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Tyrosine kinase JAK1 is associated with the granulocyte-colony-stimulating factor receptor and both become tyrosine-phosphorylated after receptor activation

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ABSTRACT **Granulocyte-colony-stimulating factor (G-CSF) stimulates the proliferation and differentiation of cells of the neutrophil lineage by interaction with a specific receptor. Early signal transduction events following G-CSF receptor activation were studied. We detected tyrosine phosphorylation of both the G-CSF receptor and the protein tyrosine kinase JAK1 following G-CSF binding to the human G-CSF receptor. *In vitro*, the kinase activity of JAK1 was increased by G-CSF stimulation. Coimmunoprecipitation of JAK1 with the G-CSF receptor suggested a physical association which existed prior to G-CSF stimulation.**

Although the granulocyte-colony-stimulating factor receptor (G-CSF-R) has been cloned (1) and is functionally active in several different cell types (2–5), little is known about the mechanism of signal transduction. G-CSF-R is believed to consist of a single chain that is activated through ligand-induced homodimerization (6) as has been shown for the erythropoietin and growth hormone receptors (7, 8). G-CSF-R does not contain an intrinsic protein kinase domain (1), although tyrosine kinase activity seems to be required for transduction of the G-CSF signal (9).

JAK kinases (10, 11) are receptor-associated proteins which are rapidly phosphorylated after receptor activation. In particular, Tyk2 is phosphorylated following interferon α (IFN-α) receptor activation (12), and JAK2 is phosphorylated following the binding of erythropoietin (13), growth hormone (14), and interleukin 3 (15) to their respective receptors. Since G-CSF-R belongs to the same hemopoietin receptor family as the latter three receptors, these observations suggested that JAK kinases might also be involved in G-CSF signal transduction. Therefore, we have examined the role of JAK1 in early signal transduction events resulting from the association of G-CSF with its receptor.

MATERIALS AND METHODS

Cell Lines. The cell line CHO-6A11 was generated by transfection with the pEE6.HCMV.GS expression vector (16, 17) carrying a full-length cDNA encoding the G-CSF-R (1), kindly provided by S. Nagata (Osaka Bioscience Institute). These cells expressed ≈60,000 receptors per cell (as determined by binding of ^{125}I-labeled G-CSF). AML-193 is a human monocytic leukemic cell line (American Type Culture Collection CRL 9589) which proliferates in response to G-CSF (18).

Antisera. Rabbit polyclonal antiserum, designated R55, was generated by immunization with a pGEX/G-CSF-R bacterial fusion protein encompassing the hemopoietin receptor domain. A *Bam*HI fragment of the receptor cDNA encoding amino acids 17–345 was inserted into the *Bam*HI site of pGEX-1 (19). The monoclonal antibody to G-CSF-R, LMM741, was raised against CHO-6A11 cells. It was coupled to CNBr-activated Sepharose at 2.5 mg of antibody per 0.5 ml of Sepharose. Rabbit antiserum M7 was generated by immunization with a pGEX/JAK1 bacterial fusion protein spanning the first kinase-like domain of JAK1 (amino acids 576–825) (10). When compared with JAK2 antiserum, M7 detects a protein which migrates more slowly in SDS/polyacrylamide gels than JAK2, suggesting that M7 is specific for JAK1 despite the close homology of domain 1 between JAK1 and JAK2 (unpublished results and ref. 20) (our results were obtained with anti-JAK2 serum which did not recognize phosphorylated JAK2). Anti-phosphotyrosine antibody 4G10 was obtained from Upstate Biotechnology. It was used at 1 μg/ml for immunoprecipitation and 0.1 μg/ml for Western blotting.

Proliferation Assays. Cells were plated in 96-well plates at 50,000 (CHO-6A11) or 20,000 (AML-193) cells per well, serum-starved (CHO-6A11) or serum- and growth factor-starved (AML-193) overnight, and incubated with recombinant human (rh)G-CSF (Amgen) for 24 hr (CHO-6A11) or 6 days (AML-193). Cells were then incubated with [methyl-^{3}H]thymidine (0.5 μCi per well; 1 μCi = 37 kBq) and harvested onto filter paper.

Cell Lysis. Cells were serum- and growth factor-starved for 24 hr before incubation with rhG-CSF (100 ng/ml). They were then lysed in either buffer A or buffer B with the addition of 0.1 mM Na_3VO_4, 1 μM pepstatin, 50 μM 3,4-dichloroisocoumarin, 1 mM phenylmethylsulfonyl fluoride, 1 mM 1,10-phenanthroline, leupeptin (10 μg/ml), and aprotonin (10 μg/ml). Buffer A is 50 mM Tris·HCl, pH 7.5/150 mM NaCl/1% (vol/vol) Triton X-100/1 mM EDTA; buffer B is 0.5 M Tris·HCl, pH 7.5/137 mM NaCl/0.1% Triton X-100/2 mM EDTA. Protein concentration in all experiments was determined by Bradford assay (Bio-Rad) using bovine serum albumin as standard and was adjusted to be equivalent in each sample. The cell lysates were precleared with rabbit anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark) or normal rabbit serum and protein A-Sepharose (Pharmacia).

Immunoprecipitation and Western Blotting. Cell lysates were incubated with the appropriate antibodies for 2 hr at 4°C. Antigen–antibody complexes were recovered by using rabbit anti-mouse immunoglobulin and protein A-Sepharose or protein A-Sepharose alone. Immunoprecipitates were analyzed by SDS/6% polyacrylamide gel electrophoresis (PAGE) under reducing conditions. Protein was then electrophoretically transferred to nitrocellulose. Antibody binding was visualized with either peroxidase-conjugated swine

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Abbreviations: G-CSF, granulocyte-colony-stimulating factor; G-CSF-R, G-CSF receptor; IFN, interferon.
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anti-rabbit immunoglobulin (Dakopatts) or peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts) and the enhanced chemiluminescence (ECL) system (Amersham). To reprobe, the nitrocellulose was first stripped of antibodies by 62.5 mM Tris·HCl, pH 6.7/2% SDS/100 mM 2-mercaptoethanol.

***In Vitro* Kinase Assay (15).** Immunoprecipitates were washed and suspended in an equal volume of kinase assay buffer (50 mM NaCl/5 mM $MgCl_2$/5 mM $MnCl_2$/0.1 mM Na_3VO_4/10 mM Hepes, pH 7.4) containing [γ-^{32}P]ATP (0.25 μCi/ml) for 30 min at room temperature. Immunoprecipitates were analyzed by SDS/8% PAGE. Radioactive bands were visualized and quantitated with IMAGEQUANT software on a PhosphorImager system (Molecular Dynamics).

Protein was electrophoretically transferred to poly(vinylidene difluoride) and the ^{32}P-labeled protein was excised and subjected to phospho amino acid analysis (21).

RESULTS

Antibody Detection of G-CSF-R and Proliferative Response to G-CSF. To generate a model system for analyzing signal transduction by human G-CSF-R, CHO-K1 cells were transfected with human G-CSF-R cDNA (CHO-6A11). Monoclonal and polyclonal antibodies to G-CSF-R were produced to enable detection of receptor-associated proteins.

Monoclonal antibody LMM741 and rabbit serum R55 detected the receptor in cell lysates from CHO-6A11 and the human monocytic leukemic cell line AML-193 (Fig. 1*A*). Two proteins of apparent molecular mass 130 and 110 kDa were detected in CHO-6A11 lysates (Fig. 1*A*, lane D) and were not present in lysates of untransfected CHO-K1 cells (lane C) or when nonimmune rabbit serum was used (lanes A and B). Three proteins of 145, 135, and 115 kDa were detected in AML-193 cells (lane E). Bands below 66 kDa were also present with nonimmune rabbit serum. The major band was due to the IgG heavy chain. AML-193 cells "proliferated" (incorporated [^{3}H]thymidine) in response to G-CSF, whereas CHO-6A11 cells did not (Fig. 1*B*). The CHO-6A11 cells did not proliferate in response to G-CSF under other conditions tested, including extending the incubation time to 4 days and incubating in the presence of fetal bovine serum or insulin, transferrin, and selenium.

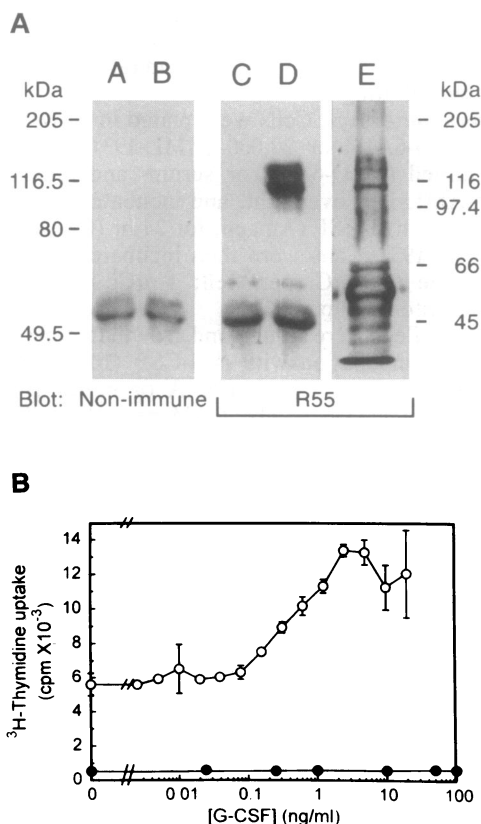

FIG. 1. (*A*) Detection of the G-CSF-R. Untransfected CHO cells (lanes A and C); CHO-6A11 cells (lanes B and D), and AML-193 cells (lane E) were lysed and G-CSF-R protein was immunoprecipitated with monoclonal antibody LMM741. Samples were immunoblotted with either nonimmune rabbit serum (lanes A and B) or with rabbit anti-receptor serum R55 (lanes C–E). (*B*) G-CSF-induced proliferation. CHO-6A11 cells (●) and AML-193 cells (○) were incubated with rhG-CSF for 24 hr (CHO-6A11) or 6 days (AML-193). Incorporated cpm are expressed as the mean ± SD of triplicates.

Phosphorylation of JAK1 in Response to G-CSF. Stimulation of CHO-6A11 cells with G-CSF induced a tyrosine-phosphorylated band of 130 kDa which was detected by antiserum to JAK1 (M7) (Fig. 2*A*). Phosphorylation of JAK1 was also observed by immunoprecipitation with M7 and immunoblotting with anti-phosphotyrosine (Fig. 2*B Upper*). When this blot was stripped and reprobed with M7, the result (Fig. 2*B Lower*) showed that there was equal sample loading in all lanes. Tyrosine phosphorylation of JAK1 was observed after 1 min of G-CSF stimulation, was maximal between 10 and 20 min, and still evident after 60 min (Fig. 2*B*). Tyrosine phosphorylation of JAK1 in response to G-CSF was also observed in the AML-193 cells (Fig. 2*C*).

***In Vitro* Kinase Activity of JAK1.** An *in vitro* kinase assay was used to test whether the increased tyrosine phosphorylation of JAK1 correlated with an increase in intrinsic kinase activity. When incubated with [γ-^{32}P]ATP, JAK1 showed an increased capacity to autophosphorylate in response to G-CSF stimulation (Fig. 3*A*). Incorporation of ^{32}P was maximally induced, 3.9-fold (mean of three experiments), at 30 min. A higher molecular weight band of unknown identity was also phosphorylated. This was not consistently seen in all experiments. Bands at other molecular weights were also occasionally observed. Phospho amino acid analysis from kinase assay samples at 0 and 30 min after G-CSF stimulation confirmed that JAK1 was phosphorylated on tyrosine residues (Fig. 3*B*). Some increase in phosphorylation on serine residues was also observed.

Association of JAK1 with G-CSF-R. To determine whether JAK1 is physically associated with G-CSF-R, a receptor immunoprecipitate was analyzed by immunoblotting with M7. JAK1 coimmunoprecipitated with G-CSF-R both before and after G-CSF treatment of CHO-6A11 cells (Fig. 4, lanes A and B). This band was not observed when G-CSF-R immunoprecipitates were blotted with M7 preimmune serum (data not shown). The amount of JAK1 coimmunoprecipitate remained constant for at least 20 min (data not shown) and appeared to be a relatively small percentage of total cytoplasmic JAK1 (Fig. 4, lanes C and D). Receptor immunoprecipitates also showed *in vitro* kinase activity corresponding to a 130-kDa protein (data not shown).

Phosphorylation of G-CSF-R. Stimulation of CHO-6A11 cells with G-CSF also resulted in phosphorylation of G-CSF-R on tyrosine within 2 min of G-CSF stimulation (Fig. 5). The phosphorylated receptor migrated at a higher apparent molecular mass (150 and 135 kDa) than the unphosphorylated receptor (130 kDa and 110 kDa). When the reverse experiment was performed, immunoprecipitating with anti-phosphotyrosine and blotting with either LMM741 or R55, the phosphorylated protein was detected at the same molecular mass, confirming this result (data not shown).

DISCUSSION

This study has established that JAK1 is associated with G-CSF-R and that both proteins are phosphorylated in response to G-CSF stimulation.

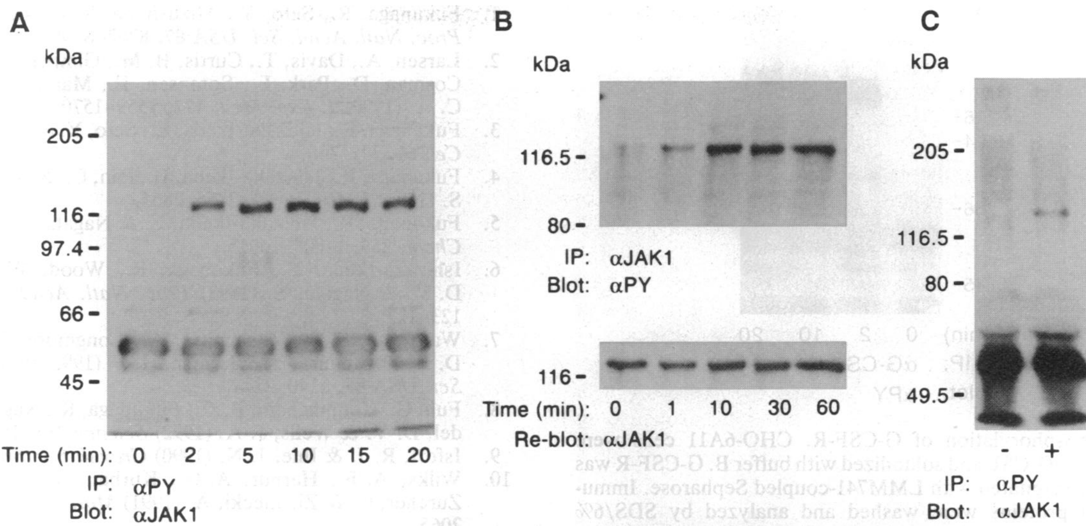

FIG. 2. Phosphorylation of JAK1 in response to G-CSF. (*A*) CHO-6A11 cells were incubated with rhG-CSF for the times indicated and lysed with buffer A. Phosphorylated proteins were immunoprecipitated (IP) with the anti-phosphotyrosine antibody 4G10 (αPY). Precipitates were analyzed by Western blot with protein A-purified rabbit anti-JAK1 IgG (M7). (*B*) CHO-6A11 cells were incubated with rhG-CSF for the times indicated and lysed using buffer A. JAK1 proteins were immunoprecipitated using purified M7 and subjected to Western blot analysis using 4G10 (*Upper*). The nitrocellulose was stripped of antibodies and reprobed with M7 (*Lower*). (*C*) AML-193 cells were incubated with G-CSF (+) or medium (−) for 10 min and lysed. Lysate proteins immunoprecipitated with 4G10 were subjected to Western blot analysis with M7.

The rapid tyrosine phosphorylation of JAK1 strongly indicates its involvement in intracellular events triggered by G-CSF. Increased levels of phosphotyrosine have proved to be an excellent index of the involvement of particular protein-tyrosine kinases in signal transduction pathways (22). Phospho amino acid analysis of JAK1 following an *in vitro* kinase assay revealed ^{32}P incorporation into both tyrosine and serine residues. This is in contrast to the observation that JAK2 incorporates ^{32}P into tyrosine and threonine residues (13). The elevated kinase activity of JAK1 upon activation of CHO-6A11 cells by G-CSF underscores the likely involvement of this molecule in G-CSF-mediated signal transduction. The apparent disparity between the times of maximum phosphorylation of JAK1 and maximum kinase activity is at present unexplained. It is possible that JAK1 is in fact phosphorylated by another, as yet unidentified, protein-tyrosine kinase.

Several forms of G-CSF-R were identified by specific antibodies in both the transfected CHO cells and the AML-193 cells. The different-sized proteins observed are likely to be due to differently glycosylated forms of the receptor (4).

The phosphorylated receptor migrated more slowly than unphosphorylated receptor on SDS/PAGE. Differential migration of phosphorylated protein has been observed with other proteins—for example, STAT91 (23) and middle-sized tumor antigen (24). Phosphorylation of murine G-CSF-R in a transfected hemopoietic cell line (32D) has also been detected recently by Pan *et al.* (25).

G-CSF-R shows considerable homology with gp130 (46.3% amino acid similarity) (4), the signal-transducing component of the interleukin 6, oncostatin M, leukemia inhibitory factor, ciliary neurotrophic factor, and interleukin 11 receptors. Of interest, two of the three highly conserved regions of homology between gp130 and G-CSF-R have been shown to be

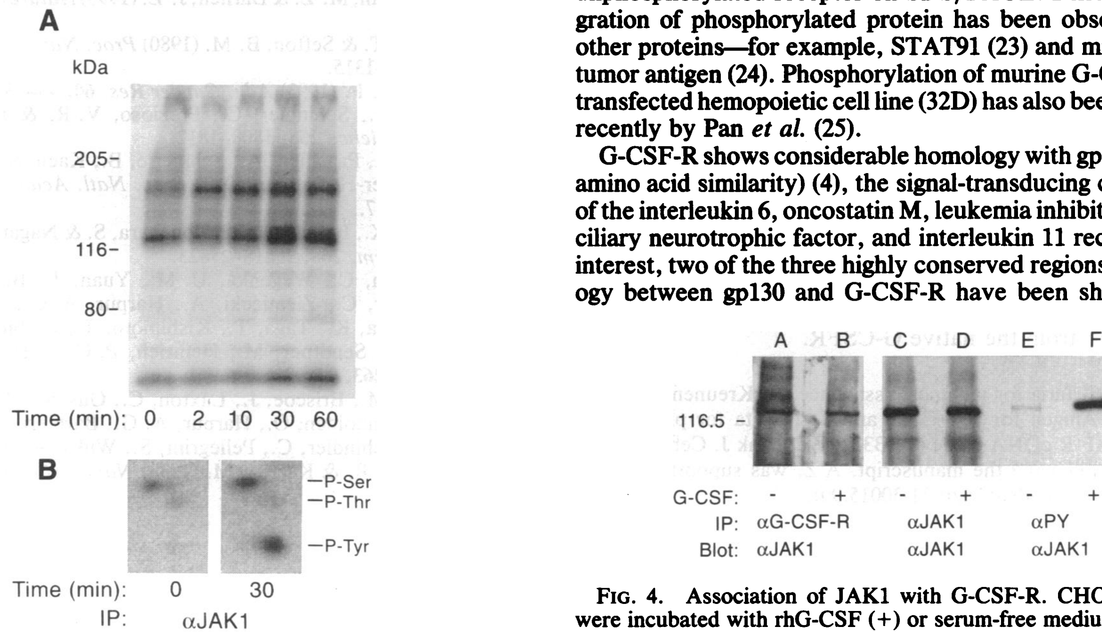

FIG. 3. Autophosphorylation of JAK1 in an *in vitro* kinase assay. (*A*) CHO-6A11 cells were incubated with rhG-CSF for the times indicated. Cells were lysed with buffer A and immunoprecipitated with purified M7. (*B*) Phospho amino acid analysis. ^{32}P-labeled JAK1 protein at 0 and 30 min from a representative experiment as described in *A* was electrophoretically transferred to poly(vinylidene difluoride) and subjected to phospho amino acid analysis.

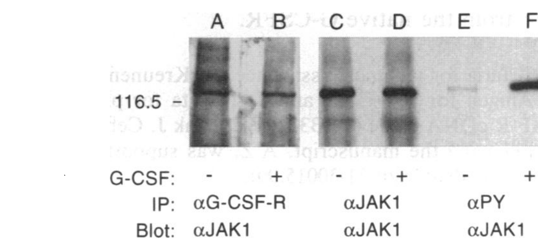

FIG. 4. Association of JAK1 with G-CSF-R. CHO-6A11 cells were incubated with rhG-CSF (+) or serum-free medium alone (−) for 10 min. Cells were lysed with buffer A. Proteins in lanes A and B were immunoprecipitated with LMM741-coupled Sepharose and analyzed by Western blot with purified M7. Exposure time was 30 min. For lanes C and D, JAK1 proteins were immunoprecipitated with M7 serum and analyzed by Western blot with purified M7. For lanes E and F, cell lysates were immunoprecipitated with 4G10. After transfer to nitrocellulose, proteins were probed with M7. Lanes C–F were exposed for 1 min.

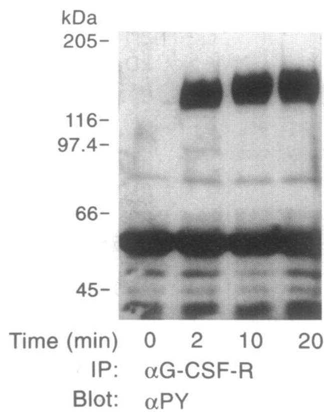

FIG. 5. Phosphorylation of G-CSF-R. CHO-6A11 cells were incubated with rhG-CSF and solubilized with buffer B. G-CSF-R was then immunoprecipitated with LMM741-coupled Sepharose. Immunoprecipitated proteins were washed and analyzed by SDS/6% PAGE. After electrophoretic transfer to nitrocellulose, phosphorylated G-CSF-R was detected with anti-phosphotyrosine (4G10).

necessary for transduction of the G-CSF growth signal, most notably a 99-amino acid region of the cytoplasmic domain (4). Consistent with the notion that this homology indicates similar function, Lutticken *et al.* (26) have recently observed JAK1 association with gp130 after stimulation with interleukin 6 and leukemia inhibitory factor.

The data presented here are strongly suggestive of an important role for JAK1 in G-CSF-R signal transduction pathways. The coprecipitation of G-CSF and JAK1 demonstrates a close association between these molecules. Furthermore, phosphorylation of both within 2 min of G-CSF binding makes it likely that JAK1 is the kinase responsible for G-CSF-R phosphorylation.

A recent report (27) indicates that JAK1 is interdependent with at least one other JAK family kinase in signaling pathways for IFN-α and IFN-γ receptors. In a JAK1-defective, IFN-unresponsive cell line, JAK1 restored both the response to IFN and phosphorylation of JAK2 and Tyk2 in the IFN-γ and IFN-α pathways, respectively (27). Similar studies will be necessary to fully establish the importance of JAK1 in the G-CSF pathway. The possible role of other members of the JAK family (in the G-CSF signaling pathway) remains to be investigated.

Although the CHO-6A11 system is an artificial one, it is clear that AML-193 cells, which proliferate in response to G-CSF, share the same JAK1 response (Fig. 2*C*). This suggests that these observations can also be applied to signal transduction from the native G-CSFR.

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