Induction of Sp1-p53 DNA-binding Heterocomplexes during Granulocyte/Macrophage Colony-stimulating Factor-dependent Proliferation in Human Erythroleukemia Cell Line TF-1*

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The involvement of Sp1 in regulating cell proliferation in myeloid leukemia cells was determined by measuring the levels and DNA binding activity of Sp1 in TF-1 cells, a human erythroleukemia cell line dependent on granulocyte/macrophage colony-stimulating factor (GM-CSF) for viability and cell growth. DNA binding of Sp1 to a specific double-stranded oligodeoxynucleotide was increased markedly in a dose-dependent manner in proliferating cells in response to GM-CSF compared with growth-arrested or apoptotic cells. Competition experiments and mobility shift interference assays with antibodies against Sp1 as well as wild-type or mutant p53 indicated that GM-CSF-inducible DNA-binding complexes contained both Sp1 and p53 and that these heterocomplexes bound to both p53- and Sp1-binding sequences with high affinity. Immunoprecipitation of nuclear extracts with a p53 antibody indicated that Sp1 was associated as a heterocomplex with p53. Formation of this complex was dependent on the level of p53 since p53 was more abundant in proliferating cells and decreased upon induction of growth arrest and apoptosis by withdrawal of GM-CSF while Sp1 levels remained unchanged. These results suggest that the association of Sp1 with p53 may represent a novel mechanism of growth regulation in cytokine-dependent leukemia cells.

Specific protein-DNA interactions play a major role in regulating the pattern of gene expression (Mitchell and Tjian, 1989). In particular, the expression of inducible genes in response to extracellular signals frequently involves the activation of preexisting transcription factors or the synthesis of new regulatory proteins (Maniatis et al., 1987). In hematopoietic cells, the pleiotropic response elicited by proliferating and differentiating agents is often accompanied by changes in the levels and/or activity of transcription factors (Curran and Franza, 1988; Griffin et al., 1989; Stein et al., 1989; Shabo et al., 1990; Sherman et al., 1990; Nakamura et al., 1991; Adunyak et al., 1991).

Transcription factor Sp1 regulates the transcription of a variety of viral and cellular genes in vitro (Dyanan and Tjian, 1985), and multiple binding sites of varying affinity have been characterized in the SV40 (Dyanan and Tjian, 1983), HIV-1 (Jones et al., 1986), herpes simplex virus (Jones et al., 1985), metallothionein IIα (Mitchell et al., 1987; Imagawa et al., 1987), and MDR1 (Ueda et al., 1987) promoters. Sp1 can act by binding to a distal enhancer as well as a proximal promoter and can form multimers that mediate superactivation and transcriptional synergism between Sp1 sites (Courey et al., 1989; Pascal and Tjian, 1991). Sp1 also appears to act in conjunction with other transcription factors such as AP-2 (Mitchell et al., 1987), CTF/NF-1 (Mitchell et al., 1987; Santoro et al., 1988), OTF-1 (Janson and Peterson, 1990) and with the viral E2 enhancer protein (Li et al., 1991), and these interactions modulate the activity of the promoters involved. A recent report by Kim et al. (1992) indicates that the tumor suppressor Rb can also affect transcription of target genes through direct or indirect interactions with Sp1. Since no common regulatory features have been identified among the promoters bearing actual or putative Sp1 binding sites, it is possible that Sp1 provides a basal level of transcription which is subsequently modulated by its interaction with other regulatory factors.

We reported recently that expression of the c-fes protooncogene in myeloblast cell line K562 confers to these cells the ability to undergo myeloid differentiation (Yu et al., 1989) and is accompanied by a marked activation of the DNA binding and transcriptional activity of transcription factor Sp1 (Borellini et al., 1991). The selection of a doxorubicin-resistant variant of promyelocytic leukemia cell line HL-60 is similarly accompanied by an increase in the DNA binding activity as well as the level of Sp1 (Borellini et al., 1990). Because of the ability of Sp1 to cooperate with a wide array of regulatory proteins and to modulate the transcriptional activity of a variety of promoters, changes in the levels and activity of Sp1 may directly affect the expression of several genes that mediate cell differentiation and proliferation in response to extracellular signals.

In the present study, the human erythroleukemia cell line TF-1 was chosen as a model system in which to investigate the activity of transcription factor Sp1 during GM-CSF-mediated proliferation. Our data show that GM-CSF-dependent proliferation is accompanied by the formation of DNA-binding heterocomplexes between Sp1 and a conformational mutant of the tumor suppressor p53.

EXPERIMENTAL PROCEDURES

Materials—Reagents were obtained from the following sources: [γ-32P]ATP (3,000 Ci/mmol), from Du Pont-New England Nuclear;
poly(dI-dC)-poly(dI-dC), phenylmethylsulfonyl fluoride, and gentamicin, from Sigma; proteinase K, from Bethesda Research Laboratories; RNase A, from Boehringer Mannheim; T4 polynucleotide kinase, from New England Biolabs; human recombinant SP1, from Promega. Double-stranded oligonucleotides for SP1 and p53 binding were obtained from Gibco BRL. Monoclonal antibodies PAb1801 (Banks et al., 1986), PAb421 (Lane and Crawford, 1979), and PAb240 (Gannon et al., 1990) were from Oncogene Science. PAb1801 recognizes an amino-terminal epitope common to wild-type and mutant forms of p53. PAb240 recognizes a conformational epitope present in mutant p53 but not in wild-type p53 in its native form. PAb241 recognizes an epitope in the carboxyl-terminal region of p53. GM-CSF and interleukin 4 (IL-4) were generously provided by Dr. Paul Trotta, Schering-Plough Research Institute. A rabbit polyclonal SP1 antibody raised against Escherichia coli recombinant SP1 was kindly provided by Dr. Krystyna Kilomanski, University of California at Berkeley.

Cell Culture—Human erythroleukemia cell line TF-1 (Kitamura et al., 1989) was kindly provided by Dr. Toshio Kitamura, DNAX, Inc. and was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 40 mM Heps (pH 7.4), 2 mM glutamine, 50 µg/ml gentamicin, and 10 ng/ml GM-CSF at a density of 105-106 cells/ml. In some experiments, the amounts of GM-CSF and interleukin 4 were varied to study the effects of cytokine deprivation on the growth of TF-1 cells. TF-1 cells provide an excellent model system for the study of transcriptional regulation of cytokine-dependent hematopoiesis. A concentration of 1 ng/ml GM-CSF was sufficient to maintain cell viability, although the highest rate of cell proliferation was observed in the presence of 10 ng/ml GM-CSF (data not shown). Since GM-CSF and other cytokines such as erythropoietin and interleukin 3 (IL-3) can extend cell survival by suppressing apoptosis in dependent cells (Koury and Bondurant, 1990; Koury, 1992; Williams et al., 1990; Rajotte et al., 1992), we determined whether apoptosis could be induced in TF-1 cells by GM-CSF deprivation by examining the integrity of chromosomal DNA from TF-1 cells after the withdrawal of GM-CSF (Fig. 1). DNA fragmentation into an oligonucleosomal ladder typical of apoptosis (Wyllie, 1980) was clearly visible at 0.1 ng/ml GM-CSF and after withdrawal of the growth factor for 24 h but not after withdrawal.

RESULTS

GM-CSF Induces Proliferation and Suppresses Apoptosis in TF-1 Cells—The viability and proliferation of human erythroleukemia cell line TF-1 are dependent on the continuous presence of GM-CSF (Kitamura et al., 1989), and therefore, TF-1 cells provide an excellent model system for the study of transcriptional regulation of cytokine-dependent hematopoiesis. A concentration of 1 ng/ml GM-CSF was sufficient to maintain cell viability, although the highest rate of cell proliferation was observed in the presence of 10 ng/ml GM-CSF (data not shown). Since GM-CSF and other cytokines such as erythropoietin and interleukin 3 (IL-3) can extend cell survival by suppressing apoptosis in dependent cells (Koury and Bondurant, 1990; Koury, 1992; Williams et al., 1990; Rajotte et al., 1992), we determined whether apoptosis could be induced in TF-1 cells by GM-CSF deprivation by examining the integrity of chromosomal DNA from TF-1 cells after the withdrawal of GM-CSF (Fig. 1). DNA fragmentation into an oligonucleosomal ladder typical of apoptosis (Wyllie, 1980) was clearly visible at 0.1 ng/ml GM-CSF and after withdrawal of the growth factor for 24 h but not after withdrawal.

FIG. 1. DNA fragmentation induced in TF-1 cells by GM-CSF deprivation. TF-1 cells were grown for 48 h in the presence of the indicated amounts of GM-CSF or in the absence of GM-CSF for 4 or 24 h. DNA from 106 cells was extracted and analyzed as described under "Experimental Procedures." M, 123-base pair DNA marker ladder.
drawal for 4 h. IL-4 did not sustain the proliferation of TF-1 cells, although it extended cell survival, as shown by the lesser extent of DNA degradation (Fig. 1). Electron microscopy confirmed the presence of condensed chromatin and other features characteristic of apoptosis (Cohen et al., 1992) in the majority of cells grown in 0.1 ng/ml GM-CSF (Fig. 2C), but only a small fraction of the cells grown in 1 ng/ml GM-CSF (Fig. 2A) exhibited these features.

Level and DNA Binding Activity of Sp1 in TF-1 Cells—To determine whether growth stimulation or induction of apoptosis by GM-CSF deprivation would result in changes in the levels of Sp1, immunoblotting of nuclear extracts was carried out. Sp1 was characterized as a single band of 100 kDa, and its level was found to be independent of whether cells were maintained in GM-CSF, maintained in IL-4, or deprived of GM-CSF for 24 h (Fig. 3).

The DNA binding activity of Sp1 was next assessed by mobility shift assay with a DNA probe containing the consensus sequences for three Sp1 binding elements. Three DNA-Sp1 complexes were noted in all extracts from TF-1 cells. A major complex (III) was present in all extracts in comparable amounts, whereas two lower mobility complexes (I and II) appeared in cells proliferating in response to GM-CSF and decreased upon withdrawal of GM-CSF (Fig. 4).

Characterization of the GM-CSF-induced DNA-Sp1 Complexes—The DNA binding activity of Sp1 was characterized further by mobility shift competition experiments. The formation of Sp1 complexes I and II was competed very efficiently by the unlabeled Sp1 DNA probe (Fig. 5A), whereas complex III required a larger excess of competitor.

Since the tumor suppressor protein p53 has also been shown to bind to Sp1 consensus sequences adjacent to the SV40 origin of replication (Bargonetti et al., 1991), we determined whether a DNA probe containing a binding consensus for p53 (Kern et al., 1991) would compete with the Sp1 probe (Fig. 5B). The formation of complexes I and II was effectively competed by a 10-fold and an equimolar amount of the p53 competitor, respectively, whereas complex III was unaffected. To rule out the possibility that Sp1 was directly binding to the p53 probe, mobility shift assays were carried out with recombinant human Sp1, but no p53 binding activity was detected (data not shown). Therefore, these results suggest that a p53-like nuclear protein is involved in the formation of DNA-Sp1 complexes that are induced during cellular proliferation in response to GM-CSF.

To determine the identity of the protein involved in heterocomplexes with Sp1, mobility shift experiments were performed in the presence of antibodies against Sp1 as well as wild-type and mutant forms of p53 (Fig. 6). Sp1 complexes I and II were reduced or eliminated by the Sp1 antibody and by a p53 antibody against mutant p53 (PAb240) (Gannon et al., 1990). Antibody PAb1801 that recognizes both wild-type and mutant p53 (Banks et al., 1996) markedly reduced both complex I and complex II. Complex III was largely unaffected by all three antibodies.

To confirm the association of Sp1 with p53, immunoprecipitation experiments were performed (Fig. 7). Antibodies to p53 immunoprecipitated a 100-kDa protein from TF-1 nuclear extracts that reacted specifically with the Sp1 antibody. PAb240 was most effective in communoprecipitating Sp1, indicating that a mutant form or conformation of p53 is associated with Sp1 in TF-1 cells.

DNA Binding Activity of p53 in TF-1 Cells—Mobility shift assays were next conducted with the sequence-specific p53 DNA probe to determine the DNA binding activity of p53 during GM-CSF-induced proliferation (Fig. 8) Two DNA-protein complexes were detected with nuclear extracts from TF-1 cells. The lower mobility complex (I) was more abundant in cells grown in the presence of 1 or 10 ng/ml GM-CSF and less abundant in cells grown in IL-4 or 0.1 ng/ml GM-CSF (Fig. 8). Withdrawal of GM-CSF for 24 h caused a significant decrease in DNA binding activity. Complex II was not significantly affected by the concentration of GM-CSF and was more abundant when higher molar amounts of probe were used, perhaps indicating low affinity binding. The addition of a large excess of poly(dI-dC).poly(dI-dC) or bovine serum albumin did not alter the formation of either complex (data not shown).

The DNA binding activity of p53 was characterized further by mobility shift competition experiments. The unlabeled p53 probe competed efficiently with p53 complex I (Fig. 9A), whereas complex II required a larger excess of competitor. Similarly, the formation of complex I was also eliminated by a 3-10-fold molar excess of the Sp1 competitor (Fig. 9B). Surprisingly, the Sp1 probe was less effective at a 30-fold excess, indicating a biphasic effect with respect to p53 complex formation, and this effect was observed in three separate

**Fig. 2.** Electron microscopy of TF-1 cells. Panel A, cells grown for 48 h in the presence of 1 ng/ml GM-CSF. Magnification, × 6,000. Panel B, cells grown for 48 h in the presence of 5 ng/ml IL-4. The beginning of chromatin condensation is apparent in the nucleus of this cell, although most cells did not show any evidence of apoptosis. Magnification, × 6,000. Panel C, cells grown for 48 h in the presence of 0.1 ng/ml GM-CSF. Chromatin condensation is clearly visible as electron-dense areas in the nucleus. Magnification, × 6,000.
Spl -p53 Heterocomplexes Induced during Proliferation

FIG. 3. Immunoblot of Spl. TF-1 cells were grown in the presence of varying concentrations of GM-CSF. Immunoblotting was performed with a polyclonal antibody raised against recombinant Spl diluted 1:1,000. Each lane contains 25 μg of protein. The relative amount of Spl determined by densitometry in cells treated with IL-4, 0.1, 1, 0.1, 10 ng/ml GM-CSF and 24 h after withdrawal of GM-CSF is 1.0, 1.1, 1.2, 1.1, and 1.1, respectively.

FIG. 4. DNA binding activity of Spl. Mobility shift assay of nuclear extracts (1.5 μg) from TF-1 cells incubated with an Spl probe. Culture conditions are indicated above each lane. I, II, and III indicate specific protein-DNA complexes.

experiments. Binding of complex II to the p53 probe was also competed efficiently by the Spl probe. These results suggest that Spl complexes I and II (Fig. 4) and p53 complex I (Fig. 8) may represent related species. Immunoblotting of nuclear extracts with PAb1801 detected a single band of approximately 50 kDa which increased in amount in cells exposed to increasing concentrations of GM-CSF, whereas lower levels were detected in cells maintained in IL-4 or 0.1 ng/ml GM-CSF (Fig. 10). The effect of GM-CSF on p53 was selective since the total amount of protein/10^6 cells did not vary under the different culture conditions (data not shown).

FIG. 5. Specificity of Spl DNA binding activity. Nuclear extracts from TF-1 cells grown in 1.0 ng/ml GM-CSF were incubated with an Spl probe in the absence or presence of a 1 X, 3 X, 10 X, 30 X, or 100 X molar excess of unlabeled competitor as indicated. Panel A, competition with the Spl probe. Panel B, competition with the p53 probe.

FIG. 6. Effect of Spl and p53 antibodies on Spl DNA binding activity. Nuclear extracts (1.5 μg) from TF-1 cells grown in the presence of 1 ng/ml GM-CSF were preincubated with each antibody for 10 min before the binding reaction was carried out. (-), no antibody; +PIS, 1 μl of preimmune rabbit serum; +Spl Ab, 1 μl of Spl polyclonal antibody; +PAb1801, 2.5 μl of monoclonal antibody PAb1801; +PAb240, 2.5 μl of monoclonal antibody PAb240.

dose-dependent fashion in response to GM-CSF but that it was not a function of increased levels of Spl. The GM-CSF-inducible DNA binding activity was dependent on the formation of heterocomplexes between Spl and a form of p53 that exhibited a mutant conformation recognized by PAb240 (Gannon et al., 1990). These heterocomplexes bound both Spl and p53 consensus sequences with high affinity and specificity under conditions of high stringency (high salt concentration and high amounts of nonspecific DNA competitor). Although nuclear levels of Spl remained constant regardless of the proliferative status of the cells, p53 levels increased in response to growth stimulation by GM-CSF and decreased upon
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97 kDa

FIG. 7. Coimmunoprecipitation of Sp1 and p53. Nuclear extracts from TF-1 cells grown in the presence of 10 ng/ml of GM-CSF were immunoprecipitated with monoclonal antibodies PAb1801, PAb240, or PAb421, separated by SDS-polyacrylamide gel electrophoresis, and immunoblotted with a polyclonal antibody against Sp1.

withdrawal of GM-CSF and onset of apoptosis. Thus, the increased DNA binding activity of Sp1 in proliferating cells was related to the accumulation of p53, whereas culture conditions leading to growth arrest and apoptosis produced the opposite effect. The transient accumulation of p53 in response to growth stimulation has been observed in fibroblasts (Reich and Levine, 1984; Mercer et al., 1984) and appears to mediate cell entry into S phase (Mercer et al., 1982, 1984). Since the biological function of p53 is clearly related to the control of the cell cycle (Levine et al., 1991; Ullrich et al., 1992), factors that affect the levels and DNA binding activity of endogenous p53 may consequently affect cell proliferation. The present study is the first to demonstrate that the DNA binding activity of Sp1 in response to cytokine-dependent proliferation is dependent on the formation of complexes with p53.

The interaction of the heterocomplex with Sp1 binding sites may be a function of either p53 or Sp1. In our study, recombinant p53 was not available to test its binding to Sp1 binding elements; however, recombinant wild-type p53 has been shown to bind specifically to the GC boxes adjacent to the SV40 origin of replication (Bargonetti et al., 1991). Conversely, the binding of the heterocomplex to the p53 consensus sequence is likely to be a function of the p53 component, since recombinant Sp1 did not directly bind to the p53 probe.

Alternatively, Sp1 may require the presence of a cofactor present in nuclear extracts but not in purified Sp1 preparations to bind the p53 consensus. In either case, the present data showing that p53 and Sp1 consensus sequences compete with each other for binding to the heterocomplex suggest that the two oligonucleotides share a common binding site on the protein complex and indicate that the binding of both Sp1 and p53 consensus sequences is probably a function of the same protein. Sp1 complexes I and II show distinct mobilities, and although they both bind Sp1 and p53 sequences, competition studies indicate that complex I has a slightly higher affinity for the Sp1 probe. These observations suggest that Sp1 and p53 can form distinct associations that exhibit different DNA binding affinities, perhaps involving other nuclear proteins. Both Sp1 and p53 have the propensity to form large oligomeric structures in solution, with subsequent changes in their DNA binding activity (Kreiss et al., 1988, 1992; Pascal and Tjian, 1991). The biphasic competition pattern that was repeatedly observed for p53 complex I (Fig. 9B) indeed suggests that several protein monomers take part in the formation of these heterocomplexes. The saturation of an initial set of DNA binding sites may induce a re-

FIG. 8. DNA binding activity of p53. Mobility shift assay with nuclear extracts (1.5 μg) from TF-1 cells incubated with a p53 probe at room temperature for 30 min. Culture conditions are indicated above each lane. I and II denote p53-DNA complexes.

FIG. 9. Specificity of p53 DNA binding activity. Nuclear extracts from TF-1 cells grown in 1.0 ng/ml GM-CSF were used in mobility shift assays with the p53 probe in the absence (−) or presence of 1X, 3X, 10X, 30X, or 100X unlabeled competitor as indicated above each lane. Panel A, competition by the p53 probe. Panel B, competition by the Sp1 probe.

FIG. 10. Immunoblot of p53. TF-1 cells were grown in the presence of varying concentrations of GM-CSF. Immunoblotting was performed with monoclonal antibody PAb1801, which recognizes denatured wild-type and mutant p53. The chemiluminescence reaction was visualized by exposure of the blot to x-ray film for 10 min. Each lane contains 25 μg of protein. The relative amount of p53 determined by densitometry in cells treated with IL-4, 0.1, 1, or 10 ng/ml GM-CSF and 24 h after withdrawal of GM-CSF is 1.0, 2.2, 4.1, 5.3, and 4.0, respectively.
Sp1-p53 Heterocomplexes Induced during Proliferation

Although the species of p53 in TF-1 cells has not been characterized fully, partial sequencing of the p53 cDNA revealed at least one base deletion at codon 251 (Shabon et al., 1992). A single base deletion would produce a truncated protein lacking the DNA binding COOH-terminal domain (Foord et al., 1991; Ulrich et al., 1992). Although we have confirmed the deletion at codon 251 by sequencing, the protein detected by immunoblotting with two different p53 antibodies exhibited a mobility that was identical to p53 from human primary fibroblasts. These observations suggest the presence of either a second allele expressing a full-length protein or the less likely possibility of two additional base deletions to give a full-length p53 with missense mutations.

The ability of antibody PAb240 to inhibit the DNA binding of Sp1 and to coimmunoprecipitate Sp1 suggests that either a mutant form of p53 or a form of p53 resembling the mutant conformation is involved in the formation of these heterocomplexes. A recent study of p53 variants in acute myeloid leukemia patients revealed that 75% of the samples in which p53 was immunoprecipitated with PAb240 contained no mutations (Zhang et al., 1992). Moreover, this same antibody recognition pattern was demonstrated in normal lymphocytes induced to proliferate. Therefore, there is the distinct possibility that alterations in the conformation of p53 could serve as the basis for explaining the increased proliferation of myeloid leukemia cells and is consistent with our results using TF-1 cells.

In contrast to the proliferation-dependent changes in the level of p53, the levels of Sp1 did not change in response to variations in the growth conditions for TF-1 cells. Since heterocomplexes containing Sp1 and p53 were more abundant in proliferating cells than in growth-arrested or apoptotic cells, these data suggest that GM-CSF-dependent proliferation results in the recruitment of transcription factor Sp1 into heterocomplexes with p53. Conversely, growth arrest and apoptosis induced by GM-CSF deprivation would result in a decrease in the amount of p53 which would subsequently cause the release of sequestered Sp1. Thus, the interaction between Sp1 and p53 may have significant repercussions on the transcriptional regulation in leukemic cells. Heterocomplexes between Sp1 and p53 could exhibit an altered selectivity in binding to Sp1 or p53 regulatory elements or a change in their trans-activating activity. The sequestration of Sp1 could also directly affect transcription from Sp1-regulated promoters. A precedent for such a regulatory mechanism was recently reported for the tumor suppressor Rh, which was found to affect transcription of target genes through interactions with Sp1 (Kim et al., 1992). Future studies will address whether the ability of p53 to associate with Sp1 is a property shared by different forms of p53 and what the effects of such interactions are on transcription.

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