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# Dominant Negative Effects of a Carboxy-truncated Jak2 Mutant on Epo-induced Proliferation and Jak2 Activation

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Members of the Janus family of protein tyrosine kinases are emerging as primary, receptor-associated transducing factors among numerous cytokine systems. However, little is understood regarding mechanisms of recruitment of these kinases to receptor complexes and their ligand-dependent activation. To initially address these questions, we have assessed effects of ectopically expressing a carboxy-truncated form of Jak2 (Jak2-829) in Epo-responsive DAER cells. Expression of this truncation mutant at low levels efficiently inhibited both Epo-dependent activation of endogenous Jak2 and Epo-induced mitogenesis (10% to 39% of parental DAER cells). These results suggest that amino-terminal domains of Jak2 may mediate the assembly of Jak2/Epo receptor complexes and that integration of Jak2-829 into receptor complexes may effectively inhibit the activity of oligomeric Jak2/receptor assemblages. 

An expanding number of cytokines which act via cell surface receptors of the type 1 and 2 superfamily recently have been shown to activate one, or more, associated Janus protein tyrosine kinases, i.e., Jak1 [1], Jak2 [2], Jak3 (two distinct forms designated) [3, 4], tyk2 [5] and the *Drosophila* hopscotch gene product *hop* [6]. These include Epo, IL-3, IL-2, IL-4, IL-6, IL-11, CNTF, LIF, OSM, growth hormone, prolactin, interferon $\alpha/\beta$ and interferon $\gamma$ [7, 8, 9, 10, 11, 12, 13]. Thus, a common set of receptor-associated effectors is defined that potentially function immediately upstream from cytosolic transducers of both growth, and differentiation signalling

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pathways. This finding raises important questions regarding cytokine-dependent mechanisms of Janus kinase activation, and the precise nature and role (Jak homology domains JH3 - JH7) of tyrosine phosphorylated targets and/or associated effectors.

Structurally, Janus kinases are unique in several features. Each encodes both a carboxy-terminal PTK domain and an adjacent homologous kinase-like domain, as well as an extended amino-terminal region with five conserved subdomains within Jak1, Jak2, Jak3 and tyk2 [1, 2, 4, 5]. In recent studies of the role of Jak2 in Epo-induced mitogenesis, we have shown that mutation of the type VIII phosphotransferase motif within the carboxy terminal kinase domain (Jak2ΔVIII) mutant inhibits catalytic activity (i.e., autophosphorylation); and that this mutant when expressed ectopically in DAER cells inhibits both Epo-induced activation of endogenous Jak2, and Epo-induced mitogenesis [14]. Since the activation of type I cytokine receptors apparently proceeds from ligand induced dimerization (or multimerization) to the autophosphorylation of receptor-associated Janus kinases, the dominant negative effects exerted by the above Jak2ΔVIII mutant likely involve its integration into, and functional disruption of, oligomeric receptor-Jak2 complexes. To further test this notion and to initially assess which global domains of Jak2 mediate its association with Epo receptor complexes, we have studied possible effects on Epo-induced Jak2 activation and mitogenesis of ectopically expressing (in DA-ER cells) a carboxy-truncated form of Jak2 (Δaa#830-1129) which lacks the predicted PTK domain. Results indicates: i) that this Jak2 mutant (Jak2-829) likewise exerts dominant negative effects on Epo-induced Jak2 activation and mitogenesis; and ii) that amino terminal subdomains, or possibly the adjacent kinase-like domain, mediate the association of Jak2 with the Epo receptor.

## MATERIALS AND METHODS

**Cell lines and Jak2 cDNA constructs**: IL-3 dependent DA-1 cells [15] ($10^7$ in 0.8ml ) were electroporated with a pXM-EpoR DNA expression vector (50μg) [16] at 950 μF, 250 volts (IBI, geneZAPPER). Cells expressing the wild type murine EpoR were selected by growth in DMEM, 10% fetal calf serum supplemented with 5 U/ml Epo (i.e., DA-ER cells). The carboxy-terminal truncation mutant Jak2-829 was constructed by inserting a suppressible reading frame termination linker (5'-CTAGTCTAGACTAG-3') at the AvrII site of Jak2 and Jak2-829 was subcloned into expression vector pEFBOS [17]. pBOSJak2-829 (42μg) then was co-transfected with pSV2-neo (8μg) into DAER cells. Transfected cells were selected in G418 (1mg/ml, 20 days) and individual clones were obtained by dilution. Proliferation rates in response to Epo were assayed by [$^3$H]thymidine incorporation. Briefly, cells were plated in 96-well plates at 2 x $10^4$ per well. Following a 44 hour incubation at 37°C, 7.5% $CO_2$, cells were incubated with 1μCi of [$^3$H]thymidine for 2 hours, harvested onto glass-fiber filters, and [$^3$H]thymidine uptake was quantitated by scintillation counting.

**Northern blotting**. Total RNA from DAER and DAERJak2-829 cells was isolated using Trizol reagent [18](GIBCO/BRL). A 10-μg aliquot of RNA was denatured with formamide and formaldehyde, electrophored in a 1.5% agarose formaldehyde gel and transferred to nylon membrane (Magnagraph, MSI, Inc.). Baked filters were prehybridized, and hybridized at 42°C for 15 hours in 50% formamide, 0.6% SDS, 250mM NaCl, 120mM $Na_2HPO_4$, pH 7.0 using a randomly primed $^{32}P$-labeled 1.2 kb HindIII to XbaI fragment of pBOSJak2-829. Filters were washed to a final stringency of 0.2 x SSC, 0.1% SDS at 50°C and exposed to film.

**Jak2 autophosphorylation assays**. DAERJak2-829 and parental DAER cells (2 x$10^7$ in 25ml) were incubated in DMEM supplemented with 1% FCS for 13 hours prior to stimulation with Epo (10 U/ml, 8 minutes). Cells then were lysed in 0.25 ml of 1% Triton, 0.1mM sodium vanadate, 5mM EDTA, 50mM NaCl, 30mM $Na_4P_2O_7$, 50mM NaF, 20mM Tris, pH7.6, 0.5μg/ml leupeptin, 0.7μg/ml pepstatin, 50μg/ml phenylmethylsulfonyl fluoride (i.e. lysis buffer) at 0°C. Following removal of nuclei by centrifugation, Jak2 then was immunoprecipitated using 1μg purified antibody to Jak2 and 30μl of a 50% slurry of protein A Sepharose. Immune complexes then were washed twice with lysis buffer, twice with kinase buffer (50mM NaCl, 5mM $MgCl_2$, 5mM $MnCl_2$, 0.1mM $Na_3VO_4$, 10mM HEPES, pH7.4), and were resuspended in 0.1ml of this buffer. Kinase reactions were initiated by adding 20μCi [$\gamma$-$^{32}P$]ATP and were incubated at 23°C for 25 minutes. Reactions were terminated by washing gels four times with lysis buffer (0°C) and by the addition of SDS-PAGE sample buffer (100°C, 5 minutes). $^{32}P$-phosphoproteins then were analyzed by electrophoresis and autoradiography.

## RESULTS AND DISCUSSION

The Jak2 carboxy-terminal truncation mutant Jak2-829 (Figure 1) was constructed as described in Methods, and was transfected stably into DAER cells. Following selection in G418, cells were expanded in IL-3 and were assayed initially for expression of Jak2-829 transcripts by Northern blotting (Figure 2A). In DAERJak2-829 cells, but not in parental DAER cells, a pEF-BOS-derived transcript of approximate 4,000 bp was detected. Due to size, resolution for this Jak2-829 transcript was limited. Specificity of hybridization to Jak2-829 transcripts also was confirmed by Northern blotting of RNA following transfection with pBOSJak2-829 versus negative control plasmids (H.Z., S.V.P. and DMW, unpublished data). Clonal lines then were derived, and were assayed for the ability to mediate Epo-induced mitogenesis as compared to parental DAER cells (Figure 2B). For all clones assayed, rates of Epo-dependent mitogenesis were inhibited markedly (10%-39%

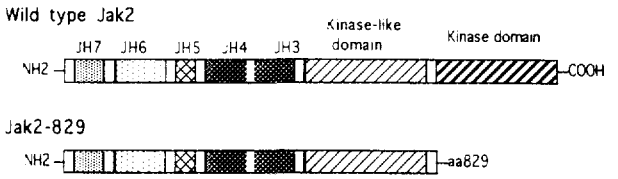


**Figure 1. Features of Jak2 and the carboxy terminal truncation mutant Jak2-829.**

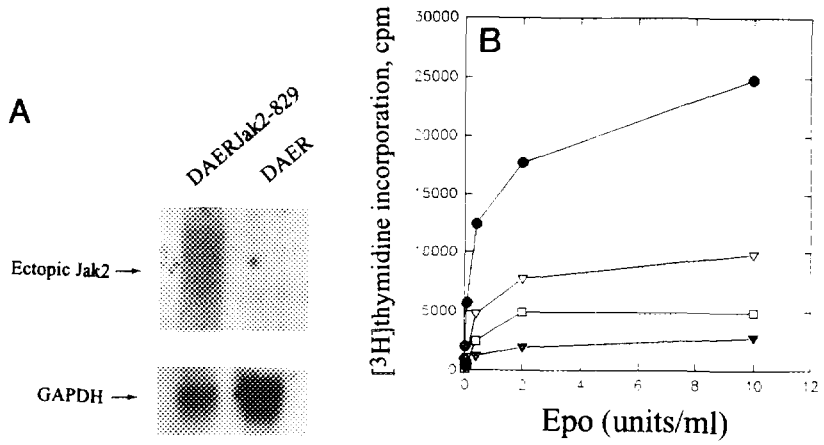


**Figure 2. Expression of Jak2-829 in DAER cells, and dominant negative effects on Epo-induced mitogenesis.** Figure 2A: Expression of Jak2-829 mutant in stably transfected G418 resistant DAER cells was confirmed by Northern blotting as described in Methods. Figure 2B: Effects of ectopic expression of Jak2-829 in DAER cells on Epo-dependent mitogenesis was assessed via assay of rates of Epo-induced [3H]thymidine incorporation (3 representative clones shown of 8 independent clones analyzed, DAER: ●; DAERJak2-829 clones: Δ, □, ▲).

versus DAER cells), suggesting that the truncation mutant Jak2-829 efficiently disrupts proliferative signal transduction.

To test whether this dominant negative effect involved direct inhibition of Epo-induced activation of endogenous Jak2, assays of Epo-induced Jak2

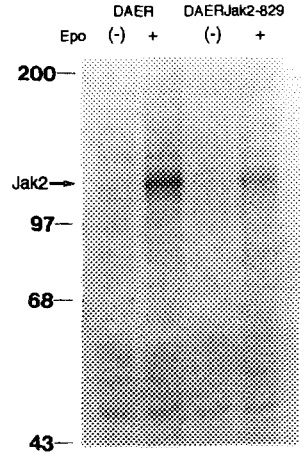


**Figure 3. Jak2-829-mediated inhibition of Epo-induced activation of endogenous Jak2.** The effect of stable ectopic expression of mutant Jak2-829 in DAER cells on Epo-induced activation of Jak2 was assessed using an in vitro Jak2 autophosphorylation assay as described in Methods.

autophosphorylation were performed using DAER-Jak2-829 cells versus parental DAER cells as a control. As shown in Figure 3, the ability of Epo to activate Jak2 in DAER-Jak2-829 cells was inhibited significantly.

Together, the above results suggest that amino terminal domains of Jak2 mediate its association with Epo receptor complexes, and that for the truncation mutant Jak2-829, this association apparently is sufficient to disrupt the assembly and/or activity of wild-type Jak2/Epo receptor complexes. Notably, evidence that activation of the Epo-receptor depends upon dimerization/ multimerization has been provided in studies by Watowich *et al* [19, 20]; and studies by Witthuhn *et al* using GST-fusion proteins suggest that Jak2 may associate constitutively with the cloned Epo receptors in the absence of ligand [21]. Further support (albeit indirect) for the notion that the Jak2-829 truncation mutants may function by disrupting activated oligomeric receptor complexes is provided by the present observation that dominant negative effects on Epo-induced growth are enforced by only low-level expression of this mutant in DAER cells. Specifically, expression levels for Jak2-829 are estimated to be at least 10-fold lower than levels of endogenous wt Jak2 . This circumstance at least suggests that amino-terminal domains of Jak2-829 may, in fact, efficiently disrupt the catalytic capacity of multimeric receptor/Jak2 assemblages. Ongoing studies aim to define possibly discrete functions exerted by selected amino terminal Jak2 homology domains.

## ACKNOWLEDGMENTS

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