rel Is Rapidly Tyrosine-phosphorylated following Granulocyte-Colony Stimulating Factor Treatment of Human Neutrophils

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Brian J. Druker†§, Manfred Neumann‡, Keiko Okuda**, B. Robert Franz** and James D. Griffin**

From the †Division of Cellular and Molecular Biology, **Division of Tumor Immunology, Dana-Farber Cancer Institute and Harvard Medical School, Boston Massachusetts 02115 and the ‡Freeman Laboratory of Cancer Cell Biology, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

Stimulation of neutrophils with granulocyte-colony stimulating factor (G-CSF) results in an enhanced respiratory burst, prolonged survival, and increased tumor cell killing. The effects of G-CSF are mediated by binding to specific, high affinity receptors. G-CSF receptors lack intrinsic tyrosine kinase activity, but activation of the receptor results in the rapid induction of tyrosine kinase activity. Antiphosphothreonine immunoblots of whole cell lysates prepared from neutrophils show that the G-CSF rapidly induces prominent tyrosine phosphorylation of a protein of a relative molecular mass of 80 kDa. Using monospecific antibodies, the 80-kDa tyrosine-phosphorylated protein has been shown to be p80"~, a proto-oncogene belonging to a family of transcriptional regulators which include NF-kB. The induction of tyrosine phosphorylation of p80"~ was unique to G-CSF in that granulocyte-macrophage colony stimulating factor which also stimulates neutrophils and induces tyrosine phosphorylation does not result in tyrosine phosphorylation of p80"~. The consequences of p80"~ tyrosine phosphorylation are not yet known; however, tyrosine-phosphorylated p80"~ is capable of binding to DNA, and G-CSF stimulation results in an increase in the amount of p80"~ which binds to DNA. These results demonstrate that one of the first biochemical events which occurs in neutrophils following G-CSF stimulation, activation of a tyrosine kinase, leads directly to the tyrosine phosphorylation of p80"~. Thus, the tyrosine kinase activated by G-CSF appears to directly transduce a signal to a protein which functions as a transcriptional regulator.

Granulocyte-colony stimulating factor (G-CSF) has diverse effects on the proliferation, differentiation, and function of mature neutrophils and their precursors (1–3). These biologic effects are mediated through binding of G-CSF to a specific, high affinity cell surface receptor. The human G-CSF receptor is a glycoprotein of relative molecular mass 100–130 kDa (4). Homodimerization of the receptor is believed to be necessary to create a high affinity binding site for G-CSF (5). However, post-receptor signal transduction mechanisms remain largely unknown. Although the G-CSF receptor does not possess intrinsic tyrosine kinase activity, one of the earliest events following G-CSF binding to its receptor is the induction of tyrosine phosphorylation of at least one and possibly several intracellular proteins (6).

Identification of the proteins which become tyrosine-phosphorylated following stimulation of neutrophils with G-CSF is likely to be important in understanding the signal transduction pathways required for neutrophil function. In this study, the major protein which is inducibly tyrosine-phosphorylated following treatment of neutrophils with G-CSF has been identified as p80"~. The p80"~ protein is encoded for by a proto-oncogene (7, 8) which belongs to a family of transcriptional regulatory proteins which include v-rel, the oncogene product of the avian reticuloendotheliosis virus strain T (rev-T) (9), the maternal effects gene dorsal of Drosophila (10–12), and the ubiquitous factor NF-kB (13–15). These results demonstrate that one of the first biochemical events which occurs in neutrophils following G-CSF stimulation, activation of a tyrosine kinase, leads directly and rapidly to the tyrosine phosphorylation of p80"~. Thus, the tyrosine kinase activated by G-CSF appears to directly transduce a signal to a protein which functions as a transcriptional regulator.

MATERIALS AND METHODS

Reagents—Highly purified recombinant human G-CSF and GM-CSF were gifts of Dr. Steven Clark and Gordon Wong, Genetics Institute, Cambridge, MA. Endotoxin, tumor necrosis factor, platelet activating factor, C5a, fMet-Leu-Phe, and phosphor ester were purchased from Sigma.

Antiserum—The antiphosphothreonine antibody 4G10 was generated using phosphotyramine as the immunogen and was used as described (16). The rel antiserum is a polyclonal rabbit antiserum prepared against a synthetic peptide corresponding to the unique carboxyl-terminal 16 amino acids of human p80"~ (17, 18).

Preparation of Human Neutrophils—Normal peripheral blood was obtained from healthy adult volunteers and fractionated on a Ficoll-Hypaque density step gradient (1.077 g/ml). The pellet was washed twice with Hank's balanced salt solution (Life Technologies, Inc.). Red blood cells were lysed by incubation for 30 min at 4 °C in ammonium chloride (8.29 g/liter, pH 7.2). The neutrophil pellet was resuspended in RPMI 1640 (Life Technologies, Inc.) containing 0.5% bovine serum albumin, Fraction V (Sigma) at 5 × 106 cells/ml.

Stimulation with Factors and Cell Lysis—Neutrophils were pre-treated with 1 mM diisopropyl fluorophosphate (Sigma) for 30 min at 4 °C to inhibit proteases before factor stimulation. Cells were then
warmed to 37 °C and treated with G-CSF at a concentration of 10 ng/ml for 0–60 min. Other factors and concentrations used for stimulation were GM-CSF (10 ng/ml), endotoxin (10 ng/ml), tumor necrosis factor (100 units/ml), platelet-activating factor (10^-6 M), C5a (10^-7 M), FeMet-Leu-Phe (10^-7 M), and phorbol ester (10^-6 M). Following stimulation, cells were washed in phosphate-buffered saline and lysed in lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40) containing 1 mM phenylmethylsulfonyl fluoride (Sigma), 0.15 unit/ml leupeptin (Sigma), and 1 mM sodium orthovanadate (Sigma) at 4 °C for 20 min at a concentration of 10^6 cells/ml. Insoluble material was removed by centrifugation at 4 °C for 10 min at 10,000 x g. Alternatively, cells were lysed in DNA affinity precipitation buffer as described below. It should be noted that we have compared lysates prepared by boiling in SDS to lysates made in NP40 with and without EDTA and have found no significant differences in tyrosine-phosphorylated proteins.

Immunoprecipitation, Gel Electrophoresis, and Immunoblotting—Whole cell lysates (5 x 10^6 cells) were mixed 1:1 with 2 x SDS buffer (19) with 2-mercaptoethanol (Life Technologies, Inc.) and heated at 95 °C for 3 min prior to one-dimensional SDS-polyacrylamide gel electrophoresis with 7.5% polyacrylamide. For immunoprecipitation, lysates containing 1 x 10^6 cells were incubated with 20 μl of 4G10 cross-linked to Protein A-Sepharose (Pharmacia LKB Biotechnology Inc.) at 2 mg/ml as described elsewhere (20) or 5 μl of polyclonal p80^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^
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The results presented here demonstrate that stimulation of neutrophils with G-CSF results in the rapid tyrosine phosphorylation of p80<sup>rel</sup>. G-CSF treatment of neutrophils not only results in the tyrosine phosphorylation of p80<sup>rel</sup> but results in an increase in the amount of p80<sup>rel</sup> which is capable of binding to a kB site. Most of this increase can be accounted for by the tyrosine-phosphorylated p80<sup>rel</sup> species.

The tyrosine phosphorylation of p80<sup>rel</sup> is specific to stimulation of neutrophils with G-CSF. Although GM-CSF is similar to G-CSF in its ability to stimulate the function of neutrophils for tumor cell killing and an enhanced respiratory burst, the proteins which become tyrosine-phosphorylated in neutrophils following GM-CSF treatment are different than those seen inducibly phosphorylated after G-CSF treatment. These data suggest that different kinases become activated following binding of ligand to their cognate receptors. Alternatively, the same kinase could have differing substrate specificity depending on the manner in which it is activated. These data also suggest that the tyrosine kinase pathway of signal transduction utilized by G-and GM-CSF may be responsible for the unique
effects of each of these factors rather than for their overlapping functions. However, it remains possible that these two pathways could converge on common downstream effectors.

The major protein which becomes tyrosine-phosphorylated following stimulation of neutrophils with G-CSF is p80c-rel. The p80c-rel protein is encoded for by a proto-oncogene which belongs to a family of transcriptional regulatory proteins which include v-rel, the oncogene product of the avian reticuloendotheliosis virus strain T (rev-T) (9). Other members of this family include the maternal effects gene dorsal of Drosophila which has a role in the establishment of dorsal/ventral polarity of the Drosophila embryo (10–12). Another well characterized member of this family is the transcription factor NF-kB which is sequestered in an inactive form in the cytoplasm through its association with IκB which in turn is associated with the cytoskeleton by virtue of ankyrin repeats (14, 26, 27). Cellular activation results in the phosphorylation of IκB, releasing NF-κB which translocates to the nucleus where it can bind to promoters or may regulate subcellular localization of p80c-rel (18). The stimulation of neutrophils results in an increase in the binding activity of NF-kB which translocates to the nucleus (18). The functional consequences of p80c-rel tyrosine phosphorylation of p80c-rel isotypes to a kB site. The transcriptional program modified by p80c-rel following G-CSF treatment of neutrophils remains to be determined as does the kinase responsible for the tyrosine phosphorylation of p80c-rel. However, this study provides some initial clues which should be helpful in the dissection of the signal transduction pathways utilized by the G-CSF receptor.

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