Microinjected cDNA encoding JAK2 protein-tyrosine kinase induces DNA synthesis in NIH 3T3 cells

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Abstract Microinjection of expression plasmids encoding either JAK2 or hyperactive (NΔ661)rJAK2 into serum-starved NIH 3T3 cells resulted in 20–30-fold induction of DNA synthesis. Control microinjections of buffer or parental pcDNA3 vector resulted in only 3–5-fold induction of DNA synthesis. Induction of DNA synthesis was blocked when plasmid encoding JAK2 was microinjected in the presence of the JAK2-selective inhibitor AG-490, whereas AG-490 did not block DNA synthesis induced by microinjected plasmid encoding (NΔ661)rJAK2. The ability of JAK2 to initiate the G_0/S cell cycle transition is comparable to that of other proto-oncogenes, and supports a mechanistic role for overexpressed Janus kinases in carcinogenesis.

Key words: Proto-oncogene; Microinjection; Protein-tyrosine kinase; Janus kinase; Tyrphostin AG-490

1. Introduction

The Janus kinases constitute a protein-tyrosine kinase family which is crucial for the propagation of cytokine-induced signal transduction [1,2]. Under normal conditions, JAK2 is converted from a latent to an active kinase by the ligand-induced activation of receptors for prolactin [3,4], erythropoietin [5], growth hormone [6], interleukin-3 [7], granulocyte-macrophage colony stimulating factor [8], cytokines such as interleukin-6 which induce gp130 homodimerization [9,10], and other cytokines in cells whose proliferation and/or differentiation is dependent upon these ligands. Erythropoietin-induced proliferation can be blocked by the expression of catalytically inactive JAK2 [11], suggesting not only that the enzyme may possess auto-regulatory domains but also that the catalytic activity of the enzyme is an essential component of the proliferative signal transduction cascade. Baculovirally mediated expression of a JAK2 mutant lacking the putative auto-regulatory domain yields an enzyme which appears to be 'hyperactive' [12]. Baculoviral expression experiments also provide evidence to suggest that as the amount of JAK2 expressed in a cell increases, the kinase becomes constitutively active [8,12,13]. Transient overexpression of JAK1 and JAK2 in human kidney 293 cells also results in the constitutive activation of these Janus kinases [14]. It has therefore been proposed that overexpression of Janus kinases, or expression of 'hyperactive' Janus kinase mutants, may result in cellular transformation.

Recent findings [15,16] substantiate this hypothesis. The *Tumorous-lethal (Tum-l)* [17] mutation is an X-linked, dominant mutation in the *Drosophila melanogaster hopscotch (hop)* [18] gene, which encodes a Janus kinase homologue. A point mutation converting amino acid residue 341 from glycine to glutamic acid has been identified as the hop^{Tum-l} defect; this mutation was linked to increased tyrosine kinase activity of the mutant Hop and the formation of melanotic tumors [15]. Further, overexpression of wild-type *hop* in larval lymph glands results in both an increase in Hop tyrosine kinase activity and in melanotic tumor formation [15]. A second line of evidence supporting the oncogenic nature of Janus kinase overexpression derives from analysis of leukaemic cells taken from the bone marrow of patients with relapse acute lymphoblastic leukaemia (ALL) [16]. Western blot analysis indicated that JAK2 was produced at a significantly higher level in the ALL cells, as compared to normal T cells, B cells, and fibroblasts, and the JAK2 was present in the ALL cells as a constitutively active kinase. The constitutive JAK2 activity was selectively inhibited by a tyrphostin, AG-490, which also inhibited proliferation of ALL cells grown in culture and of ALL cells transplanted into SCID mice.

Viral transformations which allow for cytokine-independent growth have been correlated with constitutive activation of Janus kinases [19,20]. Abelson murine leukemia virus (A-MuLV) transformation of pre-B cell lines normally requiring interleukin-7 for growth resulted in the constitutive activation of JAK1 and JAK3 [19]. CD4+ T cells infected with human T cell lymphotropic virus 1 (HTLV-1) ultimately become independent of interleukin-2, a phenomenon associated with the constitutive activation of JAK3 [20].

Each of these four experimental systems [15,16,19,20] demonstrate a strong correlation between a high level of constitutive Janus kinase activity and carcinogenesis. A classic characteristic of tumorigenic oncogenes is the ability to stimulate DNA synthesis in NIH 3T3 cells under conditions of serum starvation. In this report, we test whether catalytically active forms of rat JAK2 possess an oncogenic potential by overexpressing the kinase in NIH 3T3 cells.

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2. Materials and methods

2.1. Cell culture

NIH 3T3 cells (2×10^5) were plated onto etched glass coverslips which were immersed in 35-mm culture dishes containing Dulbecco's modified essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 U of penicillin/ml, and 50 μg of streptomycin/ml. Cells were grown to confluency at 37°C, the medium was replaced with DMEM containing 0.5% heat-inactivated FBS, and then cultures were incubated 24 h before microinjection.

2.2. Construction of expression vectors

The expression vector pcDNA3:rJAK2 was created by subcloning a 3.7 kb cDNA fragment encoding the rat JAK2 [21] into the *Not*I and *Apa*I restriction sites of pcDNA3 (Invitrogen). This vector contained a 5′ untranslated region of approximately 300 bp. The expression vector pcDNA3:(NΔ661)rJAK2 was created by digesting pcDNA3:rJAK2 with the restriction endonuclease *Bam*HI, re-ligating the plasmid digest mixture, then isolating a plasmid that lacked the *Bam*HI fragment at the 5′ end of the *jak2* coding region.

2.3. Microinjection of cells and assay for DNA synthesis

Microinjections and DNA synthesis assays were performed as previously described [22]. Briefly, plasmid DNA (100–400 μg/ml) or phosphate-buffered saline (PBS), pH 7.4, was loaded into glass capillary micropipettes made with an automatic P80/PC micropipette puller (Sutter Instruments, Co.). A specific area on the coverslip was scratched to localize the injection area, and 100–200 quiescent cells were microinjected ($3–7\times10^{-12}$ ml/cell) with coded samples using a computerized Eppendorf microinjection system. The coverslips were maintained in low fetal calf serum (FCS, 0.5%) containing media for approximately 18 h before the addition of [^{3}H]thymidine (0.5 μCi/ml, Amersham) for a 4 h pulse. The coverslips were washed with PBS, fixed in 2.5% glutaraldehyde (v/v PBS), coated with nuclear tracking emulsion (NTB2, Kodak), subjected to autoradiography for 36 h, and the cells were stained with Geisma. Every microinjected cell within the scratched area of the coverslip was identified by the computer program and scored for induction of DNA synthesis and the cells were photographed at 100×. Fold-induction of DNA synthesis is a measure of the sample's ability to drive growth-arrested G_0 fibroblasts into S phase. It is defined as the ratio of microinjected cells that incorporate [^{3}H]thymidine into DNA divided by the ratio of uninjected cells near the injected area that incorporate [^{3}H]thymidine into DNA.

In experiments involving the use of AG-490, quiescent NIH 3T3 cells were pre-incubated with 10 μM AG-490 (Calbiochem) 1 h prior to microinjection. Microinjected cells were maintained in the presence of 10 μM AG-490 in the dark for the duration of the DNA synthesis assay.

3. Results and discussion

NIH 3T3 cells which had been made quiescent by maintenance in low-serum media were microinjected with either PBS, the expression vector pcDNA3, or pcDNA3-derived expression vectors encoding either JAK2 (pcDNA3:rJAK2) or the putative hyperactive form of JAK2 (pcDNA3:(NΔ661)r-JAK2). The cells were then assayed for entry into S phase by their ability to incorporate [^{3}H]thymidine into newly synthesized DNA. A typical experimental set is shown in Fig. 1. The tabulated results of the microinjection series are presented in Table 1. Microinjection of either PBS or the expression vector pcDNA3 results in only a small, but detectable, induction of DNA synthesis. Microinjection of PBS results in a 2.6-fold induction of DNA synthesis relative to non-injected cells (Fig. 1, panel a). Microinjection of the parental pcDNA3 vector results in a comparable 4.8-fold induction of DNA synthesis (Fig. 1, panel c). In contrast, microinjection of pcDNA3:rJAK2 into quiescent NIH 3T3 cells induced approximately a 30-fold increase in DNA synthesis (Fig. 1, panel b). Quiescent NIH 3T3 cells which were microinjected with pcDNA3:(NΔ661)rJAK2, which contains the catalytic core of the kinase, also exhibited a 30-fold increase in DNA synthesis (Fig. 1, panel d). When the two forms of JAK2-expressing vectors were microinjected at high concentrations (≈0.5–2 fg DNA injected/cell), comparable increases in DNA synthesis were observed.

These results suggested that the microinjection of plasmids encoding active isoforms of JAK2 resulted in the production of active Janus kinases which in turn induced proliferation, perhaps through the phosphorylation of key signal transduction components. In order to ascertain that JAK2 kinase activity was essential for the induction of DNA synthesis, we repeated the microinjection experiments in the presence of a JAK2-selective inhibitor, AG-490 [16]. AG-490 is a tyrphostin which has an antiproliferative effect on leukaemic pre-B cells but not on normal cells. AG-490 selectively inhibits the kinase activity of JAK2, but does not inhibit other kinases such as Lck, Lyn, Btk, Syk or Src.

In Table 2 we present the results of experiments in which the expression vectors pcDNA3:rJAK2 or pcDNA3:(NΔ661)rJAK2 were microinjected into quiescent NIH 3T3 cells in the presence or absence of 10 μM AG-490. As before, there was a marked increase in DNA synthesis following the microinjection of either expression vector in the absence of AG-490. The presence of 10 μM AG-490 completely blocked the ability of the pcDNA3:rJAK2 expression vector to induce DNA synthesis. Quite interestingly, the presence of 10 μM AG-490 did not significantly inhibit the induction of DNA synthesis by microinjected pcDNA3:(NΔ661)r-JAK2. While we have not yet completely characterized this unexpected finding, it suggests that the mechanism of AG-490 inhibition may involve the putative inhibitory domain of JAK2 [12], rather than a direct interaction of the tyrphostin with the JAK2 catalytic domain.

Many of the growth factors and cytokines which induce the activation of JAK2 also induce proliferation in many different target cells. Prolactin, for example, stimulates JAK2 activation and proliferation in Nb2-11C cells, and interleukin-3 stimulates both JAK2 activation and proliferation in Baf3 cells. Growth hormone couples to the activation of JAK2 in 3T3 cells [6]. Most proliferation assays traditionally follow the entry of quiescent cells from G_0 phase into S phase via the incorporation of [^{3}H]thymidine into newly synthesized DNA. The microinjection studies presented in this paper indicate

Table 1
Summary of results from microinjection experiments

Sample	Number of injected cells	Fold-induction of DNA synthesis
PBS control	1100	2.6 (± 2.1)
pcDNA3:rJAK2	1700	29.5 (±11.3)
pcDNA3 control	1400	4.8 (± 3.1)
pcDNA3: (NΔ661)rJAK2	1700	30.1 (±10.6)

Parenthetical value is the standard deviation of measurement.

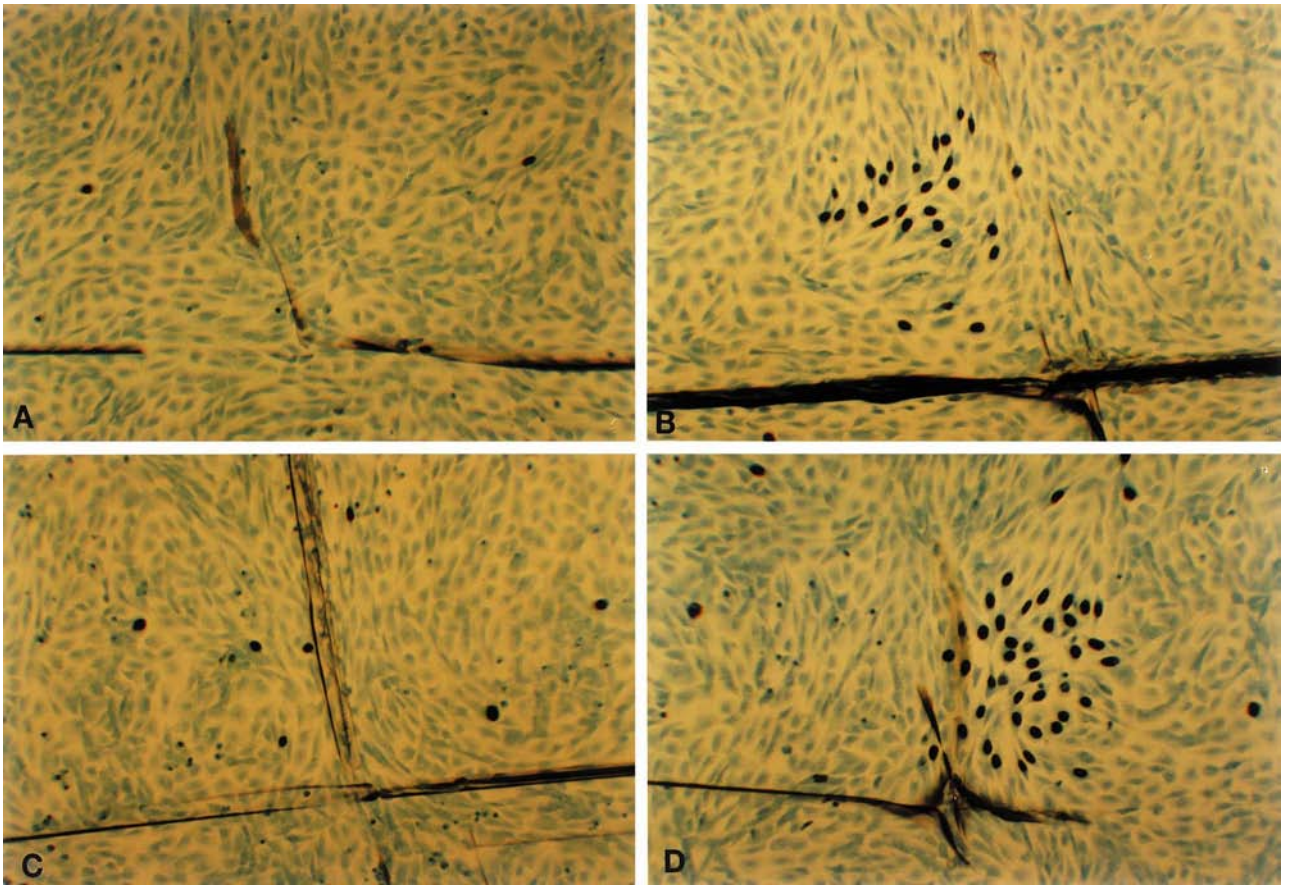

Fig. 1. rJAK2 and (NΔ661)rJAK2 induce DNA synthesis after microinjection into quiescent NIH 3T3 cells. NIH 3T3 fibroblasts were made quiescent by growth in low-serum medium (0.5% fetal calf serum) for 24 h before microinjection. Approximately 100 cells were injected with $\sim 5 \times 10^{-12}$ ml of either PBS control (a), pcDNA3:rJAK2 (b), pcDNA3 vector control (c), or pcDNA3:(NΔ661)rJAK2 (d). The concentration of microinjected DNA was 300 μg/ml. Cells were administered a 4 h pulse of [^{3}H]thymidine 18 h after microinjection, then washed, fixed, autoradiographed, stained with Geisma, and photographed at 100×.

that the presence of sufficient JAK2 in the cell may circumvent the need for an exogenous activating cytokine or growth factor in the stimulation of DNA synthesis. Based on immunocytochemical staining 8 h after microinjection, we observed a 2–4-fold increase in JAK2 protein in cells microinjected with either pcDNA3:(NΔ661)rJAK2 or pcDNA3:rJAK2 as compared to non-injected or pcDNA3-injected cells (data not shown). Constitutively active JAK2, resulting from either an overexpression of wild-type JAK2 or from a defect in the putative regulatory domain, may therefore escape normal receptor-coupled regulation and therefore be considered oncogenic. This is indeed consistent with the observations related to the *Tumorous-lethal* mutation in *hopscotch* [15], and to the potential role of JAK2 in ALL [16].

Microinjection studies of other proto-oncogenes and oncogenes report fold-increases in DNA synthesis which are comparable to the fold-increases we demonstrate here for JAK2. Both the gamma and beta isozymes of phospholipase C can induce approximately 23-fold increases in DNA synthesis [22]. The HER-2/*neu* promoter binding factor (HPBF) induces only a 12-fold induction of DNA synthesis when microinjected into quiescent NIH 3T3 cells; when HPBF is co-injected with c-Ras the combination induces a 25-fold increase in DNA synthesis [23]. While the full-length c-Raf protein did not induce DNA synthesis in quiescent NIH 3T3 cells, mutant forms of c-Raf which lacked the amino terminus did induce DNA synthesis 22–26-fold after microinjection into such cells [24]. Our results demonstrate that DNA synthesis is stimulated in serum-starved NIH 3T3 cells by the overexpression of JAK2 which apparently circumvents the normal role for cytokine or growth factor involvement in initiating the G_o/S cell cycle transition. Moreover, when JAK2 is compared with other proto-oncogenes and oncogenes which induce DNA synthesis in quiescent NIH 3T3 cells, it is comparable to those that

Table 2
Summary of results from microinjection experiments performed in the presence or absence of 10 μM AG-490

Sample	Fold-induction of DNA synthesis (without inhibitor)	Fold-induction of DNA synthesis (+10 μM AG-490)
pcDNA3:rJAK2	20.7 (±6.4)	3.8 (±1.2)
pcDNA3: (NΔ661)rJAK2	27.8 (±6.7)	22.4 (±4.5)

500–600 cells were injected in each of the four data sets. Parenthetical value is the standard deviation of measurement

induce a high level of DNA synthesis (20–30-fold induction), rather than a moderate response (10–20-fold induction). Although the breadth of the spectrum of cells which may be susceptible to JAK-mediated transformation is unknown, it appears that mutations in the *jak2* proto-oncogene resulting in either JAK2 overexpression or constitutive JAK2 activation clearly have oncogenic potential.

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