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Simultaneous Activation of JAK1 and JAK2 Confers IL-3 Independent Growth on Ba/F3 pro-B Cells

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Abstract JAK1 and JAK2 are tyrosine kinases involved in the regulation of cell proliferation, differentiation, and survival. These proteins may play a key role in mediating the effects of the cytokine IL-3 on hematopoietic cells. IL-3 induces tyrosine phosphorylation of both JAK1 and JAK2. However, it is not clear whether the activation of JAK1, JAK2, or both is sufficient to confer factor-independent growth in IL-3 dependent cells. To address this issue, fusion proteins CD16/CD7/JAK (CDJAK), comprised of a CD16 extracellular domain, a CD7 transmembrane domain, and a JAK cytoplasmic region (either a wild-type JAK or a dominant negative mutant of JAK) were constructed. We established several Ba/F3 derivatives that stably overexpress the conditionally active forms of either CDJAK1, CDJAK2, or both these fusion proteins. In this study, the autophosphorylation of CDJAK1 or CDJAK2 was induced by crosslinking with anti-CD16 antibody. We demonstrated that, like their wild-type counterparts, CDJAK1 and CDJAK2 were preassociated with the IL-3 receptor beta and alpha subunits, respectively. Furthermore, the simultaneous activation of both CDJAK1 and CDJAK2 fusion proteins, but not either one alone, led to the tyrosine phosphorylation of the IL-3 receptor beta subunit, the activation of downstream signaling molecules, including STAT5, Akt, and MAPK, and the conferring of factor-independent growth to IL-3-dependent Ba/F3 cells. Coexpression of dominant negative mutants CDJAK1KE or CDJAK2KE with wild type CDJAK2 or CDJAK1, respectively, inhibited these activation activities. These results suggest that JAK1 and JAK2 must work cooperatively and not independently and that their actions are dependent on having normal kinase activity to trigger downstream signals leading to IL-3 independent proliferation and survival of Ba/F3 cells. J. Cell. Biochem. 96: 361–375, 2005.

Key words: JAK1; JAK2; tyrosine phosphorylation; cell proliferation; IL-3

Cytokine receptors do not possess protein kinase activity, they likely associate with and require transducing signals from cytoplasmic Janus tyrosine kinases (JAKs) [Ihle et al., 1994]. JAKs (JAK1, JAK2, JAK3, and TYK2) are a family of nonreceptor tyrosine kinases [Ihle and Kerr, 1995]. One downstream effect of JAK activation is the phosphorylation of the signal transducer and activator of transcription (STAT) proteins. Phosphorylated STAT proteins then translocate into the nucleus to regulate the transcription of specific genes [Ihle et al., 1994; Ihle and Kerr, 1995]. In addition to the STAT proteins, JAKs also activate other pathways, including the phosphatidylinositol 3-kinase (PI-3K)/Akt pathway, and the mitogen-activated protein kinase (MAPK) pathway [Winston and Hunter, 1995; Burfoot et al., 1997; Takaoka et al., 1999; Oki et al., 2002]. The STAT, Akt, and MAPK pathways have been shown to regulate cell proliferation and anti-apoptosis activities [Downward, 1998; Williams, 2000; Chang and Karin, 2001].

The cytokine interleukin-3 (IL-3) regulates proliferation, differentiation, and anti-apoptosis of hematopoietic cells [Arai et al., 1990; Crompton, 1991]. IL-3 performs these functions by specifically binding to the IL-3 receptor α

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subunit (IL-3Rα) and recruiting the receptor β subunit to form a heterodimer [Miyajima et al., 1993; Orban et al., 1999]. The β subunit, known as β common (βc), is shared by cytokine receptors for IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF), whereas α subunit is specific to each cytokine. The mouse has two type of highly homologous β subunits, βc and IL-3Rβ ($\beta_{IL\text{-}3}$), while humans have one type of β subunit (βc). The IL-3 receptor (IL-3R) belongs to the cytokine receptor superfamily. Binding of IL-3 to IL-3R results in rapid tyrosine phosphorylation in JAK1 and JAK2 [Callus and Mathey-Prevot, 1998].

Past studies used biochemical approaches to form JAK2 fusion proteins with an artificial activation system to demonstrate the role of JAK2 tyrosine phosphorylation in IL-3 dependent and GM-CSF dependent cells. In these systems, it is likely that the clustering of JAK2 fusion proteins by conditional activation might have resulted in the autophosphorylation of JAK2 fusion proteins. JAK2 fusion proteins with different extracellular and transmembrane domains have showed varying effects on cell activity. The EGFR/JAK2 fusion protein, consisting of the extracellular and transmembrane domains of epidermal growth factor receptor (EFGR) and the JAK2 kinase domain, when activated by EGF, promoted cell proliferation and suppressed apoptosis in an IL-3 independent manner [Nakamura et al., 1996]. The TEL-JAK2 fusion protein, consisting of the TEL (an ETS transcription factor) and the JAK2 kinase domain, exhibited constitutive tyrosine kinase activity that transformed IL-3 dependent cells into IL-3 independent cells [Lacronique et al., 1997]. The CD16/JAK2-K fusion protein, consisting of the extracellular domain of CD16 and the transmembrane domain of CD7 (CD16/CD7) and the JAK2 kinase domain, was shown to be constitutively phosphorylated and did not to promote cell proliferation, but delayed cell death [Sakai and Kraft, 1997]. Furthermore, JAK2 fusion proteins were formed consisting of a full length JAK2 with CD16/CD7 (CD16/JAK2) [Sakai et al., 1995] or Gyrase B (GyrB/JAK2) [Mohi et al., 1998]. The fusion proteins CD16/JAK2 and GyrB/JAK2 were activated by anti-CD16 antibody and coumermycin, respectively [Sakai et al., 1995; Mohi et al., 1998]. Although these fusion proteins of full length JAK2 could transduce signals, they could not effectively facilitate cell proliferation. Other studies have revealed that the involvement of JAK2 alone is not sufficient to optimize cell proliferation. In addition, other reports have also implied that activation of both JAK1 and JAK2, leading to cell proliferation and anti-apoptosis, is correlated with IL-5 or GM-CSF activity [Kouro et al., 1996; Huang et al., 2000; Watanabe et al., 2000].

In this study, we examined how the simultaneous activation of JAK1 and JAK2 affects cell signaling and cell activity (cell proliferation and anti-apoptosis) in IL-3 dependent cells. To do this, we constructed fusion proteins of CD16/CD7 and full length JAKs or with a dominant negative mutant of JAK (JAKKE) in a conditional activation system to simultaneously activate JAK1 (or JAK1KE) and JAK2 (or JAK2KE) by anti-CD16 antibody crosslinking. The experimental results revealed that either CDJAK1 or CDJAK2 alone could be phosphorylated by antibody crosslinking. However, the simultaneous activation of both CDJAK1 and CDJAK2 were required to support signal transduction, cell proliferation, and anti-apoptosis.

MATERIALS AND METHODS

Cell Lines and Antibodies

TF-1 cells (GM-CSF- or IL-3-dependent human erythroleukemia cells) were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 50 μM β-mercaptoethanol, 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 1 ng/ml of GM-CSF. Ba/F3 cells (murine IL-3-dependent pro-B cells) were maintained in a TF-1 cell medium that was supplemented with 1% conditioned medium from WEHI-3B cells as a source of IL-3. For cytokine stimulation experiments, cells were rinsed three times with phosphate-buffered saline (PBS) and starved in 0.5% FBS medium for 12 h before being stimulated by 10 ng/ml GM-CSF or IL-3 (R&D Systems, Minneapolis, MN).

Monoclonal anti-human CD16 $F(ab')_2$ antibody clone 3G8, used in crosslinking and flow cytometric analysis, was purchased from Ancell Corporation (Bayport, MN) and clone 2H7, used in Western blot analysis of CD16, was purchased from Novocastra (England). Goat anti-mouse IgG $F(ab')_2$ antibody and rabbit IgG control antibody were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Mouse IgG1 isotype control antibody was

purchased from R&D Systems. Antibodies specific to JAK1, JAK2, JAK3, TYK2, STAT5, IL-3Rα, and β_{IL-3} were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine monoclonal antibody (4G10) and the agarose conjugate of anti-phosphotyrosine monoclonal antibody (4G10) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-phospho-MAPK antibody, anti-MAPK antibody, anti-phospho-Akt antibody, and anti-Akt antibody were purchased from New England Biolabs, Inc. (Beverly, MA). FITC-goat anti-mouse IgG was purchased from ZYMED Laboratories, Inc. (South San Francisco, CA). Monoclonal anti-α tubulin antibody and anti-mouse or anti-rabbit IgG-HRP conjugated secondary antibodies were purchased from Amersham Pharmacia Biotech, Inc. (San Francisco, CA).

Construction of Plasmids

Plasmids pKSJAK1 and pSKJAK2 contain the entire coding region (~4 kb) of murine JAK1 and JAK2, respectively. Plasmids pKSJAK1-MluI and pSKJAK2-MluI were created from pKSJAK1 and pSKJAK2, respectively, by inserting a *Mlu*I restriction site before the start codons of the respective cDNAs by PCR. The primers used to construct pKSJAK1-MluI were 5′-CGCGTCGACACGCGTACCATGCAGTATCTAAATATA-3′ (forward primer) and 5′-AGTGGAGCCGGAGAGACG-3′ (reverse primer) and for pSKJAK2-MluI, the primers used were 5′-GCCCGCGGACGCGTACCATGGGAATGGCCTGCCTTACA-3′ (forward primer) and 5′-TGCCCAAGAGAATGGTACAGA-3′ (reverse primer). The amplified *Mlu*I containing JAK1 and JAK2 PCR fragments, formed by the two sets of primers, were digested with *Sal*I and *BsmB*I and with *Sac*II and *BstX*I, respectively, allowing the fragments to be ligated with *Sal*I/*BsmB*I digested-pKSJAK1 and *Sac*II/*BstX*I digested-pSKJAK2, thus inserting the *Mlu*I site into the respective plasmids. Plasmid pCD16/CD7/Syk carries the coding regions of CD16 extracellular and CD7 transmembrane domains [Lee et al., 1999]. Plasmids pCD16/CD7/JAK1 and pCD16/CD7/JAK2 were constructed by subcloning the *Mlu*I and *Not*I of pKSJAK1-MluI and pSKJAK2-MluI into the corresponding sites in pCD16/CD7/Syk. Plasmid pKSJAK1KE-MluI was constructed by subcloning the *BsmB*I-*Bgl*II fragment of plasmid pBabehygroJAK1KE into the corresponding sites of plasmid pKSJAK1-MluI. Plasmid pSKJAK2KE-MluI was constructed by subcloning the *Stu*I-*Nhe*I fragment of plasmid pBabehygroJAK2KE into the corresponding sites of plasmid pSKJAK2-MluI. Plasmid pCD16/CD7/JAK1KE and pCD16/CD7/JAK2KE were constructed by subcloning the *Mlu*I and *Not*I fragments of plasmids pKSJAK1KE-MluI and pSKJAK2KE-MluI into the corresponding sites in pCD16/CD7/Syk. Correct PCR and cloning were confirmed by dideoxy-DNA sequencing. Plasmids pKSJAK1, pSKJAK2, and pBabehygroJAK2KE were kindly provided by Dr. James N. Ihle (St. Jude's Research Hospital, Memphis, TN) and plasmid pBabehygroJAK1KE by Dr. Hsin-Fang Yang-Yen (Academia Sinica, IMB, Taipei, Taiwan).

Transfection of Ba/F3 Cells

Ba/F3 cells were transfected by electroporation (300 V and 40 kHz) with 10 μg of plasmid DNA in a Gene Pulser II RF-Module system (BIO-RAD). Transfected cells were seeded in fresh growth medium for 24 h before G418 or G418 plus hygromycin B was added to select stable clones. CDJ1, CDJ2, or CDSTOP [Lee et al., 1999] cells were maintained in the same medium as parental Ba/F3 cells, except for the inclusion of 200 μg/ml of G418 in the medium. CDJ1 + 2, CDJ1KE + 2, and CDJ1 + 2KE cells were maintained in the same medium as Ba/F3 cells, except for the inclusion of 200 μg/ml of G418 plus hygromycin B.

Flow Cytometric Analysis

Cell surface expression of CDJAK fusion proteins was analyzed by flow cytometry. 1×10^6 rapidly growing cells were rinsed three times with ice-cold PBS. All subsequent labeling steps were performed on ice. Cells were mixed with 0.5 μg of anti-CD16 antibody and incubated for 30 min. Cells were then rinsed twice in ice-cold PBS and incubated with FITC-conjugated goat anti-mouse antibody. After 30 min, cells were rinsed three times. The fluorescent intensity of surface-bound antibodies was analyzed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Antibody Crosslinking

Cells were rinsed three times with PBS and starved for 12 h in RPMI 1640 medium supplemented with 0.5% FBS. 10^7 cells were

resuspended per 0.5 ml of original medium and incubated without (control) or with 1 μg of anti-CD16 $F(ab')_2$ antibody for 5 min at room temperature, followed by incubation with 5 μg/ml of anti-mouse IgG $F(ab')_2$ for 15 min at 37°C.

Western Blot Analysis and Immunoprecipitation

Cells were lysed in lysis buffer (1% NP-40, 0.25% sodium deoxycholate, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 mg/ml leupeptin, 1 mM Na_3VO_4, and 1 mM NaF). For Western blot analyses, lysates (100 μg total protein) were first resolved on 7.5% SDS–polyacrylamide gels, transferred onto PVDF membranes (Millipore, Bedford, MA), and then probed with primary antibodies. Bound antibodies were detected with anti-mouse or anti-rabbit IgG-HRP conjugated secondary antibodies and visualized with an enhanced chemiluminescence (ECL) detection system (MEN Life Science Products, Inc., Boston, MA). For immunoprecipitation, lysates were precleared by incubation with protein A-Sepharose beads for 30 min at 4°C and then incubated with the indicated primary antibody for 2–4 h at 4°C. Immune complexes were precipitated with protein A-Sepharose beads over 2 h at 4°C. Pelleted beads were rinsed three times with lysis buffer and then boiled for 5 min with SDS sample buffer. The eluted proteins were electrophoresed on a 7.5% SDS–PAGE gel, electroblotted, and analyzed by Western blotting as before. For coimmunopricipitation, the detergent in the lysis buffer (1% NP-40 and 0.25% sodium deoxycholate) was replaced with 0.5% NP-40.

MTT Assay

Cell proliferation was measured by means of the methyl thiazole tetrazolium (MTT) assay. Ba/F3 parental cells and Ba/F3-derived transfectants were rinsed three times with PBS and transferred to a cytokine-free medium in a flat-bottom 96-well plate (1.25×10^4 cells/100 μl/well). Ba/F3 parental cells were then incubated with or without IL-3, while Ba/F3-derived transfectants were stimulated with or without antibody crosslinking. After 48 h of incubation, 10 μl of the dye MTT (3-4, 5-dimethylthiazol-2yl-diphenyltetrazolium bromide, 5 mg/ml) was added to each well, and the plates were incubated for a further 4 h at 37°C. The MTT solution was removed without disturbing the cells and 100 μl DMSO was added to each well to dissolve the formazan crystals. After the plates had been gently shaken for 5 min to ensure that the crystals were completely dissolved, the absorbance at 570 nm was read with a microplate reader (Bio-Tek, μQuant, Winooski, VT).

DNA Fragmentation Assay

DNA fragmentation assay was performed as described previously [Huang et al., 1999]. In brief, 1×10^6 cells were cultured for 48 h in the indicated conditional medium and then rinsed, pelleted, and resuspended in 50 μl of Williams lysis buffer (50 mM Tris HCl, pH 8.0, 10 mM EDTA, 0.5% Sarkosyl, and 500 μg/ml proteinase K) and incubated at 50°C for 3 h. Ten microliters of RNase A (2 mg/ml) were then added to the samples and incubated for a further 1 h at 37°C. After the addition of 1 μl of ethidium bromide (10 mg/ml), the samples were extracted with an equal volume of phenol/chloroform (1:1). To the extracted DNA, 10 μl of 1% low melting agarose, containing 10 mM EDTA (pH 8.0), were added before storage at 4°C. For DNA gel electrophoresis, the samples were thawed at 70°C, loaded into wells, and allowed to solidify inside the wells before electrophoresis was initiated. DNA fragmentation was detected by ethidium bromide staining.

RESULTS

IL-3 Simultaneously Stimulated Tyrosine Phosphorylation of JAK1 and JAK2 in TF-1 Cells

Previous studies have suggested that the simultaneous activation of both JAK1 and JAK2 may be essential for optimizing IL-5- or GM-CSF-dependent survival in TF-1 or Ba/F3 cells [Huang et al., 2000; Watanabe et al., 2000]. Therefore, in this study, we used both these cell lines to examine further the activation of the JAK family by IL-3. To establish the level of tyrosine phosphorylation of endogenous JAK proteins in TF-1 or Ba/F3 cells, we starved these cells and then stimulated them with IL-3. As seen in Figure 1, in TF-1 cells, IL-3 stimulated tyrosine phosphorylation of both JAK1 and JAK2 but not that of JAK3 and TYK2. TF-1 cells are GM-CSF dependent cells. Therefore, stimulation of TF-1 cells with GM-CSF, leading to activation (phosphorylation) of JAK1 and JAK2, was a suitable positive control. IL-3 stimulation of Ba/F3 cells also led to tyrosine

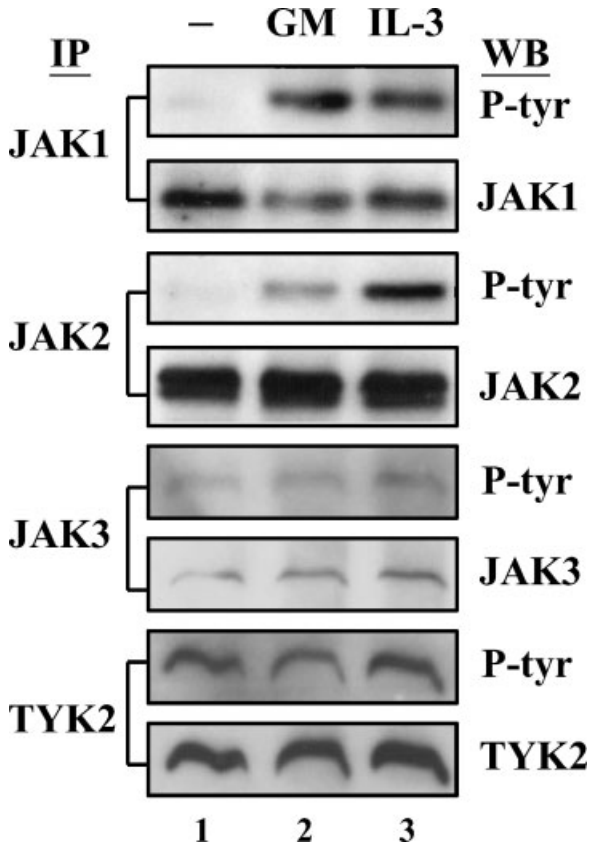


Fig. 1. IL-3 simultaneously induced tyrosine phosphorylation of JAK1 and JAK2 in TF-1 cells. TF-1 cells were cytokine-depleted for 24 h (**lane 1**) before stimulation with GM-CSF (**lane 2**) and IL-3 (**lane 3**) for 5 min. Cell lysates were prepared and subjected to immunoprecipitation (IP)-Western Blot analysis (WB). Cell lysates were immunoprecipitated with antibodies specific to JAK1, JAK2, JAK3, or TYK2. Precipitated proteins were resolved by 7% SDS–PAGE, transferred onto PVDF membranes, and Western blotted with anti-phosphotyrosine antibody (indicated as P-tyr on the right-hand side, under WB). The membranes were stripped and reprobed with anti-JAK1, -JAK2, -JAK3, or -TYK2 (as indicated on the right-hand side, under WB). The Western blot signals were visualized by ECL reaction and exposed onto X-ray film.

phosphorylation of JAK1 and JAK2 (data not shown), as has been previously reported [Callus and Mathey-Prevot, 1998].

Expression of CD16/CD7/JAK (CDJAK) Fusion Proteins in Stably Transfected Ba/F3 Cells

A previous study introduced JAK2 into Ba/F3 cells as part of a chimeric transmembrane protein containing the extracellular and transmembrane domains of CD16 and CD7, respectively. The transmembrane domain of the JAK2 fusion protein prevented homodimerization in absence of crosslinking. Clustering of the JAK2 fusion proteins, using anti-CD16 antibody crosslinking, was found to activate the JAK2 fusion protein itself [Sakai et al., 1995]. In the present study, to activate JAK1 and JAK2 simultaneously, and to study their roles in signal transduction and cell activity in the absence of cytokine, we set up the following conditional activation system. We created fusion constructs of CD16/CD7 (CD) with four different full length JAKs: CDJAK1 (CD + wild-type JAK1), CDJAK2 (CD + wild-type JAK2), CDJAK1KE [(CD + dominant-negative JAK1 (JAK1KE)], and CDJAK2KE [(CD + dominant-negative JAK2 (JAK2KE)]. These constructs were then used to generate five stable transfectants in Ba/F3 cells: CDJ1 cells expressing CDJAK1; CDJ2 cells expressing CDJAK2; CDJ1 + 2 cells coexpressing CDJAK1 and CDJAK2; CDJIKE + 2 cells coexpressing CDJAK1KE and CDJAK2; and CDJ1 + 2KE cells coexpressing CDJAK1 and CDJAK2KE. CDSTOP cells expressing CD only served as a control. The expression levels and molecular mass of the fusion proteins in the stable cell lines were determined by Western blotting. As shown in Figure 2A,B, CDJAKs and CDSTOP migrated as ~180 kDa and ~60 kDa proteins, respectively. The expression levels of CDJAK1 and CDJAK1KE fusion proteins exceeded that of endogenous JAK1 in all CDJAK1 and CDJAK1KE overexpressing transfectants, respectively, while the expression level of CDJAK1 fusion protein in CDJ1 + 2 (clone #21) cells was lower than in other CDJAK1 overexpressing transfectants. The expression levels of CDJAK2 and CDJAK2KE fusion proteins were similar to or less than endogenous JAK2 in all CDJAK2 and CDJAK2KE overexpressing transfectants. Interestingly, none of the stable transfectants of CDJAK2 or CDJAK2KE had a higher expression level of CDJAK2 or CDJAK2KE than of endogenous JAK2. Western blotting analysis with anti-CD16 antibody indicated that the expression level of CDJAK1 in CDJ1 cells was higher than that of CDJAK2 in CDJ2 cells (Fig. 2B). The CDJAKs showed two bands, possibly due to differences in post-translational modifications.

Since all the fusion proteins are membrane proteins, the cell surface expressions of these proteins were analyzed by anti-CD16 antibody labeling followed by flow cytometry. CD16 positive signals were detected in all stable

clones but not in untransfected Ba/F3 cells (Fig. 2C).

In the following experiments, cell activity was analyzed in all stable clones. For biochemical analyses, only one clone from each stable transfectant was analyzed; clone #14 for CDJ1, clone #5 for CDJ1 + 2, and clone #14 for CDJ1KE + 2.

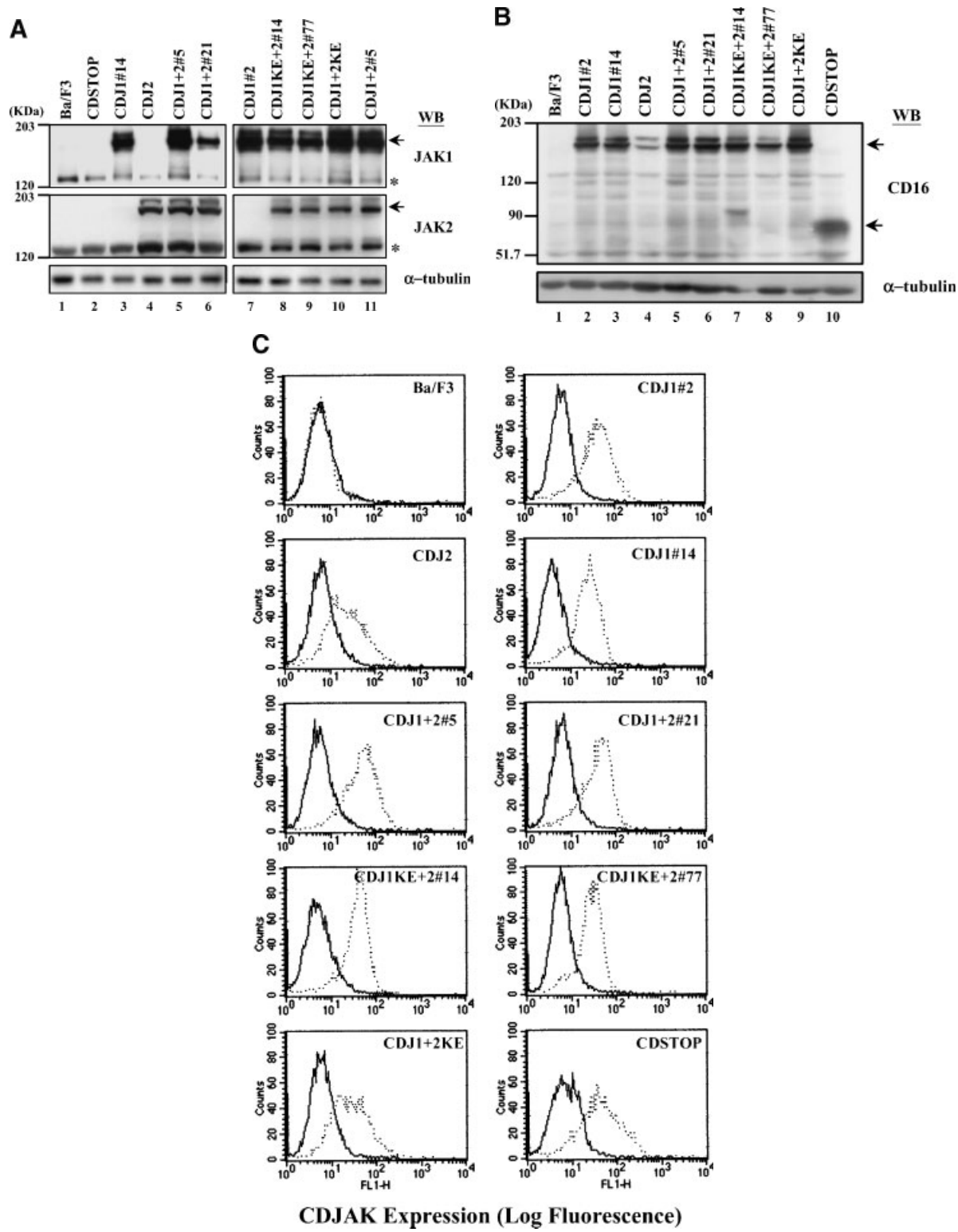


Fig. 2.

JAK2 and JAK1 Interact With IL-3Rα and β_{IL-3}, Respectively

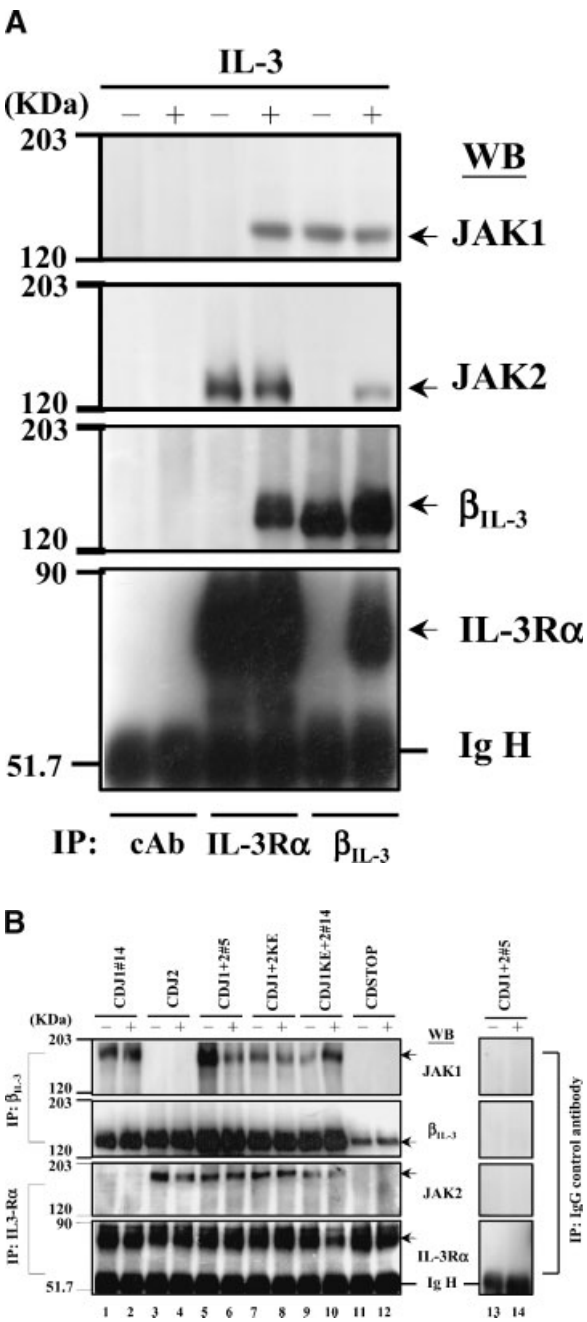
Previous reports have revealed that JAKs are preassociated with the cytokine receptor [Ogata et al., 1998]. However, the specifics of interactions between JAKs (JAK1 and JAK2) and each of the IL-3R subunits in IL-3-mediated signal transduction is unknown. Furthermore, whether CDJAK1 and CDJAK2 can also interact with the IL-3 receptor was examined. First, the circumstances under which endogenous JAK1 and JAK2 bind to the IL-3 receptor were determined. IL-3 starved Ba/F3 cells were stimulated with or without IL-3 and the respective cell lysates were then immunoprecipitated with anti-IL-3Rα or anti-β_{IL-3} antibodies followed by Western blotting with anti-JAK1 and anti-JAK2 antibodies. In Ba/F3 cells, JAK2 was coimmunoprecipitated with IL-3Rα whereas JAK1 was coimmunoprecipitated with β_{IL-3}, regardless of IL-3 stimulation (Fig. 3A). However, JAK2 was coimmunoprecipitated with β_{IL-3} and JAK1 was coimmunoprecipitated with IL-3Rα, only after IL-3 stimulation (Fig. 3B). The anti-IL-3Rα antibody was able to coimmunoprecipitate β_{IL-3} and conversely anti-β_{IL-3} antibody was able to coimmunoprecipitate IL-3Rα when the cells were stimulated with IL-3. The coimmunoprecipitation of IL-3Rα and β_{IL-3} has already been reported [Orban et al., 1999], where IL-3 induces the heterodimerization of IL-3 receptor α and β subunits. Similar experiments with Ba/F3-derived transfectants revealed that CDJAK2 and CDJAK2KE always bound with the IL-3Rα (Fig. 3B, lanes 3–10) while CDJAK1 and CDJAK1KE always bound with the β_{IL-3} regardless of antibody crosslinking (Fig. 3B, lanes 1, 2, 5–10). Antibody crosslinking was performed with or without anti-CD16 antibody, followed by incubation with anti-mouse IgG $F(ab')_2$ antibody in the absence of IL-3. These results indicate that JAK2 and JAK1 interact constitutively with IL-3Rα and β_{IL-3}, respectively.

Antibody Crosslinking Induced Tyrosine Phosphorylation of CDJAK1 and CDJAK2 Fusion Proteins

To examine whether antibody crosslinking induced tyrosine phosphorylation of CDJAK1 and CDJAK2 fusion proteins, IL-3 starved Ba/F3-derived transfectants were crosslinked with or without antibody (Fig. 4, lanes 3–16). Cell lysates were prepared and tyrosine phosphorylated proteins were immunoprecipitated by anti-phosphotyrosine antibody and identified by Western blotting with anti-JAK1 and anti-JAK2 antibodies. As shown in Figure 4, antibody crosslinking resulted in tyrosine phosphorylation of CDJAK1 in CDJ1#14 cells (lane 4), and of CDJAK2 in CDJ2 cells (lane 6). Simultaneous tyrosine phosphorylation of CDJAK1 and CDJAK2 was obtained following antibody crosslinking in CDJ1 + 2#5 cells (lanes 8 and 12). The level of tyrosine phosphorylation of coexpressed CDJAK1 and CDJAK2, however, was not increased compared to that of CDJAK1 or CDJAK2 alone. In contrast to the simultaneous tyrosine phosphorylation of JAK1 and JAK2 in CDJ1 + 2#5 cells (lane 12), tyrosine phosphorylation of CDJAK2 was reduced when dominant negative CDJAK1KE was coexpressed with CDJAK2 in CDJ1KE + 2#14 cells (lane 16). Similarly, tyrosine phosphorylation of CDJAK1 was reduced when dominant negative CDJAK2KE was coexpressed with CDJAK1 in CDJ1 + 2KE cells (lane 14). Weak tyrosine phosphorylation of dominant negative CDJAK1KE (lane 16), but not of dominant negative CDJAK2KE (lane 14), was obtained by antibody crosslinking. Antibody crosslinking did not induce tyrosine phosphorylation of endogenous JAK1 and JAK2 in these stable lines. Parental Ba/F3 cells stimulated or unstimulated by IL-3 were used as controls (lanes 1 and 2, respectively). The endogenous JAK1 and JAK2 were tyrosine phosphorylated following stimulation with IL-3 in parental Ba/F3 cells (lane 2). CDSTOP cells expressing only CD domains

Fig. 2. Expression of CD16/CD7/JAKs (CDJAKs) in Ba/F3 cells. **A**: Equivalent expression of CDJAK1 and CDJAK2 proteins in Ba/F3 cells. BaF3-derived transfectants, after antibiotic selection, were confirmed using SDS–PAGE followed by Western blotting with anti-JAK1 antibody, and reprobed with anti-JAK2 antibody. Arrows indicate CDJAK fusion proteins while asterisks indicate endogenous JAK. Size markers are in kDa. **B**: Lysates were directly subjected to SDS–PAGE followed by Western blotting with anti-CD16 antibody and reprobed with anti-α tubulin antibody as internal control. Arrows indicate CDJAK and CD fusion proteins. **C:** Flow cytometric analysis of CDJAK expression in BaF3-derived transfectants and in control parental Ba/F3 cells. Cells were stained with either IgG isotype control antibody (black line) or anti-CD16 antibody (dotted line), followed by fluorescein-tagged goat anti-mouse IgG antibody; and then analyzed by flow cytometry.

were not phosphorylated (lane 10), confirming that it was the JAK proteins that were phosphorylated in the fusion complexes and not CD domains. These results demonstrate that antibody crosslinking can induce tyrosine phosphorylation of JAK1 and JAK2, either individually or when coexpressed, when these proteins are fused to CD domains.



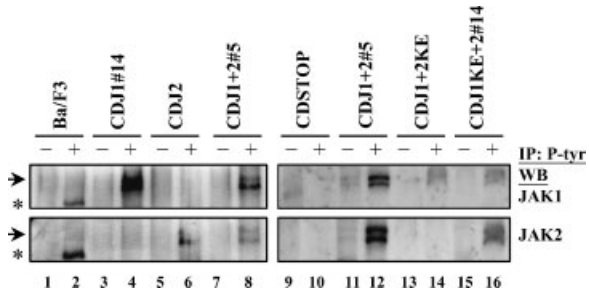


Fig. 4. Antibody simultaneously induces tyrosine phosphorylation of CDJAK1 and CDJAK2 in Ba/F3-derived transfectants. Starved Ba/F3 cells (**lanes 1** and **2**) and Ba/F3-derived transfectants (**lanes 3–16**) were stimulated by IL-3 and antibody crosslinking, respectively. Cell lysates were immunoprecipitated with anti-phosphotyrosine (P-tyr) antibody agarose conjugate, resolved by SDS–PAGE, and then subjected to Western blotting with anti-JAK1 antibody. The same membranes were stripped and reprobed with anti-JAK2 antibody. CDJAK fusion proteins are indicated by arrows. Endogenous JAK is indicated by an asterisk. Size markers are in kDa.

Crosslinking of Both CDJAK1 and CDJAK2 Induced Tyrosine Phosphorylation of β_{IL-3}

We demonstrated that CDJAK1 and CDJAK2 were preassociated with IL-3 receptor subunits prior to activation. Furthermore, CDJAK1 and CDJAK2 were simultaneously activated by antibody crosslinking. Next, we considered whether the tyrosine phosphorylation of CDJAK1 and CDJAK2 fusion proteins by antibody crosslinking could activate β_{IL-3} phosphorylation. Ba/F3-derived transfectants were incubated with or without antibody, immunoprecipitated with anti-β_{IL-3} antibody, and subjected to Western blotting with anti-phosphotyrosine (anti-P-tyr)

Fig. 3. JAK2 and JAK1 are constitutively associated with IL-3Rα and β_{IL-3}, respectively. **A**: Starved or IL-3 restimulated Ba/F3 cells were lysed and immunoprecipitated with anti-IL-3Rα (IL-3Rα), anti-β_{IL-3} (β_{IL-3}) or rabbit IgG control antibody (cAb) (as indicated on the underside). The immunoprecipitated proteins were analyzed on SDS–PAGE and transferred to PVDF membrane. The 203∼120 kDa region of PVDF membrane was probed with anti-JAK1 antibody, then reprobed with anti-JAK2 antibody, and reprobed again with anti-β_{IL-3} antibody (as indicated on the right-hand side, under WB). The 90∼51 kDa region was probed with anti-IL-3Rα antibody. **B**: Ba/F3-derived transfectants were crosslinked with or without antibody, and the lysates were immunoprecipitated with anti-IL-3Rα (IL-3Rα) or anti-β_{IL-3} (β_{IL-3}) antibodies (as indicated on the left-hand side) (**lanes 1–12**). Ba/F3-derived transfectant, CDJ1 + 2#5, was crosslinked with or without antibody, and the lysates were immunoprecipitated with rabbit IgG control antibody (as indicated on the right-hand side) (**lanes 13** and **14**). The immunoprecipitated proteins were separated on SDS–PAGE. The membrane was probed with anti-JAK1, -β_{IL-3}, -JAK2, and -IL-3Rα antibodies. Size markers are in kDa. The band close to the 51.7 kDa marker is Ig H.

antibody. The level of tyrosine phosphorylation of $\beta_{IL\text{-}3}$ in CDJ1 + 2#5 cells, after antibody crosslinking, was similar to that of parental Ba/F3 cells under IL-3 stimulation (Fig. 5, lanes 2 and 8). In contrast, CDJAK1KE or CDJAK2KE fusion proteins coexpressed with CDJAK2 or CDJAK1 fusion proteins, respectively, inhibited tyrosine phosphorylation of $\beta_{IL\text{-}3}$ (Fig. 5, lanes 9–12). Antibody crosslinking of CDJAK1 alone induced weak tyrosine phosphorylation of the $\beta_{IL\text{-}3}$ (Fig. 5, lane 4). These results demonstrate that antibody crosslinking of CDJAK1 and CDJAK2 together induced tyrosine phosphorylation of $\beta_{IL\text{-}3}$.

Crosslinking of Both CDJAK1 and CDJAK2 Triggered Downstream Phosphorylation Signals

Previous studies have shown that under IL-3 stimulation, $\beta_{IL\text{-}3}$ can be tyrosine phosphorylated, which, in turn, transduced signals through the STAT5, Akt, and MAPK pathways to regulate cellular growth and anti-apoptosis [Sato et al., 1993; Mui et al., 1995]. These events are believed to be triggered via the preassociated JAKs on the receptor molecules. In addition, the STAT5, PI-3K/Akt, and MAPK pathways are specific downstream targets of JAKs [Williams, 2000; Chang and Karin, 2001; Oki et al., 2002]. Hence, whether CDJAK1 and CDJAK2 fusion proteins were able to trigger downstream phosphorylation signals after antibody crosslinking was analyzed. Phosphorylation signals were examined using anti-P-tyr, anti-STAT5, anti-phospho-Akt (anti-P-Akt), and anti-phospho-MAPK (anti-P-MAPK) antibody Western blotting. The Western blots were stripped and reprobed with anti-α-tubulin, anti-Akt, or anti-MAPK antibodies as internal controls (Fig. 6A,B). The antibody crosslinking of CDJAK1 and CDJAK2 fusion proteins were

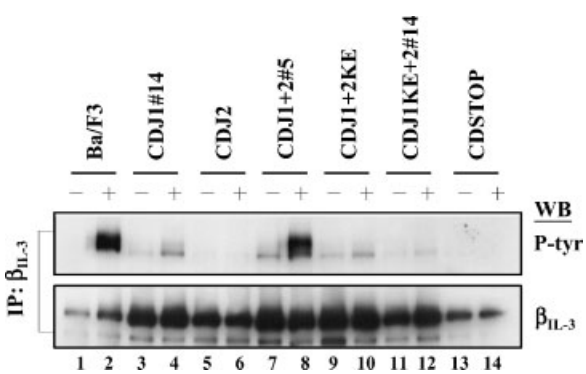


Fig. 5. Tyrosine phosphorylation of $\beta_{IL\text{-}3}$ after antibody crosslinking in CDJ1 + 2#5 cells. Starved Ba/F3 cells (**lanes 1** and **2**) and Ba/F3-derived transfectants (**lanes 3–14**) were unstimulated or stimulated with IL-3 and antibody, respectively. Immunoprecipitation was performed using anti-$\beta_{IL\text{-}3}$ antibody. Immunoprecipitates were subjected to SDS–PAGE, transferred to membranes, and Western blotted with anti-phosphotyrosine (P-tyr) antibody. The same membranes were stripped and reprobed with anti-$\beta_{IL\text{-}3}$ antibody.

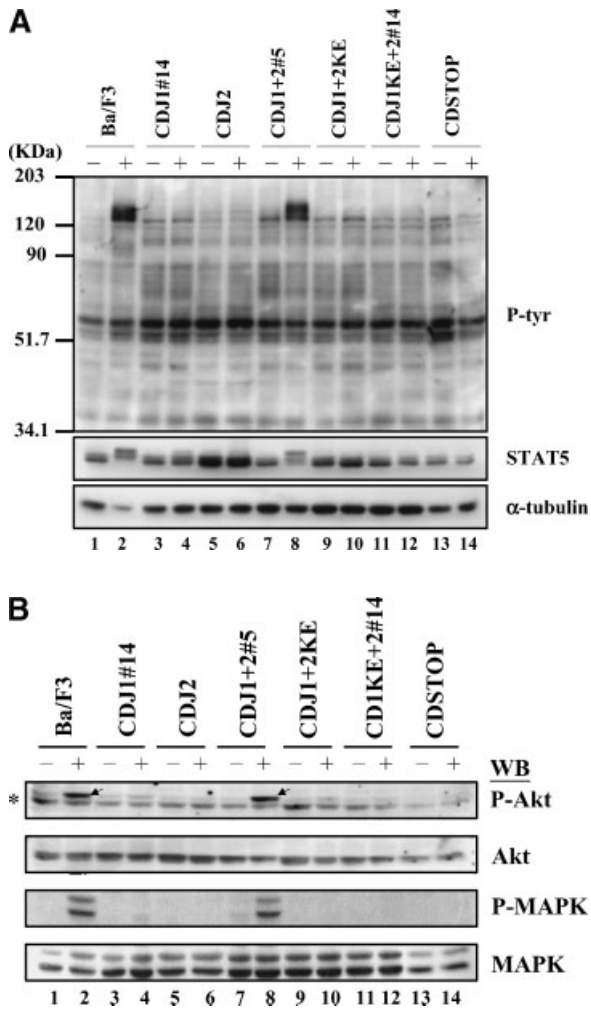


Fig. 6. Crosslinking of CDJAK1 and CDJAK2 activates tyrosine phosphorylation signals. **A**: Crosslinking of CDJAK1 and CDJAK2 activated tyrosine phosphorylation signals and STAT5 phosphorylation. Starved Ba/F3-derived transfectants were stimulated with or without antibody crosslinking (**lanes 3–14**), cell lysates were prepared and resolved by SDS–PAGE, Western blotted with anti-phosphotyrosine (P-tyr) antibody, then reprobed with anti-STAT5 or anti-α-tubulin antibody. **B**: CDJAK1 and CDJAK2 crosslinking activated phosphorylation of Akt and MAPK. Membranes probed with anti-P-Akt or anti-P-MAPK antibodies were stripped and reprobed with anti-Akt or anti-MAPK antibodies, respectively. The arrows denoted the phosphorylated form of Akt. Asterisk denotes an unknown protein that cross-reacted with anti-P-Akt antibody. Ba/F3 cells were stimulated with or without IL-3 as controls (**lanes 1** and **2**). Size markers are in kDa.

able to stimulate tyrosine phosphorylation of a specific set of proteins, especially 130~180 kDa proteins in CDJ1 + 2 #5 cells (Fig. 6A, lane 8) and the phosphorylation of STAT5, Akt, and MAPK (Fig. 6B, lane 8). Individually, CDJAK1 or CDJAK2 fusion proteins could be phosphorylated following antibody crosslinking (Fig. 3), but either of these proteins alone could not stimulate phosphorylation of the downstream proteins (Fig. 6A,B, lanes 4 and 6). Phosphorylated STAT5 migrated slower than unphosphorylated STAT5 on Western blots (Fig. 6A, lanes 2 and 8). IL-3 stimulation of parental Ba/F3 cells also resulted in the phosphorylation of the same downstream proteins (Fig. 6A,B, lane 2). CDJAK1KE and CDJAK2KE coexpressed with CDJAK2 and CDJAK1, respectively, suppressed all three downstream phosphorylations (Fig. 6A,B, lanes 9–12). These results indicate that complete signal transduction depends on the simultaneous activation of both CDJAK1 and CDJAK2 and also on the normal kinase activities of JAK1 and JAK2.

Antibody Crosslinking of CDJAK1 and CDJAK2 Is Required for Cell Proliferation and Suppression of Apoptosis

Proliferation is defined in this report as an increase in viable cell number in culture over time. A successful proliferation is controlled by increasing the number of viable cells and by suppressing apoptosis within individual cells. With the lack of either one of these capabilities, cells remain at only survival or suboptimal proliferation. To examine whether the simultaneous activation of JAK1 and JAK2 is needed for cell proliferation, the methyl thiazole tetrazolium (MTT) assay was performed. The Ba/F3-derived transfectants were stimulated with or without antibody for 48 h in IL-3 free medium. Two stable clones from each of the following transfectants were analyzed: CDJ1 (clones #2 and #14); CDJ1 + 2 (clones #5 and #21); CDJ1KE + 2 (clones #14 and #77). This was done to prove that specific cell clones screened by antibiotic selection do not affect the cell proliferation potential of Ba/F3-derived transfectants. CDJ1 + 2#5 cells exhibited optimal cell proliferation following two days of antibody crosslinking. The proliferation level of CDJ1 + 2#5 cell line was similar to that of Ba/F3 parental cells stimulated by IL-3 (Fig. 7A). Another stable clone, CDJ1 + 2#21, also exhibited proliferation after antibody crosslinking,

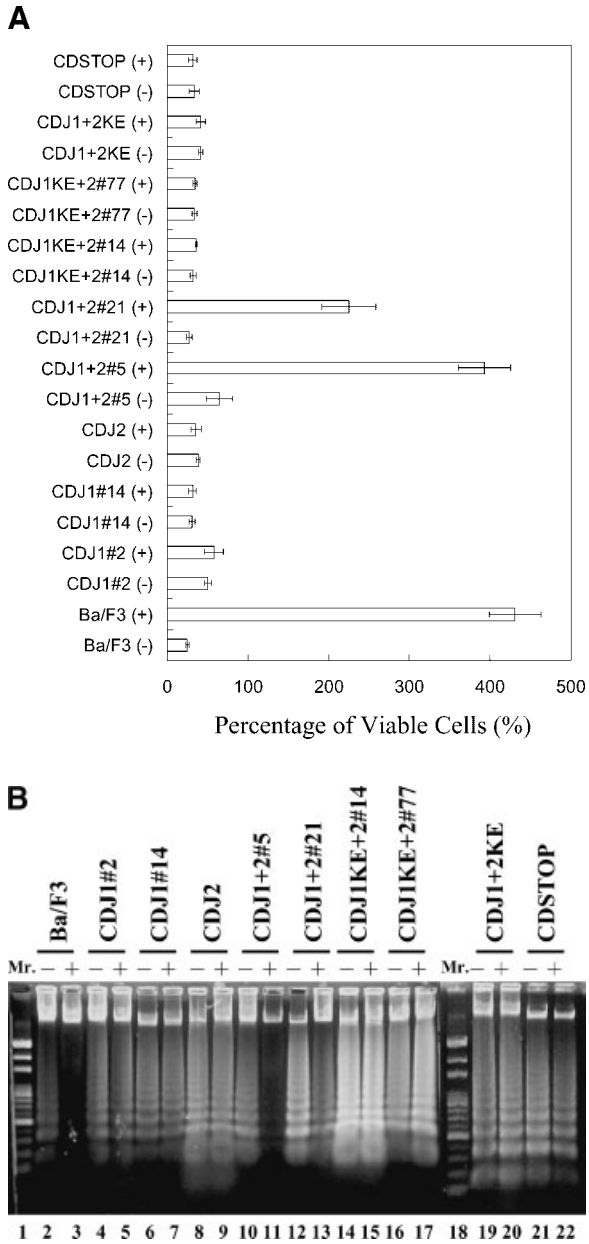


Fig. 7. Simultaneous activation effects of CDJAK1 and CDJAK2 on proliferation and anti-apoptosis of Ba/F3 cells. **A**: Crosslinking of CDJAK1 and CDJAK2 promoted cell proliferation activity in CDJ1 + 2#5 and CDJ1 + 2#21 cells. Parental Ba/F3 cells and other transfectants were washed thoroughly; cultured with or without IL-3 or antibody for 48 h. The viable cell numbers were determined by MTT assay and are shown as the percentage of the initial viable cell number. All values are averages of three independent experiments; the standard deviation is represented by error bars. **B**: Crosslinking of CDJAK1 and CDJAK2 inhibited DNA fragmentation in CDJ1 + 2#5 and partially inhibited DNA fragmentation in CDJ1 + 2#21 cells. Parental Ba/F3 cells and other transfectants were washed extensively and cultured in IL-3 or antibody for 48 h. DNA fragmentation was examined by gel electrophoresis.

although at a suboptimal level. Therefore, the simultaneous activation of CDJAK1 and CDJAK2 fusion proteins by antibody crosslinking appears necessary to support proliferation of CDJ1 + 2#5 cells in the absence of IL-3.

Next we investigated whether proliferation caused by the simultaneous activation of CDJAK1 and CDJAK2 fusion proteins by antibody crosslinking was also due to reduced apoptosis. The Ba/F3-derived transfectants were stimulated with or without antibody for 48 h in IL-3 free medium. The DNA from these cells were isolated and electrophoresed through agarose to analyze for DNA fragmentation (Fig. 7B, lanes 4–22). Parental Ba/F3 cells stimulated or not stimulated by IL-3 were used as controls (Fig. 7B, lanes 2 and 3). Figure 7B shows the crosslinking of CDJAK1 and CDJAK2 fusion proteins suppressed apoptosis in CDJ1 + 2#5 cells (lane 11 compared with lane 10). This suppression of apoptosis was similar to that seen in Ba/F3 cells under IL-3 stimulation (lane 3 compared with lane 2). Another stable clone coexpressing CDJAK1 and CDJAK2 fusion proteins, CDJ1 + 2#21, was observed to suppress apoptosis only partially after antibody crosslinking (Fig. 7B, lanes 12 and 13). In addition, other Ba/F3-derived transfectants exhibited severe DNA fragmentation, before and after antibody crosslinking (Fig. 7B lanes 4–9 and 14–22) in IL-3 free medium. These data suggest that the proliferation activity supported by the simultaneous activation of CDJAK1 and CDJAK2 fusion proteins could be due to an activation effect on anti-apoptosis activity of these fusion proteins.

These results show that two transfectants, CDJ1 + 2#5 cells and CDJ1 + 2#21 cells, have different capacities of cell proliferation and anti-apoptosis that may be due to the greater expression of CDJAK1 in CDJ1 + 2#5 cells than in CDJ1 + 2#21 cells, although the expression of CDJAK2 was similar in both cells (Fig. 2). Therefore, the simultaneous activation effect of CDJAK1 and CDJAK2 may be dose dependent.

DISCUSSION

To understand the function of the JAK2 protein independent of other growth factor signals, Sakai et al. constructed a fusion protein in which the CD16 extracellular domain, a CD7 transmembrane domain, and the entire coding region of JAK2 were fused and transiently expressed in Ba/F3 cells [Sakai et al., 1995; Sakai and Kraft, 1997]. By activating the fusion protein using anti-CD16 antibody crosslinking, they showed that the activated fusion JAK2 protein could mimic many of the signal transduction activities of IL-3 or GM-CSF stimulated endogenous JAK2, but could not promote cell proliferation in the absence of cytokine. In this study, we applied a similar approach to study the role of JAK1 and JAK2 activation in signal transduction pathways and in supporting cell proliferation. In the current report, we demonstrated that both CDJAK1 and CDJAK2 fusion proteins could be tyrosine phosphorylated independently of each other by the anti-CD16 antibody crosslinking. In the absence of IL-3, only the simultaneous activation of both CDJAK1 and CDJAK2 could trigger downstream signal transduction and support cell activity.

In our system, Ba/F3-derived transfectants overexpressing CDJAK2 fusion proteins were incapable of phosphorylating downstream targets. There have been several papers published on this topic with conflicting results. For instance, Sakai et al. reported that high levels of expression of CD16/JAK2 fusion proteins activated the phosphorylation of Shc and MAPK, but could not support cell proliferation [Sakai et al., 1995]. Liu et al. [1999] reported that a chimeric full length JAK2, β/JAK2, with the extracellular and transmembrane domains of βc subunit is constitutively activated and is sufficient for growth and survival [Watanabe et al., 2000]. Three variations come to mind for the differences between the results: clonal variation, the different extracellular and transmembrane domains of the chimeras, and the relative levels of expression. We showed two clones behaving similarly suggesting at least that these results are not the property of a single clone. However, CDJAK2 only clones expressed less fusion protein than endogenous JAK2 and become less phosphorylated upon activation (Fig. 4). Thus, an alternate explanation of our results would be that while JAK1 itself or a low level of JAK2 are not sufficient for signaling, JAK1 with a bit of JAK2 is sufficient, leaving the possibility that JAK2 expression at higher levels is sufficient for signaling.

CDJAK2 and CDJAK1 proteins were constitutively associated with IL-3Rα and βc, respectively (Fig. 3B). Our results are in contrast to that of Sakai et al., who stated that the fusion of CD domains to the N-terminal domain of JAK2

that mediates receptor association seemed to disrupt the ability of the fusion protein to interact with the receptor [Sakai et al., 1995]. We have repeated the experiments three times for this study and have showed that the fusion proteins can interact consistently with the respective receptor subunits. Antibody crosslinking may induce formation of a large functional complex consisting of CDJAK2-IL-3Rα and CDJAK1-$\beta_{IL\text{-}3}$, which may further trigger signal transduction in the absence of IL-3. Based on our results, we propose the following mechanism for the simultaneous activation of endogenous JAK1 and JAK2 proteins by IL-3. Prior to IL-3 induction, JAK2 and JAK1 proteins exist in a preassociated state with IL-3Rα and $\beta_{IL\text{-}3}$, respectively (Fig. 3A). When IL-3 binds to IL-3Rα, a large functional complex of JAK2-IL-3Rα and JAK1-$\beta_{IL\text{-}3}$ is formed. JAK1, JAK2, and $\beta_{IL\text{-}3}$ within this functional complex undergo tyrosine phosphorylation, thereby triggering further downstream signaling that result in cell proliferation and in the suppression of apoptosis. Our proposed model is consistent with the single chain cytokine receptor model (e.g., receptors for growth hormone and erythropoietin) that predicts the JAK2 protein is activated the dimerization of two cytokine receptor-JAK2 complexes after the cytokine binds to its receptor [Heldin, 1995]. The physical interaction between the IL-3 receptor and the JAKs proteins, demonstrated in our system (Fig. 3), is consistent with that seen in the IL-5 system, in which JAK2 and JAK1 proteins were shown to be constitutively associated with IL-5Rα and βc, respectively [Ogata et al., 1998]. Other studies have also reported ligand-induced JAK2 binding to the β subunit in the IL-3 or GM-CSF system [Brizzi et al., 1996; Cattaneo et al., 1996; Chin et al., 1997]. Our study, however, provides an explanation for the mechanism by which IL-3 induces heterodimerization of IL-3Rα with the $\beta_{IL\text{-}3}$, enabling JAK2-IL-3Rα to interact with the $\beta_{IL\text{-}3}$. Similarly, JAK1-$\beta_{IL\text{-}3}$ has the opportunity to interact with the α subunit (Fig. 3A).

Antibody crosslinking can independently activate CDJAK1 and CDJAK2. Activation of CDJAK2 did not induce cell proliferation or anti-apoptosis in CDJ2 cells, an observation consistent with previous reports in which CD16/JAK2 or GyB/JAK2 fusion proteins were used [Sakai et al., 1995; Mohi et al., 1998]. Although the activation of CDJAK1 and CDJAK2 did not depend on each other; the activation of CDJAK1 was observed to be correlated with that of CDJAK2 after the functional complex had been formed, as tyrosine phosphorylation of CDJAK1 was reduced after antibody crosslinking in the presence of CDJAK2KE (Fig. 4). Tyrosine phosphorylation of CDJAK2 was also reduced in the presence of CDJAK1KE; as was also seen in the IL-5 system [Ogata et al., 1998]. The inhibition of CDJAK1 by CDJAK2KE may be incomplete in our system because CDJAK1 was overexpressed while CDJAK2KE expression was weak in CDJ1 + 2KE cells (Fig. 2B,C). The expression level of CDJAK1 also affected cell proliferation and anti-apoptosis in CJ1 + 2#5 and CJ1 + 2#21 cells (Figs. 2B and 7). Therefore, JAK1 and JAK2 proteins within the functional complex might have a dose-dependent effect.

Recent study by Lacronique et al. [2000] has revealed that fusion proteins that contain the oligomerization domain of TEL and the tyrosine kinase domain JH1 (JAK homology 1) of JAK1, JAK2, JAK3, or TYK2 have similar characteristics and can effectively substitute for the survival and mitogenic signals of IL-3. That is, the tyrosine kinase domain from the four members of the JAK family does not determine the functional specificity. Therefore, the full-length proteins of JAK1 and JAK2 were studied in this report. In addition to the JH1 domain, the JAK family contains the JH2-JH7 domains. The sequence of the JH2 domain is similar to that of the JH1 kinase domain, except for the lack of kinase activity. JH2 has been suggested to have a negative regulatory effect on JAK2 kinase activity. Deletion of JH2 from JAK2 constitutively activated the cytokine receptor, independent of cytokine, but signal transduction activity was lower than that of wild-type JAK2 stimulated by cytokine [Saharinen et al., 2000]. There is much sequence variation within the N-terminal JH3-JH7 domains of the four members of the JAK family. These domains have been implicated in receptor association [Kisseleva et al., 2002] and in controlling the kinase activity of JAK3 [Zhou et al., 2001], thus implying that they could be involved in controlling JAK kinase activity and in signal transduction. Therefore, the JH2-JH7 domains may be responsible for the functional specificity of JAK, which warrants further studies on the analyses of the functions of wild-type JAK.

The β chain is the major signaling subunit of the IL-3 receptor, and its multiple functions are

believed to emerge from the recruitment of specific signaling proteins to tyrosine-phosphorylated residues in the β subunit's cytoplasmic domain [Geijsen et al., 2001]. Our results suggest that JAK1 and JAK2 are the initiators of the signal transduction cascade. Both JAK1 and JAK2 are equally important, as the complex ceased to transduce signals, to promote cell proliferation, and to suppress apoptosis when either one of the JAKs lost normal kinase activity (Figs. 5–7). The importance of JAK1 and JAK2 in IL-3 signaling has also been observed in vivo [Neubauer et al., 1998; Parganas et al., 1998; Rodig et al., 1998]. IL-3 was shown to induce colony formation of JAK1 null fetal liver cells, but it also reduced the number and the size of colonies as well as the DNA binding activity of STAT [Rodig et al., 1998]. JAK2-deficient fetal liver cells, in contrast, could not form colonies of hematopoietic progenitors, colony forming unit-mix (CFU-Mix), colony forming unit-erythroid (CFU-E), or burst forming unit-erythroid (BFU-E), in response to IL-3 [Neubauer et al., 1998; Parganas et al., 1998]. Therefore, the effect of IL-3 on cells expressing kinase negative JAK2 and/or JAK1 will be studied further to evaluate the importance of JAK/JAK2 synergism during IL3-mediated signaling.

Some of the specific downstream targets of JAK that have been identified include: STAT [Oki et al., 2002], MAPK [Chang and Karin, 2001], PI-3K/Akt [Williams, 2000], Raf-1 [Xia et al., 1996], Src homology 2 (SH2)-containing protein-tyrosine phosphatase-2 (SHP-2) [Yin et al., 1997], proline-rich tyrosine kinase 2 (Pyk2) [Yin et al., 1997], insulin receptor substrate-1 (IRS-1) [Yamauchi et al., 1998], Bruton's tyrosine kinase/Tec protein-tyrosine kinase (Btk/Tec) [Takahashi-Tezuka et al., 1997], and transcription factor II-1 (TFII-1) [Kim and Cochran, 2001]. JAK activates and directly interacts with STAT, Raf-1, SHP-2, Pyk2, IRS-1, Btk/Tec, and TFII-1. Additionally, overexpression of JAK and the downstream targets of JAK in a transient expression system indicate that JAK can also activate such downstream cytokine-independent targets as SHP-2, Pyk2, Btk, and TFII-1. However, the simultaneous activation of CDJAK1 and CDJAK2 induced phosphorylation signals, including $\beta_{IL\text{-}3}$ (Fig. 5). Hence, it is likely that the simultaneous activation of JAK1 and JAK2 may operate through $\beta_{IL\text{-}3}$-dependent and $\beta_{IL\text{-}3}$-independent signals to induce cell proliferation and suppression of cell death. Future studies in our laboratory will consist of removing the receptor binding regions from both JAK1 and JAK2, and then analyzing the simultaneous activation effects of these mutant JAKs on the transduction of $\beta_{IL\text{-}3}$-independent signals, and/or cell proliferation.

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