Activation of Distinct Transcription Factors in Neutrophils by Bacterial LPS, Interferon-γ, and GM-CSF and the Necessity to Overcome the Action of Endogenous Proteases†

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ABSTRACT: Human neutrophils can be induced to actively transcribe a number of early-response genes, in particular those encoding cytokines, chemokines, and the high-affinity surface receptor for IgG, FcγRI. Although little is known to date about the regulation of gene transcription in neutrophils, several indications point to a role for distinct transcription factors, such as members of the NF-κB and STAT families. In this study, we investigated whether these transcription factors become activated under stimulatory conditions which are known to induce gene transcription in neutrophils. Unexpectedly, we found that conventional procedures employed to prepare cellular extracts cause the release of proteolytic activities that are normally stored in intracellular granules, resulting in the degradation of various NF-κB/Rel and STAT proteins. To circumvent this problem, we developed an alternative procedure which allowed us to show that in neutrophils, LPS and TNFα induce a NF-κB DNA-binding activity which essentially consists of p50/RelA dimers, and that IFNγ promotes the binding of STAT1 homodimers to the IFNγ response region of the FcγRI promoter. Moreover, we report that neutrophil stimulation with GM-CSF results in the formation of a STAT5-containing DNA-binding activity. Collectively, the current findings open new perspectives about mechanisms that are likely to regulate gene transcription in neutrophils. In addition, the procedure described herein could prove useful in other cell types that express high levels of endogenous proteases.

Neutrophils play an important role in inflammatory responses, in view of their ability to perform a series of effector functions which collectively represent a major mechanism of host defense against injury and infection. Foremost among these functions are the phagocytosis of invading pathogens and the release of proteolytic enzymes, bactericidal proteins, toxic oxygen-derived metabolites, and lipid mediators. In light of studies conducted over the past six years, however, it has become increasingly evident that the contribution of neutrophils to host immunity extends well beyond their traditional role as professional phagocytes. Indeed, neutrophils can be induced to express a number of early-response genes whose products exert a profound influence on the evolution of inflammatory and immune reactions. Among others, these products include cell surface receptors such as FcγRI/CD64, cytokines such as TNFα[1] and IL-1β, and chemokines such as IL-8, Mip-1α, and Mip-1β (*1*−*3*).

The molecular mechanisms regulating inducible gene expression in neutrophils have only begun to be elucidated. It is known, for instance, that the accumulation of mRNA transcripts encoding FcγRI, IL-1β, IL-8, and Mip-1α essentially reflects an increased transcription of the corresponding genes, as opposed to changes in mRNA stability (*1, 4*−*6*). While the nature of the transcriptional events has yet to be determined, several indications point to a role for distinct transcription factors. For instance, a common feature of the TNFα, IL-1β, IL-8, and Mip-1α gene promoters is that they contain enhancer sequences that specifically bind transcription factors of the NF-κB family, an event which is sufficient to confer transcriptional inducibility (reviewed in ref *7*). Moreover, the transcription of the genes encoding these cytokines and chemokines is potently induced by LPS or TNFα in neutrophils (*4*−*6*), and we recently reported that both stimuli can activate NF-κB in these cells (*8*). Likewise, IFNγ strongly activates the transcription of the FcγRI gene in neutrophils (*1*), and a 39-bp region located in the FcγRI gene promoter, termed the gamma-interferon response region (GRR), has been shown to be necessary and sufficient for transcriptional induction by IFNγ in other systems (*9*). The

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[1] Abbreviations: DFP, diisopropyl fluorophosphate; EMSA, electrophoretic mobility shift assays; GRR, γ-interferon response region; GM-CSF, granulocyte-macrophage colony-stimulating factor; hSIE, human c-sis-inducible element; IFN, interferon; LPS, lipopolysaccharide; Mip, macrophage inflammatory protein; NF-κB, nuclear factor-κB; NP40, Nonidet P40; PBMC, peripheral blood mononuclear cells; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor.

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GRR typically binds members of the STAT family of transcription factors, such as STAT1-containing complexes (*9*−*11*).

In the present study, we investigated whether stimuli that induce the transcription of the genes encoding cytokines/chemokines and FcγRI in neutrophils (i.e., LPS and IFNγ, respectively) also have the ability to activate the relevant transcription factors in these cells. We now report that one major obstacle to the study of transcription factor activation in neutrophils is that these proteins become degraded (to various degrees) when neutrophil extracts are prepared following classical protocols. Nevertheless, an alternative procedure was developed, which allows for the detection of intact transcription factors in human neutrophils. Using this procedure, we show that NF-κB and STAT DNA-binding activities can be induced in these cells in response to diverse stimuli.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents. Rabbit antisera to human c-Rel (#1136, against a internal sequence downstream from the nuclear localization signal, and #265, against the C-terminus), p65/RelA (#1207 and #1226, against the N- and C-termini, respectively), p50/NF-κB1 (#1141 and #1157, against the N- and C-termini, respectively), and p52/NF-κB2 (#1267, N-terminal) were a generous gift from Dr. N. R. Rice (NCI−Frederick Cancer Research and Development Center, Frederick, MD). The specificity of these antisera has been extensively characterized (*12*, *13*). Anti-STAT1 antibodies (C1 and C2, raised against residues 741−750 and 712−728, respectively) were kindly provided by Dr. K. Ozato (National Institute of Child Health and Human Development, NIH, Bethesda, MD) (*14*). Commercial antibodies to human c-Rel (sc-70X, raised against residues 152−176), to p65/RelA (sc-372X, C-terminal), to p50 (sc-114X, against the nuclear localization signal), to RelB (sc-226X, C-terminal), to STAT1 (sc-464, against residues 613−739), to STAT3 (sc-482X and sc-483X, against residues 750−769 and 626−640, respectively), and to STAT5 (sc-835X, C-terminal) were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). In addition, antibodies to STAT1 (against residues 1−194, referred to as N1) and to STAT5 (against residues 451−649) were obtained from Transduction Laboratories (Memphis, TN). Finally, an antiserum to myeloperoxidase was obtained from Dr. R. Mantegazza (University of Trieste, Italy). Rh IκB-α (*15*) was kindly provided by Dr. R. T. Hay (Division of Cell and Molecular Biology, University of St. Andrews, Scotland). For the analysis of native NF-κB complexes, an oligonucleotide containing tandemly repeated NF-κB sites identical to those of the HIV promoter was used (5′-gatcaGGGACTTTCCgctgGGGACTTTCC-3′); an identical oligonucleotide containing mutated NF-κB sites (underlined are the altered nucleotides: 5′-AATACTTTCC) was also synthesized. For the analysis of STAT protein complexes, two oligonucleotides were used: a GAS consensus sequence corresponding to the high-affinity synthetic derivative of the human c-sis-inducible element, hSIE/m67 (5′-gtcgaCATTTCCCGTAAATCg-3′) (*16*), or the GRR of the FcγRI/CD64 gene promoter (5′-CTT TTC TGG GAA ATA CAT CTC AAA TCC TTG AAA CAT GCT-3′) (*9*). Ficoll-Paque and poly (dI-dC) were from Pharmacia (Uppsala, Sweden). Horseradish peroxidase-linked donkey anti-rabbit antibody, the enhanced chemiluminescence (ECL) detection kit, and [γ-^{32}P]-ATP were from Amersham (Little Chalfont, U.K.). RPMI 1640 was from GIBCO−BRL (Gaithersburg, MD), and low-endotoxin FCS (<6 pg/mL) was from Hyclone (Logan, UT). Rh IFNγ was kindly provided by Dr. G. Garotta (Hoffmann-LaRoche, Basel, Switzerland); rh GM-CSF was a generous gift from the Genetics Institute (Boston, MA); and rh TNFα was from Bachem (Hannover, Germany). Acetylated BSA, DFP, peroxidase-linked goat anti-mouse antibody, PMSF, and a rabbit anti-human lactoferrin antibody were from Sigma-Aldrich (St. Louis, MO). Aprotinin, bestatin, leupeptin, pepstatin, phosphoramidon, and AEBSF were from Boehringer-Mannheim (Mannheim, Germany). All other reagents were molecular biology grade, and all buffers and solutions were prepared using pyrogen-free water.

Cell Isolation and Stimulation. Neutrophils were isolated from the peripheral blood of healthy donors under endotoxin-free conditions by a modification of the method of Boyum (*17*), as described earlier (*18*). Peripheral blood mononuclear cells (PBMC) were enriched in monocytes by centrifugation over Percoll cushions, as previously described (*19*). As determined by esterase and Wright stainings, the final neutrophil suspensions consistently contained fewer than 0.5% monocytes, and the monocyte-enriched suspensions contained 15−35% contaminating lymphocytes. Cell viability exceeded 98% after 3 h in culture, as determined by trypan blue exclusion. Purified cell populations were resuspended in RPMI 1640 supplemented with 5% low-endotoxin FCS, at a final concentration of 5×10^6 cells/mL, and incubated in polypropylene tubes with occasional agitation. Cells were allowed to equilibrate for 15 min at 37 °C, prior to stimulation with LPS, IFNγ, GM-CSF, or TNFα (or their diluent, RPMI 1640), for the indicated times.

Cell Fractionations. After the desired incubation period with the stimuli, aliquots of the cell suspensions were transferred into precooled tubes containing equivalent volumes of ice-cold RPMI 1640 supplemented with DFP (2 mM, final concentration), prior to centrifugation at 2000*g* for 2 min at 4 °C. Cell pellets were washed and resuspended in ice-cold lysis buffer (10 mM HEPES pH 7.90, 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM EGTA, 0.5 mM DTT) containing an antiprotease cocktail (2 mM DFP, 1 mM PMSF, 1 mM AEBSF, 0.34 mM phosphoramidon, 40 μg/mL bestatin, and 10 μg/mL each of aprotinin, leupeptin, and pepstatin A, final concentrations), at a cell concentration of 5×10^7/mL. Following a 10-min incubation on ice, NP40 was added (0.15% final concentration), and samples were vortexed for 10 s, prior to centrifugation at 800*g* (10 min, 4 °C). The supernatants (nonnuclear fractions) were collected, and the nuclear pellets were washed in lysis buffer containing the antiprotease cocktail. Nuclear integrity was routinely verified by light microscopy. Alternatively, neutrophils were disrupted by nitrogen cavitation, using a modification of a previously published procedure (*20*). Incubations were stopped as described above, and the cells were pelleted and resuspended (at 5×10^7/mL) in ice-cold relaxation buffer (10 mM PIPES pH 7.30, 30 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1.25 mM EGTA, 0.5 mM DTT) containing the anti-protease cocktail. When the cellular fractions or extracts were to be analyzed for STAT proteins, phosphatase inhibitors (1 mM Na_3VO_4, 50 mM NaF) were also included in the lysis buffer. Cells were pressurized

under a N_2 atmosphere (350 psi, 20 min at 4 °C) with constant stirring in a nitrogen bomb (Parr Instrument Co., Moline, IL). Cavitates were spun at 1000*g* (10 min, 4 °C), to pellet most of the nuclei, and the resulting supernatants were recentrifuged (1000*g*, 10 min, 4 °C) to pellet the remaining nuclei. Both nuclear pellets were combined and washed in relaxation buffer containing the antiprotease cocktail. The 1000*g* (post-nuclear) supernatants were centrifuged at 14000*g* (15 min, 4 °C) to pellet neutrophil granules, and the resulting 14000*g* supernatants are referred to as cytoplasmic fractions. Finally, neutrophil cytoplasmic granules were isolated following nitrogen cavitation of unstimulated cells as described above, with the exception that the relaxation buffer contained no protease inhibitors. Post-nuclear supernatants were then spun at 14000*g* (15 min, 4 °C), and the resulting pellets were washed twice in relaxation buffer. Such granule preparations were kept for up to 1 h on ice before being used in binding reactions.

Extract Preparation. Nuclear extracts were prepared by a modified Dignam procedure (*21*), as follows. Washed nuclear pellets from NP40-disrupted cells were resuspended in ice-cold nuclear extraction buffer (20 mM HEPES pH 7.90, 400 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 10% (v/v) glycerol) containing the antiprotease cocktail, in a volume (in μL) equaling 1.5 times the number of million cell equivalents. Following a 20-min incubation on ice (with occasional mixing), the samples were centrifuged (14000*g*, 15 min, 4 °C); the resulting supernatants (the nuclear extracts) were aliquoted and stored at −70 °C. For cavitation-disrupted cells, washed nuclear pellets were resuspended in ice-cold relaxation buffer containing the antiprotease cocktail; NaCl was then added to 400 mM (final concentration). Samples were incubated (20 min on ice, with occasional mixing) prior to centrifugation (14000*g*, 15 min, 4 °C); glycerol was then added to the supernatants (10% final concentration), which were aliquoted and stored at −70 °C. Cytoplasmic or non-nuclear fractions were supplemented with glycerol and NaCl (10% and 150 mM final concentrations, respectively), incubated on ice for 20 min, and centrifuged (14000*g*, 15 min, 4 °C). The resulting supernatants (the cytoplasmic or non-nuclear extracts) were aliquoted and stored at −70 °C. Finally, whole-cell extracts were prepared using three different procedures. Cells were resuspended in a high-salt buffer (20 mM HEPES pH 7.90, 400 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 10% (v/v) glycerol) supplemented with the antiprotease and antiphosphatase cocktail. Cell disruption was achieved either by three freeze−thaw cycles in liquid nitrogen or by the inclusion of 0.2% Triton X-100 in the high-salt buffer, followed by a 10-min incubation on ice; samples were then centrifuged (14000*g*, 20 min, 4 °C), and the supernatants (the whole-cell extracts) were aliquoted and stored at −70 °C. Alternatively, neutrophils were resuspended in relaxation buffer containing the antiprotease and antiphosphatase cocktail, and disrupted by nitrogen cavitation; raw cavitates were supplemented with glycerol and NaCl (10% and 400 mM final concentrations, respectively), incubated on ice for 15 min, and centrifuged (14000*g*, 15 min, 4 °C). The resulting supernatants (the whole-cell extracts) were aliquoted and stored at −70 °C.

Electrophoretic Mobility Shift Assays (EMSA). The various extracts (amounts used are specified in the figure legends) were analyzed in EMSA, as described previously for NF-κB (*8*) or for STAT-containing complexes (*22*). Samples were electrophoresed on 6% gels at 4 °C in 0.5X TBE; dried gels were exposed to Kodak X-OMAT AR film at −70 °C with intensifying screens.

Electrophoreses and Immunoblots. Nuclear fractions (obtained by NP40 lysis or nitrogen cavitation) were resuspended in 100 μL of DNAse buffer (20 mM HEPES pH 7.50, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM EDTA) containing 10 units of DNAse I and the antiprotease cocktail, and incubated for 30 min at 37 °C. Reactions were stopped by the addition of an equal volume of prewarmed (80 °C) sample buffer 2X (50 mM TrisBase pH 6.80, 4% sodium dodecyl sulfate (w/v), 10% 2-mercaptoethanol (v/v), 20% glycerol (v/v)); samples were then heated for 5 min at 95 °C and used immediately or stored at −20 °C. For other subcellular fractions, as well as for the various salt extracts, concentrated sample buffer (pre-warmed at 80 °C) was directly added to yield a final concentration of 1X sample buffer, prior to a 5-min incubation at 95 °C. For the analysis of NF-κB proteins, samples were electrophoresed on 15% denaturing gels prepared according to Thomas & Kornberg (*23*), while 7.5% Laemmli gels (*24*) were used for the analysis of STAT proteins. Proteins were transferred onto nitrocellulose membranes at a constant 20 V for 60 min in a Transblot semidry transfer cell (BioRad); transfer efficiency was visualized by Ponceau Red staining. The membranes were first blocked for 60 min at 37 °C in TBS (25 mM Tris-HCl pH 7.60, 0.2 M NaCl, 0.15% Tween 20) containing 1.5% low-fat milk and further incubated (60 min, 37 °C) in the presence of the desired primary antibody. Antibody dilutions were as follows: anti-p50 (#1141, 1:1500; #1157, 1:3000); anti-p65 (#1207 or sc-372X, 1:1500); anti-c-Rel (#265, 1:20000); anti-STAT1 (sc-464, 1:500); anti-STAT3 (sc-483X, 1:2000); anti-STAT5 (from Transduction Laboratories, 1:300); and anti-lactoferrin and anti-myeloperoxidase, 1:1500. The membranes were then washed three times with 150 mL of TBS and incubated for 45 min at 37 °C in TBS containing horseradish peroxidase-linked secondary antibody (anti-rabbit or anti-mouse, diluted 1:10000 or 1:2000, respectively). After three washes, the signal was revealed with the ECL reagent, according to the manufacturer's instructions.

RESULTS

NF-κB/Rel Proteins and DNA-Binding Activities are Degraded by Proteolytic Activities in Extracts from Human Neutrophils Disrupted by Detergent Lysis. When we initially investigated whether inflammatory stimuli activate NF-κB in neutrophils, we stimulated the cells in the presence or absence of 1 μg/mL LPS, prior to NP40 lysis and EMSA analysis of the nuclear extracts. For comparison, nuclear extracts from similarly treated autologous PBMC were also analyzed. As shown in Figure 1A, a NF-κB DNA-binding activity was detected in resting neutrophils whose intensity was enhanced following LPS stimulation of the cells. A singular characteristic of this complex is that it migrated faster than the major LPS-inducible NF-κB DNA-binding activity present in nuclear extracts from PBMC (Figure 1A, arrowhead), which consisted of p50/RelA heterodimers, as previously reported (*25*). Competition experiments indicated that the neutrophil complex was specific, as its detection was

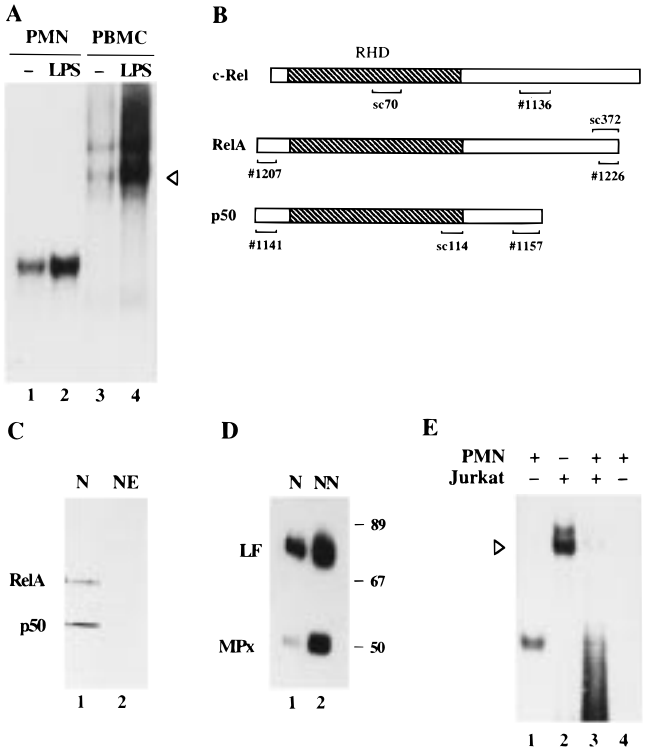

FIGURE 1: Absence of intact NF-κB/Rel proteins and DNA-binding activities in extracts from detergent-lysed human neutrophils, due to endogenous proteolytic activities. (A) Neutrophils ("PMN") or autologous PBMC were cultured in the presence or absence of 1 μg/mL LPS, for 20 min at 37 °C. Nuclear extracts were prepared following NP40 lysis of the cells and analyzed in EMSA using a consensus NF-κB probe. The amount of extract used in the binding reactions corresponded to 2.4×10^6 cell equivalents (15 μg of protein) for neutrophils and 0.3×10^6 cell equivalents (1.5 μg of protein) for PBMC. The migration of p50/RelA dimers is indicated by an open arrowhead. (B) Graphical representation of the epitopes recognized by various antibodies to NF-κB/Rel proteins. Not depicted are anti-RelB (sc-226) and anti-p52 (#1267) antibodies. (C) Neutrophils were stimulated with 1 μg/mL LPS for 20 min at 37 °C; the cells were then disrupted by NP40 lysis. The resulting nuclear fractions ("N") were either directly processed for SDS−PAGE analysis or first extracted in a high-salt buffer to yield nuclear extracts ("NE"); 10^6 cell equivalents were analyzed in each case. Immunoblots were first performed using an anti-RelA antibody (sc-372X); membranes were subsequently reblotted using an anti-p50 antiserum (#1157). (D) Unstimulated neutrophils were disrupted by NP40 lysis, and the resulting nuclear ("N") and nonnuclear ("NN") fractions were processed for SDS−PAGE (using 0.5×10^6 cell equivalents/well). Immunoblots were first performed using an anti-myeloperoxidase (MPx) antiserum; membranes were subsequently reblotted using an anti-lactoferrin (LF) antibody. Molecular weight markers are indicated (in kDa). (E) Nuclear extracts from TNF-activated Jurkat cells (5 μg of protein) or from resting neutrophils disrupted by NP40 lysis ("PMN", 0.2×10^6 cell equivalents/1.1 μg of protein) were incubated either by themselves (lanes 2 and 4, respectively) or together (lane 3), for 30 min at room temperature, prior to EMSA analysis. For comparison, a greater amount of nuclear extract from resting neutrophils (2×10^6 cell equivalents) was also included on the gels (lane 1). The migration of p50/RelA dimers is indicated by an open arrowhead. Each of the experiments depicted in this figure is representative of at least three.

competed by an excess of cold probe, whereas unlabeled mutant probe proved ineffective (data not shown). Supershift experiments were also performed, using various antibodies to NF-κB/Rel proteins; the epitopes recognized by these antibodies are graphically represented in Figure 1B. Surprisingly, none of the antibodies affected the migration or detection of the neutrophil complex (data not shown). Similarly, the inclusion of rh IκB-α in the binding mixtures (up to 200 ng) failed to compete with the detection of the neutrophil complex, whereas authentic NF-κB dimers present in nuclear extracts from TNF-activated Jurkat cells were efficiently displaced by rh IκB-α (data not shown). Finally, experiments in which the fast-migrating neutrophil band was UV-cross-linked to the NF-κB probe and resolved on SDS−PAGE revealed a radiolabeled band of about 70 kDa, a size which did not correspond to that of either c-Rel, RelA, or p50. Together, these observations strongly indicate that the fast-migrating band detected in detergent-lysed neutrophils is not NF-κB.

Our inability to detect authentic NF-κB complexes in neutrophil nuclear extracts prompted us to verify whether NF-κB/Rel proteins are present at all in the nuclei of activated neutrophils. To this end, nuclear fractions from LPS-activated cells, as well as the corresponding salt extracts, were processed for immunoblot analysis. Both RelA and p50 were clearly present in these nuclear fractions (Figure 1C) and appeared to be intact, insofar as they comigrated with the RelA and p50 bands detected in cellular extracts from Jurkat cells, as previously reported (*8*). In contrast, p50 and RelA were undetectable in neutrophil nuclear extracts prepared from the same nuclei (Figure 1C). Similarly, neither protein was detectable in the nuclear pellets recovered following salt extraction (data not shown), thereby ruling out the possibility that p50 or RelA might have been inefficiently extracted from nuclear fractions. Similar results were obtained using antisera #1141 and #1207, which recognize a different portion of p50 and RelA, or when a c-Rel antibody was used.[2] Together, these observations suggested that nuclear NF-κB/Rel proteins became degraded during the high-salt extraction step. Since neutrophils contain large amounts of proteolytic enzymes stored in intracellular granules, we investigated the distribution of selected granule constituents following NP40 lysis of the cells. To this end, immunoblots were performed on nuclear and nonnuclear fractions, using antisera raised against the azurophil granule marker, myeloperoxidase, or the specific granule marker, lactoferrin. Figure 1D shows that whereas both proteins were predominantly detected in non-nuclear fractions, substantial amounts were nevertheless present in the corresponding nuclear fractions. The latter observation indicated that a certain proportion of the proteases present in neutrophil granules might similarly associate with nuclear structures during cell lysis and subsequently degrade NF-κB/Rel proteins during nuclear extraction. To further explore this possibility, nuclear extracts from unstimulated neutrophils were co-incubated with nuclear extracts from TNF-activated Jurkat cells, prior to EMSA analysis. As shown in Figure 1E, this resulted in a nearly complete loss of the NF-κB dimers from Jurkat cells; similar results were obtained using nuclear extracts from LPS-activated neutrophils or neutrophil non-nuclear extracts.[2] The presence in both nuclear and nonnuclear extracts of proteolytic activities which degrade NF-κB dimers strongly suggested that proteases from neutrophil granules become solubilized as a consequence of NP40 lysis, and partition in both subcellular fractions. To ascertain that granule-derived proteases can indeed process

[2] P. P. McDonald, unpublished data.

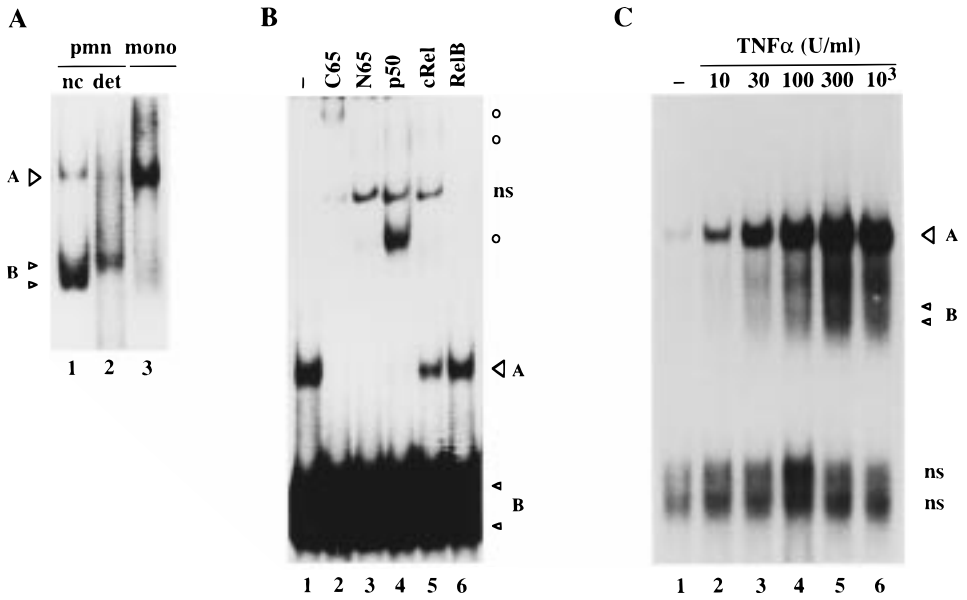

FIGURE 2: Detection and characterization of undegraded NF-κB DNA-binding activities in stimulated human neutrophils. (A) Neutrophils ("pmn") were stimulated with 1 μg/mL LPS (20 min, 37 °C); whole-cell extracts were then prepared by two different techniques and analyzed in EMSA using a consensus NF-κB probe. Lane 1: neutrophils were disrupted by nitrogen cavitation ("nc"); the cavitates were then supplemented with NaCl and glycerol (400 mM and 10%, final concentrations), yielding whole-cell extracts. Lane 2: neutrophils were disrupted by detergent lysis ("det") by resuspension in a high-salt (400 mM NaCl) buffer containing 0.2% Triton X-100. The amount of extract used in the binding reactions corresponded to 570 000 cell equivalents, representing 20 and 26 μg of protein (lanes 1 and 2, respectively). As a positive control, nuclear extracts from LPS-activated monocytes ("mono") disrupted by NP40 lysis (2 μg of protein) were also included (lane 3). The migration of p50/RelA dimers is indicated by the large arrowhead; the smaller arrowheads indicate faster-migrating bands present in the neutrophil extracts. (B) Whole-cell extracts were prepared from LPS-activated neutrophils disrupted by nitrogen cavitation, as described in part A, and analyzed in EMSA. Prior to addition of the labeled NF-κB probe, the extracts were incubated without antisera ("−"), or in the presence of antisera raised against the C-terminus of p65/RelA ("C65", #1226), the N-terminus of RelA ("N65", #1207), p50 (#1141), c-Rel (#1136), or with a purified antibody to RelB (sc-226X). The amount of extract used in the binding reactions was 40 μg of protein (1.24×10^6 cell equivalents). Supershifted complexes are indicated by an open circle; a nonspecific band ("ns") present in normal rabbit serum is also indicated. (C) Neutrophils were stimulated (20 min, 37 °C) in the absence ("−") or presence of increasing concentrations of rh TNFα. Cells were disrupted by nitrogen cavitation, and nuclear extracts were then prepared as described in Materials and Methods. The amount of nuclear extract used was 4 μg of protein (1.2×10^6 cell equivalents). Each of the experiments depicted in this figure is representative of at least four.

NF-κB dimers, we co-incubated whole-cell extracts from LPS-activated monocytes with isolated neutrophil granules disrupted by sonication, prior to EMSA analysis. This resulted in a gradual loss of the monocyte p50/RelA complex as a function of the amount of neutrophil granules included in the binding mixtures.[2]

Detection and Characterization of Authentic NF-κB Complexes in Neutrophils. To circumvent the shortcomings associated with detergent lysis of neutrophils (i.e., the release of proteases from intracellular granules), we disrupted the cells by nitrogen cavitation, a procedure which preserves granule integrity (*20*). For comparison, whole-cell extracts were also prepared from neutrophils disrupted in a high-salt buffer containing 0.2% Triton X-100. Figure 2A shows that cavitation-derived whole-cell extracts prepared from LPS-activated neutrophils contained two prominent NF-κB DNA-binding activities: a fast-migrating doublet (small arrowheads), whose upper part comigrated with the band present in whole-cell extracts from detergent-lysed cells, and a slower-migrating complex, whose mobility was similar to that of authentic p50/RelA dimers present in nuclear extracts from LPS-activated monocytes (Figure 2A, large arrowhead). Supershift experiments confirmed that this slower complex mainly consists of RelA and p50 (Figure 2B, lanes 2−4). In addition, an anti-c-Rel antiserum weakly but consistently impaired the detection of the complex (lane 5), while anti-RelB (lane 6) and anti-p52 (not shown) antibodies were ineffective. By comparison, the fast-migrating doublet present in the cavitation-derived whole-cell extracts was unaffected by any of the antisera (Figure 2B), much like the band present in extracts from detergent-lysed neutrophils. That nitrogen cavitation allowed the detection of intact NF-κB dimers in neutrophil whole-cell extracts strongly suggested that the same procedure might prove optimal for the preparation of neutrophil nuclear extracts. Neutrophils were therefore incubated with increasing concentrations of TNFα and cavitated, and nuclear extracts were analyzed in EMSA. Figure 2C shows that TNFα dose-dependently induced the formation of a slow-migrating NF-κB DNA-binding activity, a maximal effect being observed using between 100 and 1000 units/mL of the cytokine. As observed with cavitation-derived whole-cell extracts, the slow-migrating NF-κB complex present in neutrophil nuclear extracts could be supershifted by antisera to p50 or RelA, and (to a lesser extent) by anti-cRel antibodies, as previously reported (*8*). Also worthy of note is that, relative to whole-cell extracts, cavitation-derived nuclear extracts are highly enriched in specific DNA-binding activity (as they do not include a mass of cytosolic proteins), thereby allowing for a better signal-to-noise ratio when dried gels are exposed to film. As a result, we feel that the use of nuclear extracts obtained from cavitated cells represents an optimized procedure for the detection of intact NF-κB complexes in neutrophils. Collectively, the above findings emphasize the foremost impor-

tance of preserving granule integrity in order to detect intact NF-κB in neutrophil extracts. Consistent with this conclusion is that sonication of LPS-activated neutrophil cavitates prior to salt extraction (to deliberately disrupt the granules) prevented the detection of the slower-migrating NF-κB complex in EMSA.[2] Conversely, authentic NF-κB dimers were still detected when the cells were cavitated in a relaxation buffer lacking the protease inhibitors,[2] thereby confirming that the cavitation procedure is gentle enough not to disrupt cellular organelles.

STAT Proteins Are Also Subject to Cleavage by Neutrophil Proteases. We next examined whether other families of transcription factors are adversely affected by the presence of endogenous proteases in neutrophil extracts. First, we investigated whether IFNγ stimulation might induce STAT1-containing complexes in neutrophils, in view of its known ability to do so in monocytic cells (*10*, *11*, *26*). Neutrophils and autologous monocytes were cultured for 15 min in the presence or absence of IFNγ; whole-cell extracts were then prepared by repeated freeze−thaw cycles and analyzed in EMSA using a GRR oligonucleotide probe. Figure 3A shows that, in both cell types, IFNγ promoted the formation of a prominent complex; however, the mobility of this complex was noticeably faster in the neutrophil extracts. In addition, extracts from IFNγ-treated monocytes contained a slower-migrating band, which was consistently undetectable in neutrophil extracts. Similar results were obtained when the neutrophil extracts were analyzed using an hSIE/m67 probe.[2] To determine the subunit composition of these IFNγ-inducible complexes, supershift experiments were performed, using a variety of anti-STAT1 antibodies (the epitopes recognized by these antibodies are represented in Figure 3E). As shown in Figure 3B (upper panel), all antibodies efficiently supershifted the major IFNγ-inducible complex of monocyte extracts; the slower-migrating band was also supershifted by the C-terminal antibodies, but an effect of the N1 antibody toward that band proved dificult to assess, due to the presence of a supershifted complex comigrating in the same area. Antibodies to other members of the STAT family were ineffective toward either complex.[2] These results suggest that the major DNA-binding activity present in monocyte extracts consists of STAT1 homodimers, while the slower-migrating complex might represent a STAT1 tetramer, based on similar observations made in other experimental models (*27*, *28*). In contrast, the IFNγ-induced complex detected in neutrophil extracts eluded recognition by antibodies to the N-terminal and endmost C-terminal regions of STAT1, while it was supershifted by a C-terminal antibody ("C2") recognizing upstream residues spanning from 712 to 728 (Figure 3B, lower panel); the complex was unaffected by antibodies raised against other members of the STAT family.[2] Thus, the IFNγ-inducible neutrophil complex appears to represent a STAT1 homodimer whose constituent proteins are cleaved at both the N- and C-termini. In keeping with this interpretation, the neutrophil complex migrated faster than the STAT1 homodimer of monocyte extracts; similarly, the absence in neutrophil extracts of a slower-migrating band, which presumably represents a STAT1 tetramer, is consistent with the requirement of an intact N-terminus for tetramer formation (*27*, *28*). To determine whether the neutrophil complex is indeed a truncated STAT1 homodimer, whole-cell extracts from

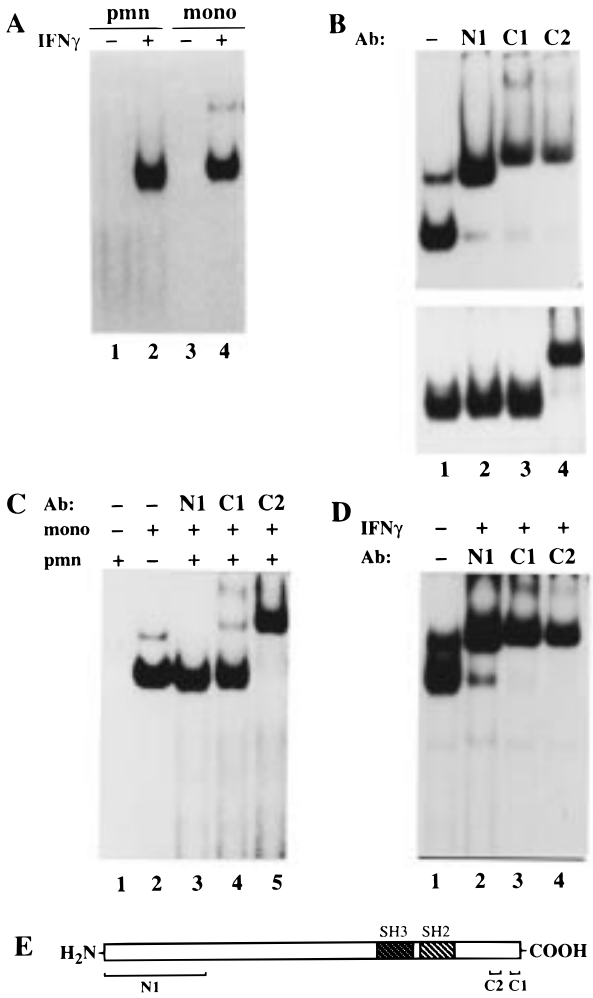

FIGURE 3: Detection and characterization of IFNγ-inducible STAT1-containing complexes in human neutrophils and monocytes. (A) Neutrophils ("pmn") or autologous monocyte-enriched suspensions ("mono") were cultured in the presence or absence of 100 units/mL IFNγ, for 15 min at 37 °C. Whole-cell extracts were then prepared by repeated freeze−thaw cycles in a high-salt buffer (400 mM NaCl), prior to EMSA analysis using a labeled GRR probe. The amount of extract used in the binding reactions was 48 μg of protein for neutrophils (1.12×10^6 cell equivalents), and 8 μg of protein for monocytes (0.34×10^6 cell equivalents). (B) Whole-cell extracts from IFNγ-activated monocytes (upper panel) or neutrophils (lower panel) disrupted by freeze−thaw cycles were analyzed in EMSA as described in part A. Prior to adding the labeled GRR probe, extracts were incubated in the absence ("−") or presence of anti-STAT1 antisera (designated N1, C1, and C2). For monocyte extracts, 13 μg of protein was loaded on the gels (0.52×10^6 cell equivalents); for neutrophil extracts, 60 μg of protein was used (1.54×10^6 cell equivalents). (C) Whole-cell extracts from resting neutrophils disrupted by freeze−thaw cycles ("pmn", 55 μg of protein) or from similarly disrupted IFNγ-activated monocytes ("mono", 8 μg of protein) were incubated either by themselves (lanes 1 and 2, respectively) or together (lanes 3−5) in the binding mixtures, prior to EMSA analysis. In some cases (lanes 4−5), anti-STAT1 antibodies ("C1, C2") were also included in the binding mixture, prior to the addition of labeled GRR probe. (D) Neutrophils were stimulated (15 min, 37 °C) with 100 units/mL IFNγ and disrupted by nitrogen cavitation, and the resulting whole-cell extracts were analyzed in EMSA. Prior to the addition of labeled GRR probe, extracts were incubated either in the absence ("−") or in the presence of anti-STAT1 antisera (N1, C1, and C2). The amount of extract used was 55 μg of protein (1.68×10^6 cell equivalents). (E) Graphical representation of the epitopes recognized by the anti-STAT1 antibodies used in the supershift experiments. Each of the experiments depicted in this figure is representative of at least three.

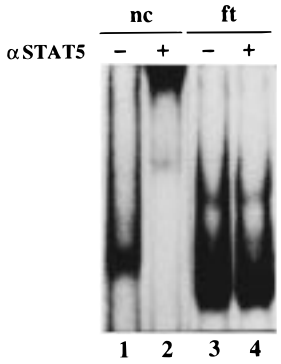

FIGURE 4: Detection of GM-CSF-inducible STAT5 complexes in human neutrophils. Cells were stimulated with 1 nM GM-CSF (15 min, 37 °C); whole-cell extracts were then prepared following disruption of the cells either by nitrogen cavitation ("nc", lanes 1−2), as described in the legend to Figure 2A, or by repeated freeze−thaw cycles in a high-salt buffer ("ft", lanes 3−4), and analyzed in EMSA. Prior to the addition of labeled GRR probe, extracts were incubated either in the absence ("−") or in the presence of an anti-STAT5 antibody ("αSTAT5", sc-835X). The amount of extract used corresponded to 730 000 cell equivalents (25 and 30.6 μg of protein for cavitation and freeze−thaw extracts, respectively). This experiment is representative of three.

resting neutrophils disrupted by freeze−thaw cycles were co-incubated with whole-cell extracts from IFNγ-activated monocytes, prior to EMSA analysis. As shown in Figure 3C, this resulted in the conversion of the major GRR-binding complex of monocyte extracts into a faster-migrating species that was no longer recognized by the C1 antibody, as well as in the loss of the slower-migrating band. Thus, neutrophil-derived proteolytic activites can process the monocyte STAT1-containing complexes into a GRR-binding activity that bears all the characteristics of the one present in extracts from IFNγ-stimulated neutrophils. In contrast, whole-cell extracts prepared following nitrogen cavitation of IFNγ-treated neutrophils contained two GRR-binding activities that were indistinguishable from those present in extracts of IFNγ-stimulated monocytes, based upon their gel mobility and antibody reactivity (Figure 3D).

Because GM-CSF was recently shown to activate STAT5 in human monocytes (*29*), similar experiments were performed in neutrophils to determine whether DNA-binding activities consisting of other STAT family members might also be affected by the cell disruption procedure. Figure 4 shows that cavitation-derived whole-cell extracts prepared from GM-CSF-stimulated neutrophils (lane 1) contained a major GRR-binding activity which appeared to represent STAT5 dimers, insofar as it was completely supershifted by an anti-STAT5 antibody (lane 2). When the same neutrophil suspensions were disrupted by repeated freeze−thaw cycles, however, one major GRR-binding activity (as well as minor ones) was detected in the resulting whole-cell extracts (lane 3), which migrated faster than its counterpart in the cavitation-derived extracts, as would be expected from a degraded complex. Accordingly, our anti-STAT5 antibody failed to recognize any of the GRR-binding activities detected in the freeze−thaw extracts (lane 4). These observations therefore lend further support to the notion that experimental procedures causing the release of endogenous neutrophil proteases lead to a partial degradation of STAT proteins.

In a final series of experiments, we investigated the effect of different cell disruption techniques on the electrophoretic

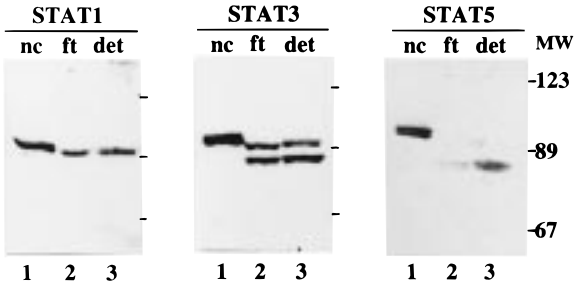

FIGURE 5: Effect of various cell disruption procedures on the detection of individual STAT proteins in human neutrophils. An equal number of unstimulated neutrophils (from the same donor) disrupted by either detergent lysis ("det"), repeated freeze−thaw cycles ("ft"), or nitrogen cavitation ("nc"). The resulting whole-cell extracts were then resolved on 7.5% SDS−PAGE, prior to immunoblot analysis. Immunoblots were performed using antibodies which specifically recognize STAT1 (sc-464), STAT3 (sc-483X), and STAT5 (against residues 451−649). For STAT1 and STAT3, 3×10^6 cell equivalents were loaded on the gels, while 1.8×10^6 cell equivalents were loaded for STAT5. This experiment is representative of three: MW, molecular weight markers (in kDa).

properties of individual STAT proteins. To this end, unstimulated neutrophils were disrupted by either nitrogen cavitation, detergent lysis, or repeated freeze−thaw cycles, and the resulting whole-cell extracts were analyzed by immunoblot. As shown in Figure 5, STAT1 was detected as a protein of about 91 kDa in the cavitation-derived extracts, whereas STAT5 was detected as a doublet of about 95 kDa, which presumably represents the STAT5a and STAT5b species, as both are recognized by our anti-STAT5 antibody. In agreement with our gel shift data, both STAT1 and STAT5 appeared to be partially degraded in whole-cell extracts prepared by either freeze−thaw or Triton X-100 lysis of the cells, as faster-migrating polypeptides were detected using the same antibodies (Figure 5). Similarly, immunoblot analysis of the same samples using an anti-STAT3 antibody revealed that the freeze−thaw and detergent extracts contained two immunoreactive bands, which had a faster mobility on SDS−PAGE than the STAT3 band detected in whole-cell extracts from cavitated neutrophils (Figure 5). These results therefore confirm that preparing neutrophil extracts using classical procedures (as opposed to nitrogen cavitation) results in the cleavage of STAT proteins by endogenous proteolytic activities.

DISCUSSION

In this study, we report that the manner in which human neutrophils are disrupted is of critical importance for the detection of undegraded transcription factors, either as individual denatured proteins or as native multimeric complexes. We indeed show that proteins belonging to the NF-κB/Rel and STAT families are proteolyzed in extracts of neutrophils disrupted by classical means (i.e., by nonionic detergent lysis or repeated freeze−thaw cycles), whereas nitrogen cavitation of the cells allowed the recovery of intact proteins and DNA-binding complexes. A major difference between these cell disruption techniques is that nitrogen cavitation preserves the integrity of neutrophil cytoplasmic granules (*20*), whereas the other two approaches cause the release of granule constituents. This is of particular consequence in view of the fact that neutrophils store considerable

quantities of proteases within their cytoplasmic granules. Accordingly, various extracts from neutrophils disrupted by classical means were found to contain active proteolytic activities that degrade NF-κB and STAT multimers, as determined in EMSA. That these proteolytic activities probably originate from the granules is supported by the finding that purified neutrophil granules efficiently degrade native NF-κB complexes.[2] This being said, it remains somewhat intriguing that NF-κB/Rel proteins underwent extensive degradation despite the inclusion of an elaborate antiprotease cocktail in all our buffers. Nevertheless, it must be emphasized that under conditions in which many millions of cells or nuclei are resuspended in a small volume of extraction buffer, the protease inhibitor concentration may not be sufficient to neutralize the massive quantities of proteases involved. Consistent with this view is that a 30-min preincubation in a buffer containing 2 mM DFP counteracted the action of isolated granules (from 30 000 neutrophils) toward authentic NF-κB dimers, whereas similarly treated granules from 2×10^6 cells retained the ability to proteolyze native NF-κB complexes.[2]

That NF-κB proteins can become proteolyzed by endogenous proteases is not without precedent. For instance, a fast-migrating NF-κB complex detected in regenerating liver cells was reported to contain an N-terminal fragment of RelA (termed p35), as well as a p50 band migrating faster than intact p50 on SDS−PAGE (*30*). Because the degradation of this NF-κB complex was partially prevented if leupeptin (and to a lesser extent, antipain or bestatin) was included in the extraction buffers, it was concluded that nuclear proteolytic activities must account for the degradation of RelA into p35 (*30*). This possibility notwithstanding, it must be stressed that the liver normally contains abundant amounts of elastase-like serine proteases (*31*), and perhaps more importantly, that elevated levels of neutrophil-derived elastase have been detected in regenerating liver (*32*). Thus, p35 formation might have partially resulted from the action of elastase and/or related serine proteases, especially since the extracts from regenerating liver were prepared in the absence of DFP (*30*). In support of this view, a fast-migrating NF-κB complex containing a similarly truncated form of RelA (i.e., lacking the C-terminal domain) was detected in cellular extracts from primary monocytes and various myelomonocytic cell lines following TNF stimulation, unless DFP was included in the extraction buffers (*33*). Finally, it was recently reported that inflammatory stimuli including LPS and TNF activate NF-κB in monocytes, but not in neutrophils; this led the authors to conclude that NF-κB activation is cell type-specific in myeloid cells (*34*). In that study, however, extracts were prepared following NP40 lysis in a buffer that contained 0.1 mM PMSF as the sole protease inhibitor, and we show herein that in neutrophils, authentic NF-κB dimers are indeed undetectable under such conditions, due to extensive degradation. Moreover, the inducible NF-κB complex detected in monocytes was incompletely supershifted by a combination of anti-p50 and anti-RelA antibodies (*34*), which is reminiscent of the properties of TNF-elicited NF-κB complexes present in monocyte extracts when DFP is omitted from the extraction buffers (*33*). Together, these various studies clearly illustrate the importance of neutralizing endogenous proteases when working with cells (or tissues) in which they are expressed at relatively high levels, and we show herein that this is especially criticical in the case of NF-κB in neutrophils.

Relative to NF-κB/Rel proteins, individual STAT proteins were only partially degraded when neutrophil extracts were prepared by conventional procedures (as determined by immunoblot), and retained the ability to dimerize and to bind DNA. Expectedly, the resulting complexes migrated faster than their intact counterparts present in cavitation-derived extracts, as shown herein for STAT1 homodimers. Similar observations were made in the case of STAT3 homodimers in extracts of G-CSF-treated neutrophils,[2] and we provided evidence that this probably applies to STAT5 dimers as well. Although few studies have been conducted to date on the expression or activation of STAT proteins in neutrophils, a re-examination of their findings in the light of the current data further supports the notion that STAT proteins become degraded during extraction. For instance, Tweardy et al. reported that, following IFNγ stimulation, whole-cell extracts from detergent-lysed neutrophils contained a DNA-binding activity which migrated faster than the STAT1 homodimer present in IFNγ-activated HepG2 cells (*35*). This is consistent with the partially proteolyzed STAT1 homodimers which we detected in detergent extracts from IFNγ-treated neutrophils. In the same study, G-CSF stimulation of neutrophils resulted in the formation of a complex which specifically bound to an hSIE/m67 probe in EMSA (*35*). Because single antibodies recognizing each of the known STAT proteins failed to interact with this complex, the authors concluded that it must contain a novel STAT-like protein expressed in neutrophils (*35*). However, in view of our finding that detergent lysis of neutrophils yields STAT complexes that are no longer supershifted by certain antibodies, a more likely explanation is that the G-CSF-elicited DNA-binding activity was a degraded complex. In keeping with this interpretation, we recently showed that, in G-CSF-treated neutrophils disrupted by nitrogen cavitation, the greater part of the inducible hSIE/m67-binding complex could be supershifted by the same anti-STAT3 antibody used in the cited study, while it was completely supershifted by a combination of anti-STAT1 and anti-STAT3 antibodies (*22*). In another study, Brizzi et al. identified an hSIE/m67-binding activity containing both STAT1 and STAT3 in GM-CSF-stimulated neutrophils disrupted by detergent lysis (*36*). Again, this complex migrated faster than authentic STAT1/STAT3 heterodimers from HepG2 cells (*36*), in keeping with our finding that both STAT1 and STAT3 are partially degraded in detergent-lysed neutrophils. Collectively, these observations add to the burden of evidence that failure to prevent granule disruption can adversely affect the conclusions of studies addressing transcription factor activation in neutrophils.

In a broader perspective, numerous studies have shown that human neutrophils can be induced to express genes encoding cytokines or cell surface receptors (among others), and evidence supporting a role for transcriptional events has been presented in several instances. In this context, the current data opens new perspectives about mechanisms that are likely to influence gene transcription in neutrophils. In particular, the ability of several neutrophil agonists (including LPS and TNFα, as shown herein) to activate NF-κB might contribute to the inducible expression of genes encoding several pro-inflammatory cytokines and chemokines, as

discussed in more detail in recent reports from our laboratory (*8*, *37*). Similarly, the ability of IFNγ to induce STAT1 activation in neutrophils is likely to participate in the onset of CD64 gene expression (*38*), as in the case of G-CSF (*22*). In addition, we found that GM-CSF elicits the formation of STAT5 dimers and (on a much smaller scale) that of m67/hSIE-binding activities containing STAT3 and STAT1.[2] Unlike IFNγ and G-CSF, however, GM-CSF does not promote CD64 gene expression in neutrophils, perhaps because it is a poor STAT1 activator in these cells[2] (*39*). In any instance, the ability of GM-CSF to activate different sets of STAT proteins underlines the complexity of the signaling pathways utilized by this cytokine in neutrophils. Finally, the current findings make it obvious that future studies will require that granule integrity be preserved when preparing neutrophil extracts, to avoid potentially misleading experimental artifacts. Indeed, the diversity and abundance of endogenous neutrophil proteases make it probable that their action will affect not only NF-κB/Rel and STAT proteins (as shown herein) but also other transcription factors. In this regard, it has been reported that a member of the ets family of transcription factors, PU.1, was detected as a fast-migrating complex in neutrophil nuclear extracts, and that co-incubation of neutrophil extracts with in vitro-translated PU.1 resulted in its degradation (*40*). Since nitrogen cavitation is a simple and reliable cell disruption procedure which allowed us to detect intact transcription factors in neutrophils, this technique could also be applied to other cell types expressing elevated protease levels.

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