RAPID COMMUNICATION

Induction of Sequence-Specific DNA-Binding Factors by Erythropoietin and the Spleen Focus-Forming Virus

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The signal transduction mechanism of erythropoietin (Epo), which regulates growth and differentiation of erythroid cells, is still unclear. Recent studies showing the activation by various ligands of a group of proteins called Stat (signal transducers and activators of transcription) proteins raised the possibility that such proteins may also be involved in the Epo signal transduction pathway. In this report, we show that Epo induces factors that specifically bind to the sis-inducible element and the gamma response region of the Fcγ receptor factor I gene in the Epo-dependent mouse erythroleukemia cell line HCD-57. These factors contain SHC proteins reacted with them. In HCD-57 cells infected with Friend spleen focus-forming virus, which now grow in an Epo-independent manner, the DNA-binding factors were constitutively activated even in the absence of Epo. These results suggest that the factors induced by Epo contain components identical or related to known Stat proteins. It is also suggested that continuous activation of these DNA-binding factors may be responsible for the ability of spleen focus-forming virus to abrogate the Epo-dependence of HCD-57 cells and cause erythroleukemia in susceptible mice. © 1995 by The American Society of Hematology.

ERYTHROPOIETIN (Epo) is a growth factor that plays a major role in the regulation of the growth and differentiation of hematopoietic cells in the erythroid lineage. The genes encoding Epo and the Epo receptor (EpoR) have been isolated, and the relationship between their structure and function has been studied extensively. However, the actual mechanism by which binding of Epo to the EpoR triggers the signal for erythroid cell growth and differentiation is largely unclear. Previous studies showed that stimulation of the EpoR by Epo induces tyrosine phosphorylation of various cellular substrates, such as the EpoR itself, the 85-kD regulatory subunit of phosphatidylinositol 3-kinase, and an unidentified 97-kD protein. Recent studies also showed that Janus kinase 2 (JAK2) is associated with a membrane-proximal region of the EpoR and phosphorylated at its tyrosine residue in response to Epo stimulation. Activation of JAK2 kinase appears to be essential for Epo-induced signal transduction.

There are a family of proteins called Stat (signal transducers and activators of transcription) proteins, which include Stat1 and Stat2, that have been identified through the study of transcriptional regulation in response to stimulation by interferon-α (IFN-α) and IFN-γ. There are an increasing number of studies showing that a variety of other cytokines, hormones, and growth factors can activate a certain set of Stat proteins, including Stat1 through Stat5 and interleukin-4 (IL-4) Stat. After ligand stimulation, specific Stat proteins appear to be phosphorylated at their tyrosine residues in the cytoplasm, to be transported into the nucleus, and to bind to certain target sequences, such as the sis-inducible element (SIE) and the gamma response region (GRR) of the Fcγ receptor factor I gene.

Because some of the protein substrates phosphorylated by Epo stimulation have molecular weights similar to those of Stat proteins and activation of JAK2 kinase was shown to be associated with phosphorylation of Stat proteins, the possibility was raised that these proteins might be involved in the Epo signal transduction pathway. Recently, it was shown that a sequence-specific DNA-binding activity that interacts with the GRR sequence was induced in the human erythroleukemia cell line TF1 in response to Epo stimulation. However, the relationship of the DNA-binding factor to Stat proteins was unclear. In this study, we show that DNA-binding factors that bind specifically to the SIE and GRR sequences are rapidly and transiently induced after Epo stimulation in the Epo-dependent mouse erythroleukemia cell line HCD-57 and that these factors contain components detected by antiphosphotyrosine antibodies and antibodies against Stat1 or Stat3 proteins. We also demonstrate that infection of HCD-57 cells with the polycythemic strain of Friend spleen focus-forming virus (SFFVα), which causes acute erythroblastosis and erythroleukemia in susceptible mice and abrogates Epo-dependent growth of HCD-57 cells, can activate both the SIE-binding and the GRR-binding factors constitutively. Our results suggest that DNA-binding factors identical or related to the known Stat proteins are involved in the Epo signal transduction pathway and that constitutive activation of these factors can cause autonomous cell growth that leads to hematopoietic hyperplasia and leukemia.

MATERIALS AND METHODS

Cells. HCD-57 cells are Epo-dependent mouse erythroleukemia cells derived from an NIH Swiss mouse infected with Friend murine leukemia virus (F-MuLV) at birth. HCD-SFFV cells are HCD-57 cells chronically infected with SFFV, which abrogates the requirement of Epo for cell growth. HCD-57 cells were maintained in Epo-containing conditioned medium as previously described. HCD-SFFV cells were maintained in the same medium without Epo.

For Epo stimulation, cells grown in the regular medium to the density of 1 × 10⁶ cells/mL were collected by centrifugation at 1,000g for 5 minutes at 4°C, resuspended in medium lacking serum.
and Epo, and incubated in 5% CO\textsubscript{2} at 37°C for 2 hours. After starvation, the cells were collected again, resuspended in regular medium with or without 100 U/mL of recombinant human Epo (Ortho Biologics, Inc., Raritan, PA; a kind gift from the R.W. Johnson Pharmaceutical Research Institute), and incubated at 37°C. In some experiments, 100 µg/mL of genistein (GIBCO-BRL, Gaithersburg, MD) was added to the media during the starvation period. EL-4 mouse lymphoma cells used as a control for the RNA analysis experiment were grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum (FCS).\textsuperscript{14}

**Cell extracts.** Cells were rinsed with ice-cold phosphate-buffered saline (PBS) containing 1 mmol/L Na\textsubscript{2}VO\textsubscript{4}, resuspended in ice-cold hypotonic buffer (20 mmol/L HEPES [pH 7.9], 10 mmol/L KCl, 1 mmol/L MgCl\textsubscript{2}, 10% glycerol, 0.5 mmol/L diethytolether (DTT), 0.5 mmol/L phenylmethylsulphonyl fluoride [PMSF], 1 µg/mL aprotinin, 1 µg/mL leupeptin, and 1 mmol/L Na\textsubscript{2}VO\textsubscript{4} with 0.2% Nonidet P-40 (NP-40), and homogenized by a Dounce homogenizer. After centrifugation at 1,000g for 5 minutes, the supernatant was separated from the nuclear pellet. Cytoplasmic extracts were prepared by removing debris from the supernatants by centrifugation (14,000g for 20 minutes) at 4°C. The nuclear pellets were rinsed with hypotonic buffer containing 0.2% NP-40, resuspended in hypotonic buffer with 300 mmol/L NaCl, and gently rocked for 30 minutes. Residual nuclei were removed by centrifugation (14,000g for 20 minutes) at 4°C and the supernatants were collected as nuclear extracts. For whole cell extracts, cells were resuspended in ice-cold extraction buffer (20 mmol/L HEPES [pH 7.9], 10 mmol/L KCl, 1 mmol/L MgCl\textsubscript{2}, 150 mmol/L NaCl, 1% Triton X-100, 0.5 mmol/L DTT, 0.5 mmol/L PMSF, 1 µg/mL aprotinin, 1 µg/mL leupeptin, and 1 mmol/L Na\textsubscript{2}VO\textsubscript{4} and gently rocked for 30 minutes. After centrifugation at 14,000g for 20 minutes at 4°C, the supernatant was collected as a whole cell extract. The protein concentration of each sample was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

**The electrophoretic mobility shift assay (EMSA).** EMSAs were performed as previously described.\textsuperscript{9} A cytoplasmic extract containing 30 µg of protein or a nuclear extract containing 10 µg of protein was mixed with 5 µg of poly(dI)(dC) (Pharmacia, Piscataway, NJ) with or without an unlaabeled competitor oligonucleotide (100-fold molar excess over labeled probe) and incubated on ice for 10 minutes. Probe DNA labeled with Klenow fragment and [\textsuperscript{32}P]dCTP was then added so that the binding reaction mixture had a total volume of 30 µL and contained a final concentration of 13 mmol/L HEPES (pH 7.9), 65 mmol/L NaCl, 1 mmol/L DTT, 0.15 mmol/L EDTA, and 8% glycerol. Then, the mixture was incubated at room temperature for 30 minutes. In some experiments, antibodies were added to the mixture of the extract and poly(dI)(dC) and incubated at 4°C for 16 hours before the labeled probe was added. After the binding reaction, samples were subjected to electrophoresis in a 5% polyacrylamide gel containing 5% glycerol with 0.2% (v/v) Tris-glycine and 0.5x TBE buffer for 2 hours at 200 V. The gels were then dried and exposed to Kodak X-5 film at −80°C. The probes used in this study were double-stranded oligonucleotides corresponding to the high-affinity binding sequence GRR (5’-GTCGACATTTCCCGTAAATC-3’). To determine if stimulation of HCD-57 cells by Epo induces DNA-binding factors that interact with the SIE and GRR sequences known to be recognized by Stat proteins, EMSAs were performed by using \textsuperscript{32}P-labeled oligonucleotide probes corresponding to these two sequences. Cells were starved for 2 hours in the medium depleted of serum and Epo, and then the medium was changed to fresh regular medium with or
without Epo. No DNA-binding activity was detected by these two probes before stimulation (Fig 1A and B, lanes 1) or after the addition of the medium without Epo (Fig 1A and B, lanes 2). However, when the medium supplemented with Epo was used, two mobility shift signals were detected by the SIE probe (Fig 1A, lane 3) and one signal was detected by the GRR probe (Fig 1B, lane 3). The mobility of the signal detected by the GRR probe was slower than that of either signal detected by the SIE probe. The shifted bands detected by the SIE probe disappeared when an excess of unlabeled SIE or GRR probe was included in the binding reaction (Fig 1A, lanes 4 and 5), whereas the same amount of an unrelated oligonucleotide had no effect (Fig 1A, lane 6). The shifted band detected by the GRR probe was completely competed by the homologous GRR competitor (Fig 1B, lane 5) and partially by the SIE probe (Fig 1B, lane 4), but not by the unrelated oligonucleotide (Fig 1B, lane 6).

To analyze the kinetics of the induction of the DNA-binding factors, we prepared cytoplasmic and nuclear extracts from HCD-57 cells stimulated by Epo for different periods of time and examined the extracts for the presence of factors that bind to the SIE or GRR probes. As shown in Fig 2A, a significant level of SIE-binding factors was detected in the cytoplasm 1 minute after Epo stimulation and in the nucleus 5 minutes after stimulation. In contrast, the GRR-binding factor was detected in the cytoplasm as early as 30 seconds after Epo stimulation and in the nucleus by 1 minute (Fig 2B). High levels of both the SIE- and GRR-binding factors could be detected in the cytoplasm and nucleus for up to 30 minutes after Epo stimulation but the levels decreased after 60 minutes. HCD-57 cells continuously maintained in the presence of Epo showed neither the SIE- nor GRR-binding factors (data not shown).

The SIE- and the GRR-binding factors react with anti-phosphotyrosine antibodies. To characterize the DNA-binding factors induced by Epo stimulation, we analyzed them for the presence of phosphotyrosine by examining the effect of antiphosphotyrosine antibodies on the mobility shift complex formation. As shown in Fig 3A, addition of 1G2 antiphosphotyrosine antibody resulted in a supershifted SIE signal but it did not show a significant effect on the complex formation by the GRR-binding factor. However, another antiphosphotyrosine antibody, 25.2G4, strongly inhibited the formation of the GRR-protein complex and caused a slight enhancement of the signal corresponding to the faster migrating SIE complex. Other antiphosphotyrosine antibodies, such as PY-20 and 4G10, failed to exert a significant effect on the complex formation by either the SIE- or the GRR-binding factors induced by Epo (data not shown). When the cells were treated before Epo stimulation with a tyrosine kinaser inhibitor, the levels of these factors were greatly reduced (data not shown).
kinase inhibitor, genistein, the SIE- and the GRR-binding factors could not be detected either in the cytoplasm or the nucleus (Fig 3B).

Anti-Stat protein antibodies react with the SIE- and the GRR-binding factors induced by Epo stimulation. To determine whether the DNA-binding factors induced by Epo stimulation are related to Stat family proteins, we tested the reactivity of anti-Stat protein antibodies with these factors. As shown in Fig 4A, both of the signals corresponding to the SIE complexes were supershifted by anti-Stat1N antibody. After addition of anti-Stat1C antibody, formation of the SIE-complexes was partially inhibited. Anti-Stat3 antibody altered the ratio of the two SIE complexes indicated by a slight decrease in the relative intensity of the band corresponding to the slower migrating complex and an increase in that corresponding to the faster migrating complex. Effects of the same antibodies on the GRR complex formation were also examined (Fig 4B). Although anti-Stat1N antibody did not have any effect, both anti-Stat1C and anti-Stat3 antibodies showed inhibitory effects on the formation of the GRR complex. We failed to detect any effect of anti-Stat2 antibody on the complex formation by any of the SIE- or GRR-binding factors, and unrelated antibodies, such as anti-p53 and antirabbit IgG antibodies, also failed to have an effect (data not shown). Because we found that anti-Stat1 antibodies reacted with the DNA-binding factors induced by Epo, we investigated whether any of the proteins recognized by the anti-Stat1 antibodies are phosphorylated in response to Epo stimulation. Whole cell extracts prepared from HCD-57 cells stimulated with or without Epo were immunoprecipitated with anti-Stat1N or anti-Stat1C antibody. Precipitates were then analyzed by immunoblotting with antiphosphotyrosine antibodies (Fig 4C). A signal corresponding to a 92-kD tyrosine-phosphorylated protein was clearly detected by anti-Stat1N and anti-Stat1C antibodies only after Epo stimulation.

Stat1 and Stat3 mRNAs are expressed in HCD-57 cells. Because the involvement of Stat family proteins in the Epo signal transduction pathway was suggested, we examined the mRNA expression of known Stat genes in HCD-57 cells by an RT-PCR analysis. Because the mouse nucleotide sequences were available only for Stat1, Stat3, and Stat4, we analyzed the expression of these three genes. The given sets of primers described in the Materials and Methods are expected to generate amplified DNA products of 687 bp, 495 bp, and 720 bp in length for Stat1, Stat3, and Stat4 mRNA, respectively. As shown in Fig 5, the expected size of signals for Stat1 and Stat3 mRNA were detected in both
HCD-57 and EL-4 cells. In contrast, the DNA product for Stat4 mRNA was detected in EL-4 cells, but not in HCD-57 cells, indicating the lack of Stat4 mRNA expression in the latter cell line. Nucleotide sequence analysis of the cloned fragment corresponding to these signals confirmed that the amplified DNA was actually derived from Stat1 and Stat3 mRNA (data not shown).

Epo-induced DNA binding factors are constitutively activated in SFFV-infected HCD-57 cells. To investigate the effects of SFFV, which abrogates Epo dependence of HCD-57 cells, on activation of the SIE- and the GRR-binding factors, we performed EMSAs by using cell extracts prepared from HCD-SFFV cells that were treated with or without Epo (Fig 6). As previously shown, HCD-57 cells expressed SIE- and GRR-binding activities only after Epo stimulation (Fig 6A and B, lanes 3). In contrast, two distinct SIE-binding activities and one GRR-binding activity were detected in SFFV-infected HCD-57 cells after starvation in serum-free medium (Fig 6A and B, lanes 4) or after incubation in complete medium without Epo (Fig 6A and B, lanes 5). When HCD-SFFV cells were treated with Epo, an increase in all DNA-binding activities was observed (Fig 6A and B, lanes 6). The DNA-protein complexes formed by the HCD-SFFV cell extracts showed the same mobilities as those formed by the Epo-stimulated HCD-57 cell extracts.

To determine whether the DNA-binding factors expressed in SFFV-infected HCD-57 cells are also related to known Stat family proteins, we tested the reactivity of anti-Stat protein antibodies with these DNA-binding factors. As shown in Fig 6C, both of the signals corresponding to the SIE complexes were supershifted by anti-Stat1N antibody, and the complex formation was partially inhibited by anti-Stat1C antibody. Anti-Stat3 antibody altered the ratio of the two SIE-complexes so that the faster migrating complex was more abundant than the slower migrating complex. Anti-Stat1N antibody did not have any effects on the GRR complex formation, whereas the complex formation was partially inhibited by both anti-Stat1C and anti-Stat3 antibodies (Fig 6D). These results observed in SFFV-infected HCD-57 cells were very similar to those observed in uninfected HCD-57 cells stimulated with Epo.

**DISCUSSION**

In this study, we showed that DNA-binding factors that specifically recognize the SIE and GRR sequences are induced by Epo stimulation in the Epo-dependent mouse erythroleukemia cell line HCD-57. These DNA-binding factors are activated in the cytoplasm immediately after Epo stimulation and, after a brief delay, the same factors are detected in the nucleus, suggesting that the activated factors are transported from the cytoplasm to the nucleus. Activated DNA-protein complexes were detected at a significant level only transiently both in the cytoplasm and the nucleus. They, therefore, might be involved in the initial rapid phase of the signal transduction pathway required for resting cells to resume their ability to proliferate.

The two SIE-binding factors and the GRR-binding factor appear to contain different components because the DNA-protein complexes formed by these factors exhibited distinct mobilities and the kinetics of their activation were different. However, both unlabeled SIE and GRR oligonucleotides could inhibit (at least partially) SIE- and GRR-binding fac-
Fig 4. Relationship of the Epo-induced DNA-binding factors to Stat proteins. Cytoplasmic extracts were prepared from HCD-57 cells stimulated with Epo for 15 minutes and incubated without (−) or with 0.25 µg of anti-Stat1N (1N), 10 µg of anti-Stat1C (1C), or 10 µg of anti-Stat3 (3) antibody for 16 hours at 4°C. The samples were then incubated with a 32P-labeled SIE (A) or GRR (B) probe and subjected to 5% polyacrylamide gel electrophoresis. The position of the SIE complex supershifted by anti-Stat1N antibody is indicated by an arrow. (C) Whole cell extracts were prepared from HCD-57 cells treated without (−) or with (+) Epo for 15 minutes and incubated with agarose-conjugated anti-Stat1N (1N) or anti-Stat1C (1C) antibody for 2 hours at 4°C. After extensive washing, the immunoprecipitated proteins were separated by 7.5% SDS-PAGE and transferred to a nitrocellulose filter. The filter was incubated with a mixture of antiphosphotyrosine antibodies (PY20 and 4G10, each diluted 1:2,500) and then with an antimouse IgG antibody conjugated to horseradish peroxidase. Antibodies bound to the filter were detected by the enhanced chemiluminescence method. The position of the immunoprecipitated 92-kD phosphotyrosyl protein is indicated by an arrow.

The DNA-binding factors induced by Epo appear to contain phosphotyrosine because antiphosphotyrosine antibodies affected complex formation by these factors. Because treatment of the cells with a tyrosine kinase inhibitor prevented induction of the DNA-binding factors by Epo, tyrosine phosphorylation of these factors may be crucial for their activity. Interestingly, different effects on complex formation were observed for different antiphosphotyrosine antibodies. This finding suggests that the conformation surrounding the phosphotyrosines in the SIE-binding factors and the GRR-binding factor is different and that each antiphosphotyrosine antibody can react with phosphotyrosine when it is present only in a certain context.

DNA-binding proteins activated by a number of growth factors and cytokines have been shown to be members of the Stat family of transcription factors. Stat1 is activated by IFN-α, IFN-γ, epidermal growth factor (EGF), IL-10, platelet-derived growth factor, and ciliary neurotrophic factor, whereas Stat3 has been shown to be activated in response to IL-6, EGF, and granulocyte colony-stimulating factor (G-CSF). Several observations from our studies suggest that the DNA-binding factors induced by Epo stimulation belong to the Stat family of DNA-binding proteins. The factors specifically bind to sequences known to be recognized by Stat proteins and the kinetics of their induction are rapid and transient like those of known Stat proteins. The proteins are phosphorylated on tyrosine and tyrosine phosphorylation appears to be required for their DNA binding activity. Finally, antibodies raised against certain known Stat proteins react with these factors. Both of the SIE-binding factors contain a component that is recognized by anti-Stat1N and anti-Stat1C antibodies and the molecular weight of the phosphotyrosyl protein detected by immunoprecipita-
tion with anti-Stat1 antibodies coincides with that of the phosphorylated form of Stat1α. In addition, Stat1α mRNA was detected by RT-PCR using specific primers in HCD-57 cells. Therefore, it is likely that Stat1α is a component of the SIE-binding factors, although involvement of a novel Stat protein antigenically related to Stat1 is also possible. The complex formation by the GRR-binding factor was inhibited by anti-Stat1C antibody, which was raised against amino acid residues 613-739 in the C-terminus of Stat1, but not by anti-Stat1N antibody, which was raised against the amino terminal 194 amino acids. In addition, antibodies raised against amino acids 607-647 or the C-terminal 39 amino acids of Stat1 were recently shown to have no effect on complex formation by the GRR-binding factor induced by Epo in human erythroleukemia TF1 cells. Therefore, it is possible that the GRR-binding factor does not include Stat1 but another protein whose antigenicity is related to that of the epitope recognized by anti-Stat1C antibody. Alternatively, the anti-Stat1N antibody used in this study and the other anti-Stat1 antibodies used in the previous study might not be able to react with the cognate epitopes of the Stat1 protein when it is incorporated in the GRR-binding complex. The SIE-binding factor complex with a slower mobility contains a component recognized by anti-Stat3 antibody, and the activity of the GRR-binding factor was also affected by the same antibody. This combined with data showing that Stat3 mRNA can be detected in HCD-57 cells by RT-PCR suggests that Stat3 may also be activated by Epo and included in the SIE- and the GRR-binding factors, although it is also possible that a distinct protein related to Stat3 is involved. Lack of reactivity of anti-Stat2 antibody with any of the DNA-binding factors suggests that Stat2 is not a component of Epo-induced DNA-binding complexes. Likewise, involvement of Stat4 in the Epo signal transduction pathway in HCD-57 cells is unlikely because Stat4 mRNA was not detected in these cells using the highly sensitive RT-PCR method. We are currently attempting to affinity-purify the Epo-induced DNA-binding proteins using agarose-conjugated probes so that we can further characterize their molecular nature. Because there is a possibility that a novel Stat protein antigenically related to Stat1 or Stat3 is contributing to the SIE- or the GRR-binding factor formation, attempts are being made to search for a novel, Stat-related gene expressed in HCD-57 cells. We are also testing other Epo-responsive hematopoietic cells, including various myeloid cell lines that have been transfected with the EpoR gene, mouse erythroleukemia cell lines, and human leukemic cell lines, for activation of Stat-related DNA-binding factors.

Studies with the erythroleukemia-inducing SFFV have clearly shown that viruses can affect the proliferation and differentiation of hematopoietic cells without carrying an oncogene. So far, it has been shown that the primary effect of SFFV on erythroid cells is induced by the product of its unique envelope gene, and the interaction of the envelope glycoprotein with the EpoR appears to be crucial for its effect on erythroid cell growth.12,41-46 However, little is known about how this interaction leads to autonomous cell growth. In this study, we showed that SFFV infection of HCD-57 cells results in the constitutive activation of DNA-binding factors that are transiently induced by Epo in uninfected cells. This result suggests that SFFV, which is a component of the erythroleukemia-inducing SFFV, might be triggering the signal that enables the erythroid cells to proliferate autonomously without Epo. It is not known how SFFV activates these DNA-binding factors without Epo, but because neither the EpoR nor the SFFV envelope glycoprotein have an effect on complex formation by the GRR-binding factor induced by Epo in human erythroleukemia TF1 cells, it is likely that they are activating these DNA-binding factors indirectly. Recently, it has been reported that JAK2 tyrosine kinase is associated with the cytoplasmic domain of the EpoR and activated after Epo stimulation.14,15 Because JAK2 kinase was shown to be responsible for the tyrosine phosphorylation of Stat proteins,29,30 it is possible that the SFFV envelope glycoprotein can generate a functionally active form of the EpoR complex more stably than Epo and that the associated JAK2 is activated constitutively, which in turn activates the DNA-binding factors. A recent report demonstrating the constitutive tyrosine phosphorylation of JAK2 in an SFFV-induced erythroleukemia cell line is in agreement with this hypothesis. Alternatively, the SFFV envelope glycoprotein might be negatively affecting the mechanism that inactivates the JAK2 kinase or the DNA-binding factors (eg, phosphatases). Further studies using the anemia-inducing strain of SFFV that does not induce Epo-independence, other recombinant SFFVs, and mutant Epo receptors will allow us to determine the structures of the viral envelope glycoprotein and the EpoR required for the constitutive activation of the DNA-binding factors. Identification of the cellular target genes that these DNA-binding factors can regulate would also give us further insights into the functional significance of these factors in the physiologic mechanism for erythropoiesis as well as the pathogenic mechanism for leukemia induction.
**Fig 6. Constitutive activation of the SIE- and the GRR-binding factors by SFFV.** Cytoplasmic extracts prepared from HCD-57 cells (lanes 1 to 3) or HCD-SFFV cells (lanes 4 to 6) after starvation (lanes 1 and 4), 15 minutes after incubation in complete medium without (lanes 2 and 5) or with (lanes 3 and 6) Epo were incubated with a 32P-labeled SIE (A) or GRR (B) probe and subjected to 5% polyacrylamide gel electrophoresis. For supershift and inhibition analysis with anti-Stat antibodies, cytoplasmic extracts were prepared from HCD-SFFV cells after 15 minutes of incubation in complete medium without Epo. The extracts were then incubated without (−) or with 0.25 μg of anti-Stat1N (1N), 10 μg of anti-Stat1C (1C), or 10 μg of anti-Stat3 (3) antibody for 16 hours at 4°C. The samples were incubated with a 32P-labeled SIE (C) or GRR (D) probe and subjected to 5% polyacrylamide electrophoresis.

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