Cytokine Signaling through Stat3 Activates Integrins, Promotes Adhesion, and Induces Growth Arrest in the Myeloid Cell Line 32D

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Abstract

Hematopoietic cell development and function is dependent on cytokines and on intercellular interactions with the microenvironment. Although the intracellular signaling pathways stimulated by cytokine receptors are well described, little is known about the mechanisms through which these pathways modulate hematopoietic cell adhesion events in the microenvironment. Here we show that cytokine-activated Stat3 stimulates the expression and function of cell surface adhesion molecules in the myeloid progenitor cell line 32D. We generated an erythropoietin receptor (EpoR) isoform (ER343/401-S3) that activates Stat3 rather than Stat5 by substituting the Stat3 binding/activation sequence motif from gp130 for the sequences surrounding tyrosines 343 and 401 in the receptor cytoplasmic region. Activation of Stat3 leads to homotypic cell aggregation, increased expression of intercellular adhesion molecule 1 (ICAM-1), CD18, and CD11b, and activation of signaling through CD18-containing integrins. Unlike the wild type EpoR, ER343/401-S3 is unable to support long term Epo-dependent proliferation in 32D cells. Instead, Epo-treated ER343/401-S3 cells undergo G1 arrest and express elevated levels of the cyclin-dependent kinase inhibitor p27Kip1. Sustained activation of Stat3 in these cells is required for their altered morphology and growth properties since constitutive SOCS3 expression abrogates homotypic cell aggregation, signaling through CD18-containing integrins, G1 arrest, and accumulation of p27Kip1. Collectively, our results demonstrate that cytokine-activated Stat3 stimulates the expression and function of cell surface adhesion molecules, indicating that a role for Stat3 is to regulate intercellular contacts in myeloid cells.

Stat proteins are principal mediators of cytokine receptor signals (1–3). They are localized in the cytoplasm, where they can be rapidly recruited to activated receptor complexes following cytokine stimulation. Stats are then phosphorylated on critical tyrosine residues, enabling homo- or heterodimerization through reciprocal SH2-domain-phosphotyrosine interactions. Activated Stats translocate to the nucleus to stimulate transcription of cytokine-dependent genes (1).

Many cytokine-specific signals are mediated by Stat proteins (1–4). For example, Stat1 is critical for interferon-α and interferon-γ signaling and animals lacking Stat1 have severely...
impaired immune responses (5,6). Similarly, Stat4 and Stat6 regulate lymphocyte responses to interleukin-12 (IL-12) and IL-4, respectively (7–10). In contrast, Stat3 and Stat5 are activated by a variety of cytokines with different biological functions (3,11), and these proteins have important roles in several hematopoietic and non-hematopoietic cell types. Stat5a and Stat5b regulate prolactin-dependent mammary gland development and growth hormone-dependent liver functions (12–14). The Stat5 proteins are also important regulators of peripheral T-cell proliferation (15,16) and erythropoietin (Epo)-dependent survival of murine fetal liver erythroid progenitors (17). Homozygous deletion of Stat3 causes early embryonic lethality (18). However, tissue-specific gene deletions have shown that Stat3 is critical for epithelial cell apoptosis and involution in the post-lactating mammary gland (19), skin remodeling and keratinocyte migration (20), and macrophage deactivation, down-regulation of inflammatory cytokines, and a balanced Th1 cell response (21).

Identification of gene targets for activated Stats is elucidating the mechanisms through which they elicit specified in vivo functions. For example, Stat1 regulates the interferon-α-inducible transcription of several immunomodulatory proteins, including major histocompatibility complex class I and II proteins (5,22), whereas Stat5 activates the expression of a number of proteins involved in cell proliferation and regulation of apoptosis (17,23–27). Stats also stimulate the expression of members of the SOCS/CIS/Jab protein family, which participate in a negative feedback system to down-regulate cytokine signals through Jak and Stat proteins (28–30). Overall, however, the nuclear targets of activated Stats are not well defined, and it is unclear how Stat-regulated genes contribute to the development and function of hematopoietic cells.

In vivo, the growth, development, and function of hematopoietic cells is also profoundly influenced by intercellular contacts in the microenvironment mediated by cell surface adhesion molecules (for review, see Ref. 31). For example, during development in the bone marrow or thymus, immature hematopoietic and lymphoid cells require contacts with resident stromal cells to fully complete their developmental program. In addition, several mature hematopoietic cell types require specific cell-cell contacts to perform their specialized functions. The adhesion events that control the development, trafficking, and function of hematopoietic cells are regulated by integrins, selectins, and mucins (31,32). Cytokine signaling can modify the activity of hematopoietic cell adhesion molecules and cellular responses to adhesion events (31). However, little is known about the mechanisms that regulate these processes and, in particular, the role of cytokine-activated Stat proteins.

The goal of this study was to investigate the function of Stat3 in myeloid cells. Since cytokine receptors that naturally activate Stat3 also stimulate other Stat proteins, such as Stat1 or Stat5, we generated a chimeric EpoR that selectively activated Stat3. This system enabled us to define specific, cytokine-dependent, functions of Stat3. Results from our studies demonstrate a new role for Stat3. We show that sustained activation of Stat3 in 32D cells stimulates the expression and function of hematopoietic cell adhesion molecules. These results suggest that Stat3 regulates cytokine-dependent cell adhesion events in myeloid progenitor cells.

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1The abbreviations used are: IL, interleukin; Epo, erythropoietin; EpoR, Epo receptor; PCR, polymerase chain reaction; FCS, fetal calf serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; EMSA, electrophoretic mobility shift assay; SIE, Sis-inducible element; WT, wild type; PI3K, phosphoinositol 3-kinase; ICAM-1, intercellular adhesion molecule 1; G-CSF, granulocyte colony-stimulating factor; G-CSFR, G-CSF receptor.
EXPERIMENTAL PROCEDURES

Generation of EpoR Mutants
The murine EpoR (33) was mutagenized by a polymerase chain reaction (PCR)-based strategy, which substituted the Stat5 binding/activation sites at positions 343 and 401 (34,35) with the Stat3 binding/activation site from gp130 (36). To introduce the substitution at residue 343, two PCR fragments (A and B) were generated from the EpoR cDNA by amplification with primer pairs 1 and 2 or 3 and 4 (Table I). Fragments A and B were mixed and amplified by PCR using primers 1 and 4 to generate a PCR fragment (C) containing EpoR nucleotides 990–1235, with the Stat3 binding site substituted for nucleotides 1183–1197. Fragment C was digested with BamHI and HindIII and subcloned into the BamHI/HindIII sites of the EpoR 3′ region. The substitution at residue 401 was generated by a similar strategy using primers 5, 6, 7, and 8 (Table I). This yielded a PCR fragment containing EpoR nucleotides 1209–1561 with the Stat3 binding site substituted for nucleotides 1357–1371. This fragment was subcloned into the HindIII/AvrII sites of the EpoR cDNA containing the substitution at position 343 to generate a receptor containing both substitutions (ER343/401-S3). A similar strategy employing primers 9, 10, 11, 12 (Table I) was used to generate the mutant ER-Y343/401F. Regions of PCR amplification were verified by dideoxy sequence analysis. ER343/401-S3 and ER-Y343/401F were subcloned into the mammalian expression vector pMEX.

Cell Lines, Culture Conditions, and Transfections
The 32D cell line is an IL-3-dependent myeloid progenitor cell line (37,38). 32D cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and 5% conditioned medium from the WEHI 3B cell line (IL-3 source) (RPMI/FCS/WEHI). Cells were electroporated with linearized plasmids as described previously (39) and selected in RPMI/FCS/WEHI containing 750 μg/ml G418 (EpoR isoforms, granulocyte colony-stimulating factor receptor (G-CSFR)) or 5 μg/ml puromycin (SOCS3) (39,40). EpoR and G-CSFR expression was verified by immunoprecipitations from metabolically labeled cells or by immunoprecipitation and immunoblotting (not shown). SOCS3 expression was verified by immunoblotting of whole cell lysates (not shown).

Growth Factor-dependent Proliferation Assays and Cell Cycle Analysis
Cells growing in RPMI/FCS/WEHI were washed three times with RPMI containing 10% FCS (RPMI/FCS), then plated in RPMI/FCS supplemented with Epo (concentrations indicated in the text) and cultured for up to 2 weeks. Cell density was maintained under 10^6 cells/ml by dilution in fresh medium. Viable cells, as judged by trypan blue dye exclusion, were counted every 2–3 days, and total cell numbers were graphed to determine doubling times. All cell lines were strictly growth factor-dependent and died after 24 h in RPMI/FCS (data not shown).

For cell cycle analysis, 10^6 cells were collected after 3 d of growth in medium containing Epo or IL-3. The media was removed by aspiration, and cells were resuspended in 75% ethanol and stored at −20 °C for up to 1 week. To stain with propidium iodide, the cells were washed once with PBS and resuspended in PBS containing 50 µg/ml propidium iodide (Sigma) and 25 µg/ml RNase A and incubated for 60 min at 4 °C. The cells were analyzed by flow cytometry on a Coulter Epics Profile (Miami, FL) machine. Cell cycle profiles were generated by MultiCycle (Phoenix Flow System, San Diego, CA) software.

Antibody Stain and Flow Cytometry; Aggregation and Spreading Assays
For flow cytometry analysis, cells (~10^6) were incubated in 1% goat serum in PBS for 60 min at 4 °C. Antibodies specific for murine ICAM-1 (YN1, ATCC, Manassas, VA) or CD18 (M18/2, PharMingen, San Diego, CA) were added at a concentration of 1 µg/ml, and cells were
incubated for 60 min at 4 °C. Cells were then washed three times with goat serum/PBS and incubated in goat serum/PBS containing anti-rat fluorescein isothiocyanate-conjugated secondary antibody (1:1000 dilution) (Sigma) for 60 min at 4 °C. Controls were incubated with secondary antibody alone. The cells were fixed in 2% formaldehyde/PBS and washed twice with PBS. The stained cells were analyzed by flow cytometry on a Coulter Epics Profile machine.

For aggregation assays, cells were cultured in RPMI/FCS/WEHI or RPMI/FCS containing 0.5 units/ml Epo as indicated, then dispersed by pipetting and plated in the indicated conditions (see Fig. 6B for details). Cells were incubated for 60 min at 37 °C in a humidified incubator containing 5% CO₂ and photographed as described below.

For cell spreading assays, antibodies were diluted to 10 μg/ml in 0.1 M NaHCO₃, pH 9.0, added to the wells of a 96-well EIA/RIA plate (Costar, Cambridge, MA) (50 μl/well) and incubated overnight at 4 °C. Wells were then blocked with PBS containing 1% BSA for 2 h at room temperature. For BSA controls, some wells were incubated only with 1% BSA in PBS. The wells were then washed 3× with PBS at room temperature and used in spreading assays. Cells were treated as indicated (see figure legends for details), then dispersed by pipetting and plated in antibody- or BSA-containing wells. Cells were incubated for 60–90 min at 37 °C in a humidified incubator containing 5% CO₂. The cells were fixed in 2% formaldehyde/PBS for 15 min at room temperature, and plates were stored at 4 °C. Cells were photographed at 200× magnification (Olympus IX70 microscope) using a SPOT digital camera. The resulting digital images were manipulated with Adobe Photoshop (Adobe Systems, Inc., San Jose, CA).

Epo Stimulations, Immunoprecipitations, Preparation of Whole Cell Lysates, SDS-Polyacrylamide Gel Electrophoresis, and Immunoblot Analysis

EpoR antisera has been described previously (41). Antiserum specific for Stat5, Stat3, Stat1, or p27kip1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and antiserum specific for Jak2 was obtained from Upstate Biotechnology (Lake Placid, NY). Cells were treated with Epo as indicated in the text, and detergent cell extracts were made as described previously (39) using Buffer A (1% w/v Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride, and 10 μl/ml aprotinin). Lysates were cleared by incubation with protein A-agarose beads (30–120 min at 4 °C) and used for immunoprecipitations as described (39). Proteins were separated by one-dimensional SDS-polyacrylamide gel electrophoresis and were electrophoretically transferred to nitrocellulose filters. Whole cell lysates were made by sonicating cells in protein gel sample buffer, as described previously (39); 5 × 10⁵ cell equivalents were separated by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose filters. Immunoblotting assays were performed as described previously (39,40).

Iodination of Cell Surface Proteins

Cells (5 × 10⁶/sample) were radioiodinated as described (42). The iodinated cells were lysed in buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM MgCl₂, and 1 mM phenylmethylsulfonyl fluoride for 1 h on ice before being clarified by centrifugation in a microcentrifuge at 10,000 rpm for 10 min at 4 °C. One-fifth of the total lysate, or 1 × 10⁶ cell equivalents, was used for each immunoprecipitation, as described above.

Electrophoretic Mobility Shift Assay (EMSA)

Cells were treated as described in the figure legends. To prepare nuclear extracts, cells were resuspended in ice-cold Buffer B (150 mM NaCl, 100 mM sucrose, 1.5 mM MgCl₂, 3% glycerol) and lysed by the addition of an equal volume of ice-cold Buffer C (Buffer B containing 10 μl/ml Nonidet P-40). The lysate was layered on a cushion of Buffer D (25% glycerol, 10
mM Tris-HCl, pH 7.4, 1.5 mM MgCl₂), and the nuclei were pelleted in a room temperature microcentrifuge at 3000 rpm for 5 min. The nuclei were washed with cold PBS, then lysed in high salt lysis buffer (20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM EDTA pH 8.0, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride) for 20 min at 4 °C while shaking. The nuclear extract was clarified by centrifugation for 5 min at 14,000 rpm in a microcentrifuge, and the protein concentration in the extract was determined as described (43) using commercially available reagents (Pierce). DNA binding activity was assayed with 8 μg of nuclear protein (per sample), and a radiolabeled, double-stranded oligonucleotide containing the Stat5 or SIE (Sis-inducible element) consensus binding sequence (Santa Cruz Biotechnology, Santa Cruz, CA). Binding reactions were performed for 20 min at room temperature in the presence or absence of antibodies specific for Stat3 or Stat1, as indicated in the figure legends. The reaction mixtures were separated on 5% polyacrylamide gels, and migration of the nucleoprotein complexes were determined by autoradiography.

RESULTS

Construction and Expression of EpoRs with Altered Stat Specificity

A schematic diagram of EpoRs containing modified Stat binding sites is shown in Fig. 1. 32D cell lines expressing the WT EpoR or chimeric EpoRs isoforms were established. Metabolic pulse-chase labeling experiments showed that the introduced mutations did not detectably alter the synthesis or processing of the EpoR (data not shown). Cells expressing WT or mutant EpoRs expressed similar levels of cell surface receptors (4000–5300 receptors/cell) and displayed similar ligand binding properties (Kd ~1.2 nM; data not shown), indicating that the Stat binding site substitutions did not alter the affinity of the receptor for Epo.

Activation of Downstream Signaling Proteins from EpoRs with Altered Stat Binding Sites

To examine the pattern of Epo-dependent Stat protein activation in transfected 32D cell lines, Stat tyrosine phosphorylation and DNA binding activity were assayed. Stat5 was activated by the WT EpoR during short term stimulation of growth factor-deprived cells with 0.5 units/ml Epo or saturating (5.0 or 50.0 units/ml) concentrations of Epo (Fig. 2A; upper panel, lanes 1–4). The majority of Stat5 tyrosine phosphorylation was abrogated in Epo-stimulated ER343/401-S3 or ER-Y343/401F cells (Fig. 2A; upper panel, lanes 5–8 and 9–12, respectively), particularly in response to 0.5 units/ml Epo (Fig. 2A, upper panel, lanes 6 and 10). Equivalent levels of Stat5 were present in the immunoprecipitation samples, as determined by immunoblotting with Stat5 antibody (data not shown). EMSAs showed that the pattern of Stat5 DNA binding activity was similar to that of Stat5 protein tyrosine phosphorylation (Fig. 2A, lower panel). During long term culture in 0.5 units/ml Epo, Stat5 activation was sustained in cells expressing WT EpoR and markedly decreased in ER343/401-S3 cells (Fig. 2B, upper and lower panels, lanes 2, 3, 5, and 6). Stat5 tyrosine phosphorylation was correspondingly decreased in ER-Y343/401F cells during long term culture in Epo (Fig. 2B, upper panel, lanes 8 and 9), although some active Stat5 was detected after 48 h in Epo by EMSA (Fig. 2B, lower panel, lanes 8 and 9). In all cell lines, Stat5 was activated when cells were grown in IL-3, as expected (44,45) (Fig. 2B, upper and lower panels, lanes 1, 4, and 7). The levels of Stat5 in the immunoprecipitation samples were similar, as judged by anti-Stat5 immunoblots (Fig. 2B, middle panel, lanes 1–9).

Stat3 was not tyrosine-phosphorylated in mock-stimulated cells, in IL-3-treated cells, or in cells expressing WT EpoR or ER-Y343/401F, even in saturating concentrations of Epo (Fig. 3A, upper panel, lanes 1–4; Fig. 3B, upper panel, lanes 1–3 and 7–9). However, Stat3 was activated in ER343/401-S3 cells following short term stimulation of growth factor-deprived
cells with 0.5 units/ml Epo or saturating concentrations of Epo (Fig. 3A; upper panel, lanes 6–8). Furthermore, activated Stat3 was readily detected during long term culture (24–48 h) of these cells in 0.5 units/ml Epo (Fig. 3B, upper panel, lanes 5 and 6). The levels of immunoprecipitated Stat3 were similar in all samples (Fig. 3B, middle panel). EMSAs with the SIE oligonucleotide, which contains the binding site for Stat3 and Stat1, showed the presence of activated SIE-binding proteins in Epo-stimulated ER343/401-S3 cells but not in WT or Y343/401F cells (Figs. 3A and B, lower panels). To determine if both Stat1 and Stat3 were activated by the EpoR substitutions or if only Stat3 was activated, supershift EMSA assays were performed using Stat1- or Stat3-specific antibodies. These experiments showed that the SIE binding activity in Epo-treated ER343/401-S3 cells was due to Stat3 only (Fig. 3C). Collectively, these results demonstrate that EpoR isoforms with modified Stat binding sites have altered Stat signaling specificity. Epo stimulation of ER343/401-S3 cells results in sustained activation of Stat3 and an abrogated Stat5 response, whereas Epo-treated ER-Y343/401F cells have reduced Stat5 activity.

Epo-dependent Jak2 and EpoR tyrosine phosphorylation is correlated with kinase activation and EpoR signal transduction, respectively (46,47). The pattern of Epo-dependent Jak2 tyrosine phosphorylation was similar in WT, ER343/401-S3, and ER-Y343/401F cells (Fig. 4; upper panel; data not shown). The pattern of EpoR tyrosine phosphorylation was also similar in WT and ER343/401-S3 cells (Fig. 4; lower panel). These results demonstrate that the substitution of Stat3 binding sites for Stat5 binding sites do not have a marked effect on Jak2 activation or receptor tyrosine phosphorylation, indicating that the mutations do not significantly alter the conformation or activation of the EpoR.

**EpoR-mediated Stat3 Activation Inhibits Epo-dependent 32D Cell Proliferation, Leading to Accumulation of p27^Kip1 and G1 Arrest**

To determine the effect of Epo-dependent Stat3 activation on 32D cell proliferation, cells expressing WT or mutant EpoRs were grown in media containing IL-3 or 0.5 or 5 units/ml Epo. Representative growth curves are shown in Fig. 5A. All cell lines proliferated at a comparable rate (doubling time of 10–12 h) in media containing IL-3 (Fig. 5A, top panel). Cells expressing WT EpoR or ER-Y343/401F proliferated similarly in media containing 0.5 or 5.0 units/ml Epo (doubling time of 10–12 h). In contrast, ER343/401-S3 cells proliferated well only during the first 24–48 h in Epo. Their growth rate then slowed dramatically, and viable cell numbers decreased over time (Fig. 5A, middle and lower panels). All cell lines demonstrated strict dependence on exogenously added cytokine for survival and died within 24–48 h of cytokine withdrawal (data not shown). Therefore, although ER343/401-S3 supports Epo-dependent proliferation over a short term period (24–48 h) and viability over several days, it is unable to support long term proliferation of 32D cells.

To examine whether Epo-treated ER343/401-S3 cells arrested in a particular stage of the cell cycle, cell cycle profiles were analyzed by propidium iodide staining and flow cytometry (Fig. 5B). The distribution of cells in the various stages of the cell cycle was similar for all cell lines cultured in IL-3 as well as for WT EpoR and ER-Y343/401F cells cultured in Epo. Approximately 30–40% of the cells were in the G1 phase, 45–65% were in S phase, and 5–15% were in the G2/M phases, whereas only 2–4% of the cells were present in the subdiploid population (Fig. 5B; data not shown). In contrast, when ER343/401-S3 cells were cultured in Epo for 3 days, a greater percentage of the population was found in the G1 phase (45–60%) and a lesser percentage in S phase (20–35%) (Fig. 5B). Consistent with this, elevated levels of the G1 cyclin-dependent kinase (cdk) inhibitor p27^Kip1 were detected in ER343/401-S3 cells after culture for 2 days in Epo-containing media. The level of p27^Kip1 remained elevated in ER343/401-S3 cells through 6 days of culture in Epo-containing media, in contrast to Epo-treated WT or ER-Y343/401F cells, which contained only low levels of p27^Kip1 (Fig. 5C; data not shown).
not shown). When maintained in IL-3-containing media, all three cell lines (WT, ER343/401-S3, and ER-Y343/401F) also contained low levels of p27Kip1 (Fig. 5C, lanes 1 and 5; data not shown). Therefore, in these cells activation of Stat3 leads to an accumulation of p27Kip1 and G1 arrest.

**Epo Stimulation of ER343/401-S3 Cells Induces Homotypic Cell Aggregation and the Increased Expression of ICAM-1, CD18, and CD11b**

We observed a morphology change in Epo-treated ER343/401-S3 cells, concomitant with their growth arrest. After 48 h of Epo culture, the majority of ER343/401-S3 cells were found in large aggregates, and a small number of cells (less than 1% of the population) had attached and spread on the bottom of the tissue culture dish. In contrast, WT and Y343/401F cells continued to grow as single cells in suspension (Fig. 6A; data not shown). Homotypic aggregation of hematopoietic cells is typically mediated by interactions between integrins and their counterligands (48,49). To explore the possibility that an integrin/counterligand interaction mediates Epo-dependent ER343/401-S3 homotypic aggregation, we took advantage of the fact that integrin binding activity in hematopoietic cells is tightly regulated by intracellular signaling pathways, or “inside-out” signals, often through phosphoinositol 3-kinase (PI3K) (50–53). In addition, integrin binding activity is dependent on divalent cations. Therefore, we determined if ER343/401-S3 homotypic aggregation was dependent on PI3K and divalent cations by analyzing reaggregation in the presence of EDTA or the PI3K inhibitor wortmannin. ER343/401-S3 cell aggregates, which had formed after 48 h in Epo culture, were disrupted and allowed to reform in media containing or lacking inhibitors. ER343/401-S3 cells reaggregated by 60 min in the absence of inhibitors. In contrast, reaggregation was significantly inhibited in the presence of EDTA or wortmannin (Fig. 6B). These results suggest that an integrin-counterligand interaction may mediate ER343/401-S3 cell homotypic aggregation.

Once hematopoietic cell integrins have been activated by inside-out signals, they are competent to bind their counterligands on other cells or substrates in the extracellular matrix (32). Interaction with these ligands can activate integrins to signal to the cytoskeletal network, resulting in cytoskeletal rearrangements and changes in cell morphology. Therefore, the ability of hematopoietic cells to undergo morphological changes in response to interactions with integrin substrates or anti-integrin antibodies provides an indication of integrin signaling activity. To determine if integrin signaling was activated by Epo treatment, cells were plated on immobilized antibody specific for murine CD18 (β2 integrin subunit), a component of the αLβ2, αMβ2, and αxβ2 integrins. WT cells remained spherical and did not spread on the antibody (Fig. 6C, upper left panel), indicating that CD18-containing integrins were not in an active signaling state. In contrast, Epo-treated ER343/401-S3 cells spread on immobilized CD18 (Fig. 6C, lower left panel) or CD11b (αM integrin subunit) antibodies (data not shown). In wells containing immobilized BSA, WT cells remained as single cells, whereas ER343/401-S3 cells reaggregated (Fig. 6C, right panels). Treatment of WT or ER343/401-S3 cells with IL-3 did not stimulate cell spreading on immobilized CD18 antibody (data not shown). Therefore, signaling through CD18-containing integrins is activated by Epo treatment of ER343/401-S3 cells.

Next, we analyzed the expression of selected adhesion molecule proteins beginning with ICAM-1, since it is a counterligand for CD18-containing integrins, and its promoter is known to be Stat-responsive (54). Low levels of ICAM-1 were detected on the surface of WT cells cultured in IL-3 or Epo or in ER343/401-S3 cells cultured in IL-3, as judged by immunoprecipitation of radiolabeled cell surface proteins (Figs. 7, A and B, lanes 3, 4, and 7) or flow cytometry (Fig. 7C). The expression of cell surface ICAM-1 was increased significantly (approximately 10-fold) by Epo treatment of ER343/401-S3 cells (Fig. 7B, lane 8, and Fig. 7C).
CD18 was detected on the surface of WT and ER343/401-S3 cells grown in IL-3, as judged by immunoprecipitation of iodinated cell surface proteins or flow cytometry. Epo treatment led to an increase in CD18 levels at the cell surface in both cell types, with CD18 levels significantly higher on ER343/401-S3 cells relative to WT cells (Fig. 7A, lanes 1 and 2; Fig. 7B, lanes 5 and 6; Fig. 7C). Strikingly, the amount of CD11b was also increased in Epo-treated ER343/401-S3 cells relative to Epo-treated WT cells or WT and ER343/401-S3 cells cultured in IL-3-containing media (Figs. 7, A and B; data not shown). RNase protection assays confirmed these results, demonstrating that the levels of ICAM-1, CD18, and CD11b mRNAs were increased in Epo-treated ER343/401-S3 cells relative to cells grown in IL-3 or to Epo-treated WT or ER-Y343/401F cells (data not shown). In contrast, expression levels of CD49d (α4 integrin subunit), which associates with the β1 and β7 integrin chains, did not change in WT or ER343/401S3 cells in response to Epo (data not shown), indicating that the expression of only specific adhesion molecules is stimulated by Epo treatment of ER343/401-S3 cells. Thus, cytokine-dependent activation of Stat3 stimulates the expression of ICAM-1, CD18, and CD11b in 32D cells.

Activated Stat3 Is Required to Stimulate CD18 Signaling, Homotypic Aggregation, and Cell Cycle Arrest

To determine if Stat3 activation was required for the Epo-induced alterations in ER343/401-S3 cell morphology and growth, we first tested whether these effects were reversed by culturing in IL-3-containing media. ER343/401-S3 cells were cultured for 48 h in Epo-containing media and then were collected and split into two samples. One sample was returned to Epo-containing media and cultured an additional 24 h for a total of 72 h culture in Epo (Epo culture). The second sample was placed in IL-3-containing media and cultured for 24 h (Epo-WEHI culture). The cell cycle arrest induced by Epo treatment of ER343/401-S3 cells was released after 24 h of culture in IL-3-containing media, as evidenced by the down-regulation of p27Kip1 (Fig. 8B, compare lanes 2 and 3) and the decreased percentage of cells in G1 phase (30–50% for Epo-WEHI cultures) relative to cells maintained in Epo for 72 h (60–70% in G1 phase) (data not shown). In addition, homotypic aggregation of ER343/401-S3 cells was inhibited after 24 h of culture in IL-3-containing media (Fig. 9A, upper right panel), and CD18 signaling activity was down-regulated as determined by cell spreading assays on immobilized CD18 antibody (data not shown).

Expression of the negative regulators SOCS-1 and SOCS-3 was strongly induced in Epo-treated ER343/401-S3 cells. In contrast, Epo-treated WT cells activated the expression of CIS and SOCS-1 (data not shown). This suggested to us that SOCS3 may be regulated by Stat3 and may function to down-regulate Stat3 activity. To test this, we generated an ER343/401-S3 cell line with constitutive SOCS3 expression. As shown in Fig. 8A, Epo-dependent Stat3 tyrosine phosphorylation was inhibited in ER343/401-S3 cells constitutively expressing SOCS3. We then determined the effect of constitutive SOCS3 expression on Epo-induced changes in ER343/401-S3 cell growth and morphology. Accumulation of p27Kip1 and G1 arrest was abrogated by SOCS3, with approximately 60% of ER343/401-S3 cells present in G1 after 72 h of culture in Epo-containing media compared with only 40% of ER343/401-S3 cells containing SOCS3 (Fig. 8B, compare lanes 2 and 5; data not shown). Homotypic aggregation was inhibited by SOCS3, as evidenced by a reduction in the number of cell aggregates and smaller aggregate size (Fig. 9A, compare upper and lower left panels). Signaling through CD18-containing integrins was also abrogated by SOCS3, as shown by a reduction in the number of spread cells and their decreased branching pattern on immobilized CD18 antibody (Fig. 9B, compare upper and lower left panels). Thus, constitutive expression of SOCS-3 blocks Stat3 activation as well as the growth arrest and morphological changes that occur in Epo-treated ER343/401-S3 cells.
G-CSF Stimulates 32D Cell Homotypic Aggregation and Signaling through CD18-containing Integrins

To determine if a cytokine receptor that naturally activates Stat3 stimulates cell adhesion and CD18 signaling, we generated a 32D cell line expressing the murine G-CSFR. G-CSFR cells formed aggregates between 3 and 7 d of culture in G-CSF-containing media (Fig. 10A and data not shown). G-CSF treatment also stimulated CD18 signaling activity, as determined by cell spreading assays on immobilized CD18 antibody (Fig. 10B). Collectively, our data demonstrate that Stat3 plays an essential role in activating signaling through CD18-containing integrins and promoting adhesion in 32D cells.

DISCUSSION

The results of this study demonstrate a new role for the cytokine-inducible Stat3 protein. We show that Stat3 stimulates the expression and activation of adhesion molecules in 32D cells. This finding suggests that Stat3 may play an important role in mediating cytokine-dependent cell adhesion events in myeloid progenitor cells.

Several lines of evidence demonstrated that Stat3 stimulated 32D cell homotypic aggregation and growth arrest. The evidence also suggested that these two events are coupled. First, down-regulation of Stat3 activity by enforced SOCS3 expression in ER343/401-S3 cells abrogated Epo-induced homotypic aggregation and growth arrest. Second, when Epo-treated ER343/401-S3 cells were returned to IL-3-containing media for 24 h, homotypic cell aggregation was decreased, and the cells re-entered the cell cycle, indicating that sustained activation of Stat3 was necessary for cell aggregation and growth arrest. In addition, Epo-treated ER-Y343/401F cells, which exhibited abrogated Stat5 activity and no Stat3 activity, proliferated at a rate similar to WT cells and did not undergo homotypic aggregation, indicating that the loss of Stat5 signaling is not sufficient to stimulate cell adhesion and growth arrest. Therefore, the specific activation of Stat3 initiates a gene expression program in 32D cells, which stimulates intercellular contacts and inhibits cell proliferation.

The ICAM-1 promoter contains a Stat-responsive element, which appears to be activated by Stat1 (54). Our results suggest that ICAM-1 expression may be transcriptionally regulated by Stat3 also, since the levels of ICAM-1 mRNA were increased in ER343/410-S3 cells within 2 h of Epo treatment. G-CSF treatment also rapidly induced ICAM-1 mRNA, although the direct contribution of Stat3 is unclear since G-CSF stimulates Stats 1, 3, and 5 in G-CSFR cells (data not shown). In contrast, CD18 and CD11b mRNAs were induced between 24 and 48 h of Epo treatment in ER343/401-S3 cells (data not shown), suggesting that they may not be directly regulated by Stat3.

The signaling activity of CD18-containing integrins was stimulated by cytokine-dependent Stat3 activation, as demonstrated by the ability of Epo-treated ER343/401-S3 cells or G-CSF-treated G-CSFR cells to spread on immobilized CD18 antibody. Since ICAM-1 expression was also induced in these cells, an interaction between ICAM-1 and CD18-containing integrins may mediate the homotypic cell aggregation observed in both cases.

Cells expressing WT EpoR, ER343/401-S3, or G-CSFR display surface integrins when cultured in media containing IL-3, yet they grow as individual cells in suspension and not as cell aggregates, indicating that the integrins are not in a functionally active state and/or their counterligands are not available for binding. Since integrin activity is tightly regulated in hematopoietic cells, two events may be required for cytokine-dependent homotypic cell aggregation. First, expression of a cell-associated integrin counterligand, such as ICAM-1, must be stimulated. Second, the cell surface integrin(s) must be functionally activated by an inside-out signal to bind its counterligand. Homotypic aggregation of Epo-treated ER343/401-
S3 cells is inhibited by wortmannin, indicating that PI3K plays a critical role in the activation pathway of cell adhesion.

The role of Stat3 in the process of integrin activation is unclear. Interferon-α-activated Stat3 has been shown to directly bind the p85 subunit of PI3K, targeting the enzyme to the receptor complex at the plasma membrane and stimulating PI3K activity (55). Therefore, it is possible that Stat3 activated through ER343/401-S3 could serve a similar function in directly activating PI3K. Alternatively, Stat3 may induce the expression of proteins that regulate PI3K and integrin activity, such as chemokines and/or chemokine receptors. Further work is necessary to elucidate the function of Stat3 in cell adhesion events. In addition, it is important to acknowledge that although our data are consistent with the proposal that an interaction between ICAM-1- and CD18-containing integrins mediates ER343/401-S3 cell aggregation, they do not rule out the possibility that other integrin/counterligands or other adhesion molecules, such as cadherins, might be involved.

Many adherent cell types require contacts with substrates in the extracellular matrix for proliferation; however, high cell density and increased levels of cell-cell interactions can result in growth arrest. This contact inhibition is accompanied by the accumulation of p27^Kip1, which regulates growth arrest by blocking G1 cdk activity (56–60). In contrast, many hematopoietic cell lines, such as 32D, normally grow in suspension and require exogenous cytokines rather than extracellular matrix contacts to sustain proliferation. Our results suggest that proliferation of 32D cells may also be regulated by intercellular contacts in certain circumstances (e.g. homotypic aggregation). Similar to contact-inhibited adherent cells, p27^Kip1 levels accumulate in 32D cells during conditions of increased intercellular contact. In our system, p27^Kip1 appears to be regulated post-transcriptionally, since p27^Kip1 mRNA levels do not change in response to Epo-treatment (data not shown), despite increases in the levels of p27^Kip1 protein. Therefore p27^Kip1 does not appear to be a direct Stat3 target gene in Epo-treated ER343/401-S3 cells. The accumulation of p27^Kip1 may be controlled by signaling through cell surface adhesion molecules in ER343/401-S3 cell aggregates, similar to its mode of regulation in adherent cell models of contact inhibition (58,61,62). In agreement with this, engagement of β1 integrins on normal human CD34+ hematopoietic progenitor cells results in the accumulation of p27^Kip1 and growth arrest (63).

Furthermore, accumulated p27^Kip1 is down-regulated when Epo-treated ER343/401-S3 cells are switched to IL-3-containing media. This indicates that IL-3-dependent signaling pathways can regulate p27^Kip1 expression. Similar results have been obtained in studies of normal human hematopoietic progenitor cells in which the growth arrest induced by β1 integrin engagement can be overcome by supraphysiological concentrations of IL-3 or stem cell factor (63).

Interestingly, physiological concentrations of either cytokine, however, were not sufficient to overcome β1 integrin-mediated growth arrest (63). Therefore, a complex relationship exists between adhesion molecule signaling and cytokine signaling. Further investigation is necessary to understand the extent of cross-talk between adhesion molecules and cytokine receptors in hematopoietic cells and how these pathways regulate hematopoietic cell development and function.

Recent studies demonstrate that the granulocyte colony-stimulating factor receptor (G-CSFR) is essential for stimulating G-CSF-mediated mobilization of hematopoietic progenitor cells or mature neutrophils from the bone marrow and for neutrophil chemotaxis in response to IL-8 (64,65). Interestingly, these cell trafficking processes could not be stimulated by EpoR-dependent signals, as determined by gene targeting studies, which substituted a chimeric G-CSFR/EpoR receptor for the endogenous G-CSFR (65). These experiments suggest that a specific G-CSFR-dependent signaling pathway regulates G-CSF-stimulated progenitor cell and neutrophil migration from the bone marrow. One of the primary differences in the EpoR
and G-CSFR signaling pathways is the Stat proteins they activate, with Stat5 as the major EpoR-responsive Stat, and Stats 1, 3, and 5 as G-CSFR-responsive Stats. Since our studies have indicated a role for Stat3 in cell adhesion molecule expression and function, it will be of interest to determine the in vivo function of Stat3 in mediating G-CSFR-responsive migration of hematopoietic cells.

Acknowledgements

We thank Hong Lu and Ling Zhang for excellent technical support, Steve Magid and Tim Lee for assistance with plasmid constructions, Brad McIntyre for generous gifts of many reagents, Doug Hilton for providing SOCS cDNAs, and Joan Egrie and Steve Elliot (Amgen) for the generous gift of recombinant Epo. In addition, we thank Henry Chan, David McConkey, Matthew Harbison, and members of the Watowich laboratory for their suggestions throughout the course of this work, and we thank Brad McIntyre, Rebecca Wells, and Peter Murray for their advice and critical review of the manuscript.

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Fig. 1. Schematic diagram of EpoRs with altered Stat binding regions
The cytoplasmic region of the WT EpoR is diagrammed on the left. The positions of the Jak2 binding domain (stippled box) and sequences surrounding the tyrosine residues required for Stat5 activation (Tyr-343 and Tyr-401) are indicated. Other tyrosine residues in the receptor cytoplasmic region are not shown. ER343/401-S3 (343/401-S3) has the sequence GYMPQ (Stat3 binding site) substituted for residues 342–346 and 400–404 of the WT EpoR, as indicated. The substitutions preserve Tyr-343 and Tyr-401 but change residues at the −1, +1, +2, and +3 positions. ER-Y343/401F (Y343/401F) has phenylalanine substitutions at positions 343 and 401, as indicated. All other residues are identical to WT EpoR.
Fig. 2. Epo-dependent Stat5 activation in 32D cells expressing WT or mutant EpoRs

A. 32D cells expressing WT, ER343/401-S3 (343/401), or ER-Y343/401F (Y343/401F) were starved of growth factors for 4 h, then stimulated for 8 min at room temperature with 0.5, 5.0, or 50.0 units (U)/ml Epo or were left unstimulated, as indicated. **Upper panel (αPY blot)**, Stat5 tyrosine phosphorylation was analyzed by immunoprecipitation and immunoblotting. **Lower panel (Stat5 EMSA)**, Stat5 DNA binding activity was determined by gel mobility shift assay using a Stat5-specific oligonucleotide.

B. cells were maintained in RPMI/FCS/WEHI (W) or in RPMI/FCS containing 0.5 units/ml Epo for 24 h (24) or 48 h (48). **Upper panel (αPY blot)**, Stat5 tyrosine phosphorylation was analyzed by immunoprecipitation and immunoblotting. **Middle panel (αStat5 blot)**, antiphosphotyrosine immunoblots were stripped and reprobed with Stat5-specific antisera. **Lower panel (Stat5 EMSA)**, Stat5 DNA binding activity was analyzed by EMSA. Control (C) contained extract from Epo-treated WT cells incubated with radiolabeled probe and a 100-fold excess of unlabeled probe. Digital images displaying the Stat5 polypeptide (~95 kDa) or Stat5-DNA complexes are shown.
Fig. 3. Epo-dependent Stat3 activation in 32D cells expressing WT or mutant EpoRs
A, growth factor-deprived cells were stimulated with the indicated concentrations of Epo as described in Fig. 2A. Upper panel (αPY blot), Stat3 tyrosine phosphorylation was analyzed by immunoprecipitation and immunoblotting. Lower panel (Stat3 EMSA), Stat3 DNA binding activity was determined by gel mobility shift assays using an SIE-containing oligonucleotide. B, cells were maintained as described in the legend to Fig. 2B. Upper panel (αPY blot), Stat3 tyrosine phosphorylation was analyzed by immunoprecipitation and immunoblotting. Middle panel (αStat3 blot), antiphosphotyrosine immunoblots were stripped and reprobed with a Stat3-specific antisera. Lower panel (Stat3 EMSA), Stat3 DNA binding activity was determined as described above. Control (C) contained extract from Epo-treated ER343/401-S3 cells incubated with radiolabeled probe and a 100-fold excess of unlabeled probe. C, ER343/401-S3 cells were grown in media containing IL-3 or 0.5 units (U)/ml Epo as described in panel B. EMSAs were performed with an SIE-containing oligonucleotide in the absence of antibody (343/401) or in the presence of Stat3-specific (343/401+aStat3) or Stat1-specific (343/401+aStat1) antibodies. Stat-DNA binding complexes and supershifted complexes are indicated by solid or open arrows, respectively. Digital images of the Stat3 polypeptide (~95 kDa) or the Stat3-DNA complexes are shown.
Fig. 4. Epo-dependent tyrosine phosphorylation of Jak2 and EpoR in 32D cells expressing WT and ER343/401-S3
Growth factor-deprived cells were stimulated with the indicated concentrations of Epo as described in the legend to Fig. 2A. Jak2 (upper panel) and EpoR (lower panel) tyrosine phosphorylation (PY) was analyzed by immunoprecipitation and immunoblotting. Digital images of the Jak2 (~130 kDa) or EpoR (~70 kDa) polypeptides are shown. U, units.
Fig. 5. Epo-dependent proliferation, cell cycle profiles, and p27Kip1 expression

A, cells expressing WT (circles), ER343/401-S3 (squares), or ER-Y343/401F (diamonds) were grown in medium containing IL-3 (upper panel), 0.5 units (U/ml Epo (middle panel), or 5.0 units/ml Epo (lower panel) for 8 days as described under “Experimental Procedures.” A plot of total cell numbers for a representative experiment is shown. B, cells expressing WT or ER343/401-S3 (343/401) were grown for 3 days in medium containing IL-3 (white bars) or 1 unit/ml Epo (black bars). Cells were stained with propidium iodide, and DNA content was analyzed by flow cytometry. The percentage of cells in G1 (upper panel) or S phase (lower panel) is indicated for each cell type and culture condition for a representative experiment. C, cells expressing WT or ER343/401-S3 were cultured in media containing IL-3 (W) or 0.5 units/ml Epo for 2, 4, or 6 days (as indicated), and expression of p27Kip1 was determined by immunoblotting. A digital image of p27Kip1 signal (~27 kDa) is shown.
Fig. 6. Morphology of WT and ER343/401-S3 cells

A, WT and ER343/401-S3 (343/401) were grown in media containing IL-3 (WEHI) or 0.5 units/ml Epo (Epo) for 48 h and then photographed. An arrow marks the position of a representative ER343/401-S3 cell aggregate. B, following 48 h of Epo culture, ER343/401-S3 cell aggregates were disrupted by pipetting and were plated in media containing 0.5 units/ml Epo with or without 100 nM wortmannin or 2.5 mM EDTA, as indicated. Cells were incubated for 60 min at 37 °C and photographed. NT, not treated. C, cell-spaying assays were performed with Epo-treated (48 h) WT or ER343/401-S3 cells on immobilized anti-CD18 (CD18) antibody or BSA, as indicated. Arrows mark the position of spread cells.
Fig. 7. Expression of ICAM-1 and CD18 in WT and ER343/401-S3 cells
Cells expressing WT or ER343/401-S3 (343/401) were grown for 48 h in media containing 0.5 units/ml Epo (E) or IL-3 (W). A and B, iodinated cell surface proteins were immunoprecipitated with an ICAM-1-specific antibody or a murine CD18-specific antibody, as indicated. A digital image of the autoradiogram from WT (A) or ER343/401-S3 (B) cells is shown. The migration positions of ICAM-1, CD11a/CD11b, and CD18 polypeptides are indicated by arrows. C, WT (upper panel) or ER343/401-S3 (lower panel) cells were grown in media containing Epo as described above. The cells were stained with ICAM-1-specific antibody (gray histograms), CD18-specific antibody (black histograms), or secondary antibody alone (white histograms) and analyzed by flow cytometry.
Fig. 8. Stat3 activation and p27^Kip1^ levels in ER343/401-S3 cells constitutively expressing SOCS3 or switched to IL-3-containing media

A, ER343/401-S3 (343/401) cells or ER343/401-S3 containing SOCS3 (343/401+SOCS3) were deprived of growth factors then stimulated with 0.5 units/ml Epo, as described in the legend to Fig. 2A. Stat3 tyrosine phosphorylation (PY) was analyzed by immunoprecipitation and immunoblotting. B, cells were grown in IL-3-containing media (W) or media containing 0.5 units (U)/ml Epo (E) for 72 h. Parallel cultures were maintained in media containing 0.5 units/ml Epo for 48 h, then switched to IL-3-containing media for an additional 24 h (E/W). Whole cell extracts were analyzed for p27^Kip1^ content by immunoblotting.
Fig. 9. Homotypic aggregation and signaling through CD18-containing integrins in ER343/401-S3 cells with abrogated Stat3 activity

A, ER343/401-S3 (343/401) cells or ER343/401-S3 constitutively expressing SOCS3 (Socs3) were cultured for 72 h in media containing 0.5 units/ml Epo (Epo) or for 48 h in media containing 0.5 units/ml Epo, then 24 h in media containing IL-3 (Epo-WEHI). Cells were photographed, and digital images of 100× phase contrast pictures are shown. B, cells were cultured for 72 h in media containing 0.5 units/ml Epo (Epo), and spreading assays were performed on immobilized anti-CD18 (CD18) antibody or BSA, as indicated. Digital images of 200× phase contrast pictures are shown.
Fig. 10. G-CSF stimulates homotypic aggregation and CD18 signaling

G-CSFR cells were cultured in media containing IL-3 or G-CSF (25 ng/ml), as indicated. A, cells were photographed (upper panel) after 7 d. An arrow marks the position of a cell aggregate. B, cells were used in spreading assays on immobilized CD18 antibody or BSA after 7 d, as indicated. Arrows mark the position of spread cells. Digital images of 200× phase contrast pictures are shown.
### Table I

#### Sequence of oligonucleotide primers

The sequence of oligonucleotide primers used to generate mutant EpoRs are shown, with regions encoding the mutations underlined.

| Primer name | Sequence |
|-------------|----------|
| 1 (990–1006) | 5′-CTTCCCTGAGGATCCAC-3′ |
| 2 (1235–1216) | 5′-GATGTTTCTGAAGCTTCATC-3′ |
| 3 (a343-S3) | 5′-CTGAAGCAATGTACCGGTCCCAGTGCTCACTGCC-3′ |
| 4 (343-S3) | 5′-GAGTTACGTGCTCAAGGATAGTGTTGCTGGCCCAGG-3′ |
| 5 (1209–1228) | 5′-GACTATGAGAAGCTTCAG-3′ |
| 6 (1561–1541) | 5′-CCGACCTCTAGGATCCTCGTCC-3′ |
| 7 (a401-S3) | 5′-CTGAGCAATGTACCCGATCGAATGGTCCGGATG-3′ |
| 8 (401-S3) | 5′-GGTTACGTGCTCAAGGACCCCAGCTCTAGCTCGT-3′ |
| 9 (aY343F) | 5′-CAATACCAAGAGGTGCTTG-3′ |
| 10 (Y343F) | 5′-CAGGACACTCTTGGATTGGTG-3′ |
| 11 (αY401F) | 5′-CAGGATGGAATCAAGCTGTGG-3′ |
| 12 (Y401F) | 5′-CAGCTTTGAGTTACCATCTCGT-3′ |