB-α. Proc. Natl. Acad. Sci. USA **90:**2532–2536.
12. **Campanero, M. R., R. Pulido, M. A. Ursa, M. Rodríguez-Moya, M. O. de Landázuri, and F. Sánchez-Madrid.** 1990. An alternative leukocyte homotypic adhesion mechanism, LFA-1/ICAM-1-independent, triggered through the human VLA-4 integrin. J. Cell Biol. **110:**2157–2165.
13. **Chandler, L. A., C. P. Ehretsmann, and S. Bourgeois.** 1994. A novel mechanism of Ha-*ras* oncogene action: regulation of fibronectin mRNA levels by a nuclear posttranscriptional event. Mol. Cell. Biol. **14:**3085–3093.
14. **Chiao, P. J., S. Miyamoto, and I. M. Verma.** 1994. Autoregulation of IκBα activity. Proc. Natl. Acad. Sci. USA **91:**28–32.
15. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18:**5294–5299.
16. **Clark, B. D., K. L. Collins, M. S. Gandy, A. C. Webb, and P. E. Auron.** 1986. Genomic sequence for human prointerleukin 1 beta: possible evolution from a reverse transcribed prointerleukin 1 alpha gene. Nucleic Acids Res. **14:** 7897–7914.
17. **Cordle, S. R., R. Donald, M. A. Read, and J. Hawiger.** 1993. Lipopolysaccharide induces phosphorylation of MAD3 and activation of c-rel and related NF-κB proteins in human monocytic THP-1 cells. J. Biol. Chem. **268:**11803–11810.
18. **Cosman, D.** 1987. Control of messenger RNA stability. Immunol. Today **8:**16–17.
19. **Decker, C. J., and R. Parker.** 1994. Mechanisms of mRNA degradation in eukaryotes. Trends Biochem. Sci. **19:**336–340.
20. **de Martin, R., B. Vanhove, Q. Cheng, E. Hofer, V. Csizmadia, H. Winkler, and F. H. Bach.** 1993. Cytokine-inducible expression in endothelial cells of an IκBα-like gene is regulated by NFκB. EMBO J. **12:**2773–2779.
21. **Dhawan, J., A. C. Lichtler, D. W. Rowe, and S. R. Farmer.** 1991. Cell adhesion regulates pro-alpha 1 (I) collagen mRNA stability and transcription in mouse fibroblasts. J. Biol. Chem. **266:**8470–8475.
22. **Eierman, D. F., C. E. Johnson, and J. S. Haskill.** 1989. Human monocyte inflammatory mediator gene expression is selectively regulated by adherence substrates. J. Immunol. **142:**1970–1976.
23. **Frantz, B., E. C. Nordby, G. Bren, N. Steffan, C. V. Paya, R. L. Kincaid, M. J. Tocci, S. J. O'Keefe, and E. A. O'Neill.** 1994. Calcineurin acts in synergy with PMA to inactivate IκB/MAD3, an inhibitor of NF-κB. EMBO J. **13:**861–870.
24. **Fraser, I., D. Hughes, and S. Gordon.** 1993. Divalent cation-independent macrophage adhesion inhibited by monoclonal antibody to murine scavenger receptor. Nature (London) **364:**343–346.
25. **Gerritsen, M. E., and C. M. Bloor.** 1993. Endothelial cell gene expression in response to injury. FASEB J. **7:**523–532.
26. **Gillis, P., and J. S. Malter.** 1991. The adenosine-uridine binding factor recognizes the AU-rich elements of cytokine, lymphokine, and oncogene mRNAs. J. Biol. Chem. **266:**3172–3177.
27. **Gilmore, T. D., and P. J. Morin.** 1993. The IκB proteins: members of a multifunctional family. Trends Genet. **9:**427–433.
28. **Green, M. R.** 1991. Biochemical mechanisms of constitutive and regulated pre-mRNA splicing. Annu. Rev. Cell Biol. **7:**559–599.

28a. **Haskill, S.** Unpublished data.

29. **Haskill, S., A. A. Beg, S. M. Tompkins, J. S. Morris, A. D. Yurochko, A. Sampson-Johannes, K. Mondal, P. Ralph, and A. S. Baldwin.** 1991. Characterization of an immediate-early gene induced in adherent monocytes that encodes IκB-like activity. Cell **65:**1281–1289.
30. **Haskill, S., C. Johnson, D. Eierman, S. Becker, and K. Warren.** 1988. Adherence induces selective mRNA expression of monocyte mediators and proto-oncogenes. J. Immunol. **140:**1690–1694.
31. **Haskill, S., A. Peace, J. Morris, S. A. Sporn, A. Anisowicz, S. W. Lee, T. Smith, G. Martin, P. Ralph, and R. Sager.** 1990. Identification of three related human *GRO* genes encoding cytokine functions. Proc. Natl. Acad. Sci. USA **87:**7732–7736.
32. **Hemler, M. E., F. Sánchez-Madrid, T. J. Flotte, A. M. Krensky, S. J. Burakoff, A. K. Bhan, T. A. Springer, and J. L. Strominger.** 1984. Glycoproteins of 210,000 and 130,000 M.W. on activated T cells: cell distribution and antigenic relation to components on resting cells and T cell lines. J. Immunol. **132:**3011–3018.
33. **Henkel, T., T. Machleidt, I. Alkalay, M. Krönke, Y. Ben-Neriah, and P. A. Baeuerle.** 1993. Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB. Nature (London) **365:**182–185.
34. **Hynes, R. O.** 1992. Integrins: versatility, modulation, and signaling in cell adhesion. Cell **69:**11–25.
35. **Isaacs, K. L., R. B. Sartor, and S. Haskill.** 1992. Cytokine messenger RNA profiles in inflammatory bowel disease mucosa detected by polymerase chain reaction amplification. Gastroenterology **103:**1587–1595.
36. **Ito, C., and A. S. Baldwin.** Personal communication.
37. **Juliano, R. L., and S. Haskill.** 1993. Signal transduction from the extracellular matrix. J. Cell Biol. **120:**577–585.
38. **Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. Le Bail, M. B. Urban, P. Kourilsky, P. A. Baeuerle, and A. Israël.** 1990. The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the *rel* oncogene product. Cell **62:**1007–1018.
39. **Kiledjian, M., and T. Kadesch.** 1991. Post-transcriptional regulation of the human liver/bone/kidney alkaline phosphatase gene. J. Biol. Chem. **266:** 4207–4213.
40. **Kishimoto, T., T. Taga, and S. Akira.** 1994. Cytokine signal transduction. Cell **76:**253–262.
41. **Le Bail, O., R. Schmidt-Ullrich, and A. Israël.** 1993. Promoter analysis of the

gene encoding the IκB-α/MAD3 inhibitor of NF-κB: positive regulation by members of the rel/NF-κB family. EMBO J. **12:**5043–5049.
42. **Lenardo, M. J., and D. Baltimore.** 1989. NF-κB: a pleiotropic mediator of inducible and tissue specific gene control. Cell **58:**227–229.
43. **Lofquist, A. K., J. S. Morris, K. Mondal, L. K. Price, and J. S. Haskill.** Unpublished observations.
44. **Malim, M. H., and B. R. Cullen.** 1993. Rev and the fate of pre-mRNA in the nucleus: implications for the regulation of RNA processing in eukaryotes. Mol. Cell. Biol. **13:**6180–6189.
45. **Mellits, K. H., R. T. Hay, and S. Goodbourn.** 1993. Proteolytic degradation of MAD3 (IκBα) and enhanced processing of the NF-κB precursor p105 are obligatory steps in the activation of NF-κB. Nucleic Acids Res. **21:**5059–5066.
46. **Osipovich, O. A., K. V. Fegeding, N. I. Misuno, T. S. Kolesnikova, I. K. Savostin, A. B. Sudarikov, and N. N. Voitenok.** 1993. Differential action of cycloheximide and activation stimuli on transcription of tumor necrosis factor-a, IL-1b, IL-8, and P53 genes in human monocytes. J. Immunol. **150:** 4958–4965.
47. **Read, M. A., M. Z. Whitley, A. J. Williams, and T. Collins.** 1994. NF-κB and IκBα: an inducible regulatory system in endothelial activation. J. Exp. Med. **179:**503–512.
48. **Rice, N. R., and M. K. Ernst.** 1993. *In vivo* control of NF-κB activation by IκBα. EMBO J. **12:**4685–4695.
49. **Rice, N. R., M. L. MacKichan, and A. Israël.** 1992. The precursor of NF-κB p50 has IκB-like functions. Cell **71:**243–253.
50. **Sachs, A. B.** 1993. Messenger RNA degradation in eukaryotes. Cell **74:**413–421.
51. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
52. **Savant-Bhonsale, S., and D. W. Cleveland.** 1992. Evidence for instability of mRNAs containing AUUUA motifs mediated through translation dependent assembly of a >20S degradation complex. Genes Dev. **6:**1927–1939.
53. **Schütze, S., K. Potthoff, T. Machleidt, D. Berkovic, K. Wiegmann, and M. Krönke.** 1992. TNF activates NF-κB by phosphatidylcholine-specific phospholipase C-induced "acidic" sphingomyelin breakdown. Cell **71:**765–776.
54. **Schwartz, M. A.** 1992. Transmembrane signalling by integrins. Trends Cell Biol. **2:**304–308.
55. **Scott, M. L., T. Fujita, H.-C. Liou, G. P. Nolan, and D. Baltimore.** 1993. The p65 subunit of NF-κB regulates IκB by two distinct mechanisms. Genes Dev. **7:**1266–1276.
56. **Shaw, G., and R. Kamen.** 1986. A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell **46:**659–667.
57. **Smith, C. W. J., J. G. Patton, and B. Nadal-Ginard.** 1989. Alternative splicing in the control of gene expression. Annu. Rev. Genet. **23:**527–577.
58. **Song, M.-K. H., B. Dozin, D. Grieco, J. E. Rall, and V. M. Nikodem.** 1988. Transcriptional activation and stabilization of malic enzyme mRNA precursor by thyroid hormone. J. Biol. Chem. **263:**17970–17974.
59. **Sporn, S. A., D. F. Eierman, C. E. Johnson, J. Morris, G. Martin, M. Ladner, and S. Haskill.** 1990. Monocyte adherence results in selective induction of novel genes sharing homology with mediators of inflammation and tissue repair. J. Immunol. **144:**4434–4441.
60. **Sun, S.-C., P. A. Ganchi, D. W. Ballard, and W. C. Greene.** 1993. NF-κB controls expression of inhibitor IκBα: evidence for an inducible autoregulatory pathway. Science **259:**1912–1915.
61. **Sun, S.-C., P. A. Ganchi, C. Béraud, D. W. Ballard, and W. C. Greene.** 1994. Autoregulation of the NF-κB transactivator RelA (p65) by multiple cytoplasmic inhibitors containing ankyrin motifs. Proc. Natl. Acad. Sci. USA **91:**1346–1350.
62. **Ulmer, A. J., and H.-D. Flad.** 1979. Discontinuous density gradient separation of human mononuclear leukocytes using Percoll as gradient medium. J. Immunol. Methods **30:**1–10.
63. **Wallis, W. J., P. G. Beatty, H. D. Ochs, and J. M. Harlan.** 1985. Human monocyte adherence to cultured vascular endothelium: monoclonal antibody-defined mechanisms. J. Immunol. **135:**2323–2330.
64. **Wang, A., M. Doyle, and D. Mark.** 1989. Quantitation of mRNA by the polymerase chain reaction. Proc. Natl. Acad. Sci. USA **86:**9717–9721.
65. **Wright, S.** 1993. Regulation of eukaryotic gene expression by transcriptional attenuation. Mol. Biol. Cell **4:**661–668.
66. **Yurochko, A. D., D. Y. Liu, D. Eierman, and S. Haskill.** 1992. Integrins as a primary signal transduction molecule regulating monocyte immediate-early gene induction. Proc. Natl. Acad. Sci. USA **89:**9034–9038.