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Kinase-negative mutants of JAK1 can sustain interferon-γ-inducible gene expression but not an antiviral state

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The receptor-associated protein tyrosine kinases JAK1 and JAK2 are both required for the interferon (IFN)-γ response. The effects of expressing kinase-negative JAK mutant proteins on signal transduction in response to IFN-γ in wild-type cells and in mutant cells lacking either JAK1 or JAK2 have been analysed. In cells lacking endogenous JAK1 the expression of a transfected kinase-negative JAK1 can sustain substantial IFN-γ-inducible gene expression, consistent with a structural as well as an enzymic role for JAK1. Kinase-negative JAK2, expressed in cells lacking endogenous JAK2, cannot sustain IFN-γ-inducible gene expression, despite low level activation of STAT1 DNA binding activity. When expressed in wild-type cells, kinase-negative JAK2 acts as a dominant-negative inhibitor of the IFN-γ response. Further analysis of the JAK/STAT pathway suggests a model for the IFN-γ response in which the initial phosphorylation of JAK1 and JAK2 is mediated by JAK2, whereas phosphorylation of the IFN-γ receptor is normally carried out by JAK1. The efficient phosphorylation of STAT 1 in the receptor–JAK complex may again depend on JAK2. Interestingly, a JAK1-dependent signal, in addition to STAT1 activation, appears to be required for the expression of the antiviral state.

Keywords: antiviral/interferons/JAKs and STATs/mutants/signal transduction

Introduction

Signal transduction through JAK/STAT pathways is activated by a wide range of cytokines and growth factors. The activation of STATs (signal transducers and activators of transcription) by the JAKs (Janus kinases) occurs in receptor–JAK complexes at the cell membrane and provides a direct mechanism for transducing extracellular signals to the nucleus (reviewed in Darnell *et al.*, 1994). There are four known mammalian members of the JAK family of receptor-associated protein tyrosine kinases, JAK1 (Wilks, 1989), JAK2 (Harpur *et al.*, 1992; Silvennoinen *et al.*, 1993), JAK3 (Johnston *et al.*, 1994; Witthuhn *et al.*, 1994) and Tyk2 (Firmbach-Kraft *et al.*, 1990). Each is ~130 kDa and has a C-terminal protein tyrosine kinase domain, an adjacent kinase-related domain and five further domains extending towards the N-terminus with similarity between members of the family (Wilks *et al.*, 1991). Seven STATs (including STATs 5A and B) have been cloned (Fu *et al.*, 1992; Schindler *et al.*, 1992a; Akira *et al.*, 1994; Hou *et al.*, 1994; Wakao *et al.*, 1994; Yamamoto *et al.*, 1994; Zhong *et al.*, 1994; Quelle *et al.*, 1995). Each contains an SH2 and putative SH3 domain. Activation of the STATs involves phosphorylation of a conserved tyrosine just C-terminal to the SH2 domain (Shuai *et al.*, 1993; Improta *et al.*, 1994), with subsequent homo- or heterodimerization to yield, with or without additional polypeptides, a spectrum of transcription factors with differing affinities for related DNA response elements (reviewed in Darnell *et al.*, 1994; Schindler and Darnell, 1995).

Different cytokines activate different spectra of JAKs and STATs (reviewed in Ihle, 1995; Ihle and Kerr, 1995). JAK–receptor association is constitutive but may also increase in response to ligand binding. The interferon (IFN)-α receptor consists of at least two subunits, IFNAR1 and ABR, which associate with Tyk2 and JAK1 respectively (Uze *et al.*, 1992; Abramovich *et al.*, 1994; Colamonici *et al.*, 1994; Novick *et al.*, 1994). The IFN-γ receptor also comprises at least two chains—IFNγR α and AF1—in association with JAK1 and JAK2, respectively (Aguet *et al.*, 1988; Hemmi *et al.*, 1994; Soh *et al.*, 1994; Sakatsume *et al.*, 1995). Ligand binding induces receptor dimerization/oligomerization, resulting in tyrosine phosphorylation/activation of the JAKs and receptor components. The specificity of JAK activation is determined by interaction with particular cytokine receptors. Specificity of STAT activation is determined by the receptor–JAK/STAT complex in part through STAT SH2 group interaction with specific phosphorylated tyrosines in receptor subunits (Greenlund *et al.*, 1994; Heim *et al.*, 1995; Stahl *et al.*, 1995). A kinase deletion mutant of Tyk2 can sustain a very low level induction of the 6-16 gene in response to high doses of IFN-α (Velazquez *et al.*, 1995) and probably, therefore, can sustain activation of the JAK/STAT pathway, albeit extremely inefficiently. In addition, cells lacking Tyk2 or JAK1 show reduced IFN-α and -β binding (Pellegrini *et al.*, 1989; Müller *et al.*, 1993a). Accordingly, it is likely that the JAKs play a structural as well as an enzymic role in the correct assembly and function of the cell surface receptors.

Mutant cell lines defective in one or other or both of the major IFN-α/β or IFN-γ response pathways and which lack individual JAKs or STATs have been isolated and characterized (Pellegrini *et al.*, 1989; John *et al.*, 1991;

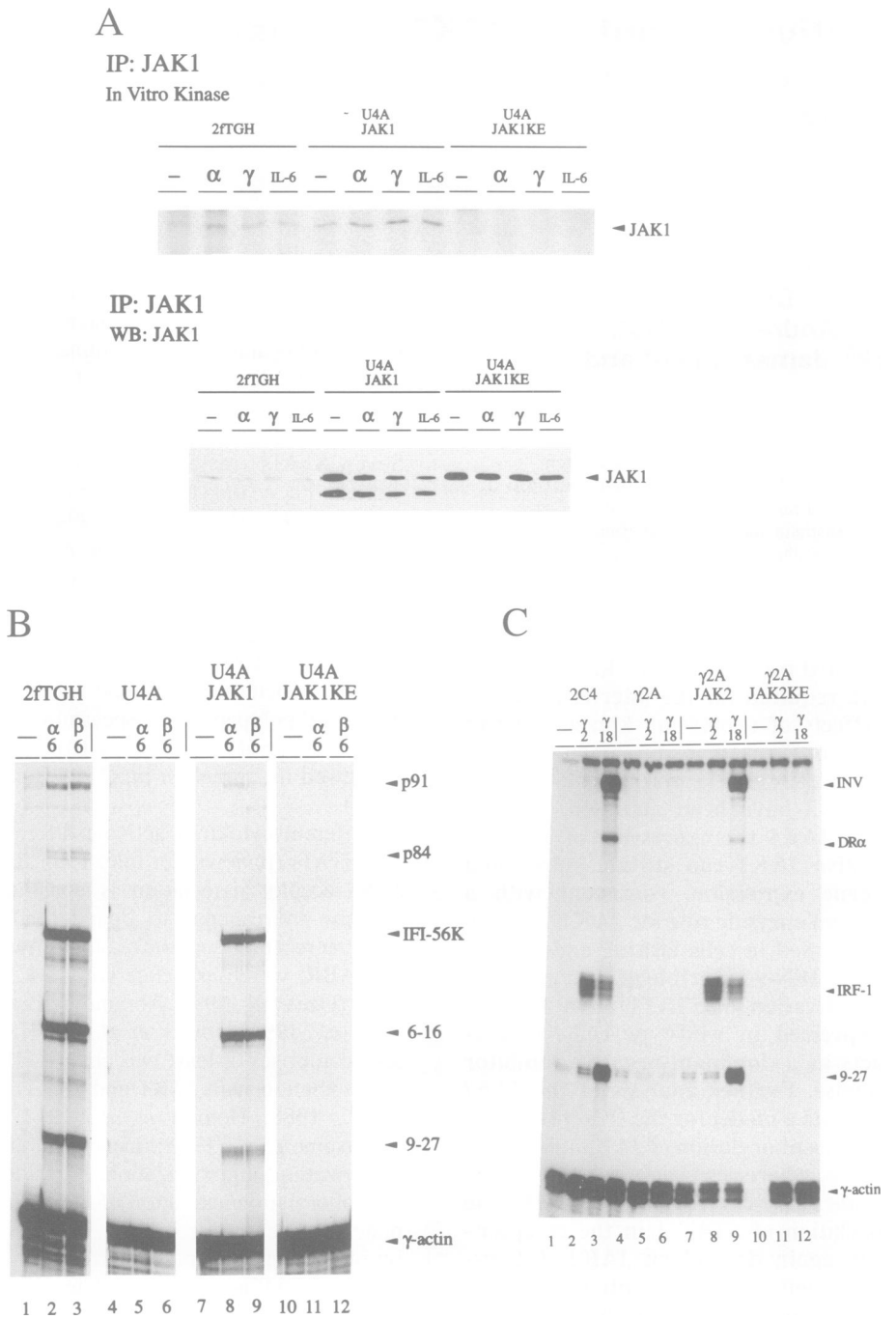

Fig. 1. Kinase-negative mutants of JAK1 and JAK2. (**A**) *In vitro* kinase activity. Wild-type and mutant JAK1s were immunoprecipitated from 2fTGH, U4A/JAK1 or U4A/JAK1.KE cells which had been pre-treated for 10 min at 37°C with 1000 IU/ml of α or γ IFNs or with IL-6 (see Materials and methods) and the immune precipitates were assayed for JAK autokinase activity. Each sample was assayed for the incorporation of ^{32}P from [γ-^{32}P]ATP by SDS–PAGE (upper panel) and by Western transfer with anti-JAK1 antibody to monitor expression levels and as a loading control (lower panel). (**B** and **C**) Kinase-negative mutants of JAK1 (B) cannot support IFN-α/β- and of JAK2 (C) cannot support IFN-γ-inducible gene expression. The IFN-inducible mRNAs indicated to the right (B and C) and the probes utilized to detect them were described previously (Müller *et al.*, 1993b; INV, invariant chain of HLA class II; DRα, HLA class II DRα; p91/p84, STAT1α/β; 2–5A, 2–5A synthetase). They were analysed by RNase protection (see Materials and methods) utilizing 10 μg of cytoplasmic RNA from each of the indicated cell lines, with the protection of γ-actin as a loading control. IFN treatment of the cells was with 1000 IU/ml for 6 h for IFNs-α and -β (B) and 2 and 18 h for IFN-γ (C). The second, faster-migrating, JAK1-specific band (lanes 4–8 and Figure 3B) consistently observed with extracts from U4A cells expressing transfected wild-type JAK1 has not been analysed and presumably represents an aberrant or breakdown product.

McKendry *et al.*, 1991; Velazquez *et al.*, 1992; Müller *et al.*, 1993a,b; Watling *et al.*, 1993). JAK1, Tyk2 and STATs 1 and 2 are required for the major IFN-α/β response (although a residual response to IFN-β, for example, is observed in the absence of Tyk2; Pellegrini *et al.*, 1989) and JAKs 1 and 2 and STAT1 for the IFN-γ response. In both the major IFN-α/β and the IFN-γ pathways the kinases are interdependent: in the absence of one kinase activation of the other is not observed, thus precluding a simple sequential kinase cascade (Müller *et al.*, 1993a).

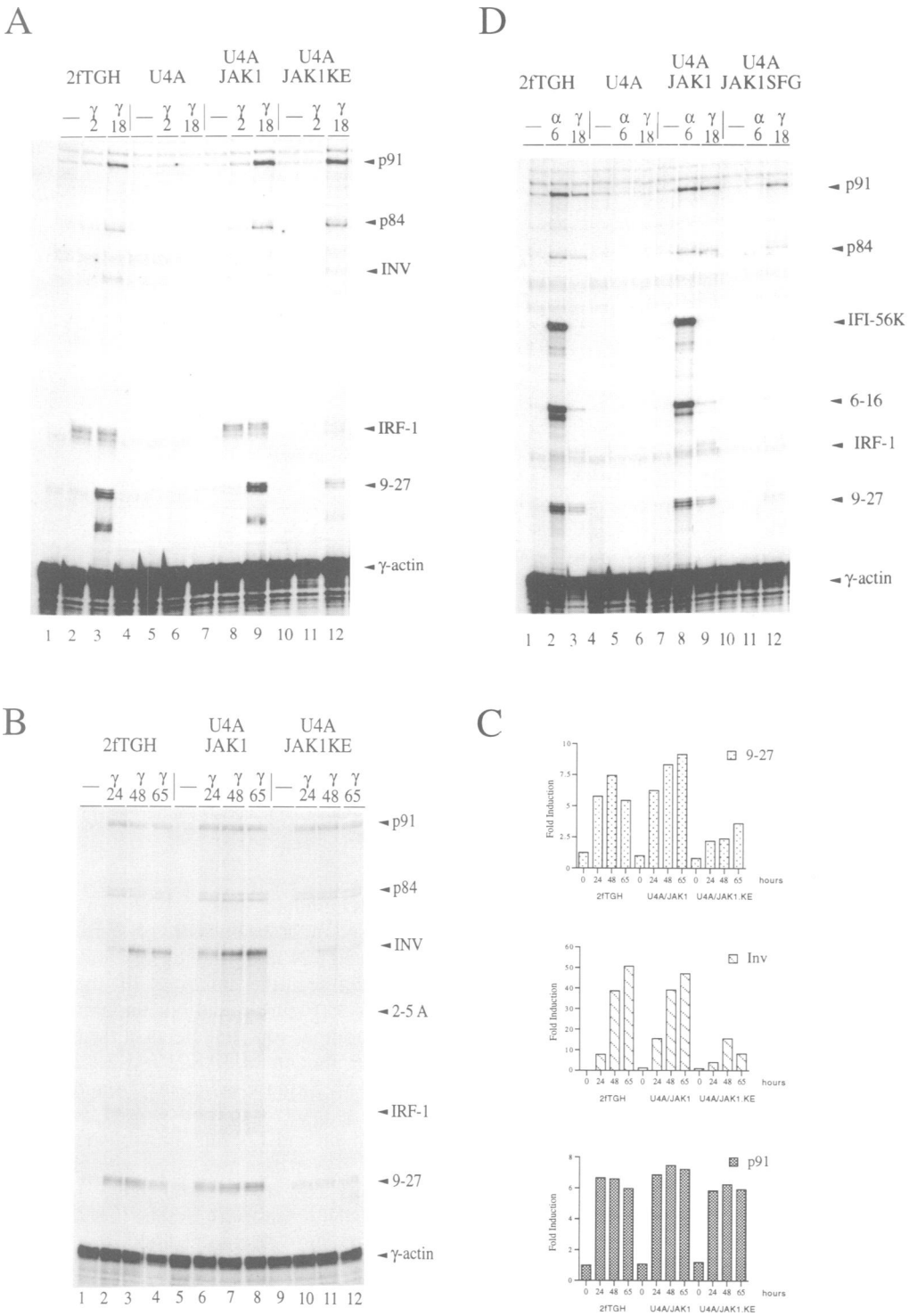

Fig. 2. Kinase-negative mutants of JAK1 can sustain IFN-γ-inducible gene expression. For the JAK1.KE mutant (**A**) early (2 and 18 h) and (**B**) more prolonged (24, 48 and 65 h) expression of inducible mRNAs was monitored by RNase protection (see Figure 1 and Materials and methods). (**C**) Quantitation of the data in (B) by PhosphorImager analysis. Fold induction was calculated after correction for the γ-actin loading control. (**D**) A second mutant of JAK1, JAK1.SFG, also supports IFN-γ- (18 h) but not IFN-α- (6 h) inducible gene expression monitored, as above, by RNase protection.

Here, kinase-negative mutants of JAK1 and 2 have been stably expressed in wild-type and JAK1- or JAK2-deficient mutant cell lines, and the response to IFN-γ analysed. In cells lacking endogenous JAK2, the introduced mutant JAK2 can sustain low level receptor and STAT1 phosphorylation but not IFN-γ-inducible gene expression. In wild-type cells it can act as a dominant-negative inhibitor of the IFN-γ response. In cells lacking endogenous JAK1, the introduced mutant JAK1 can sustain substantial IFN-γ-inducible gene expression but not an antiviral state. Moreover, in wild-type cells, introduced kinase-negative JAK1 is without detectable effect on JAK/STAT activation

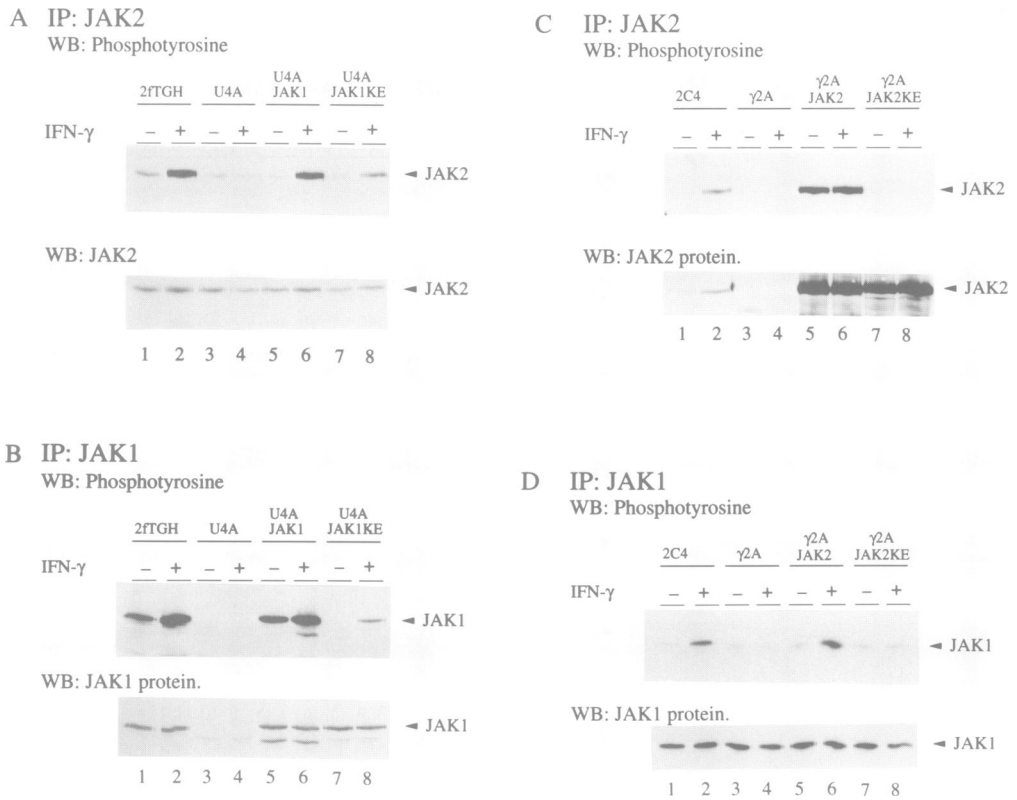

Fig. 3. JAK phosphorylation in response to IFN-γ is sustained by kinase-negative JAK1 (**A** and **B**) but not by kinase-negative JAK2 (**C** and **D**). IFN-γ-inducible tyrosine phosphorylation of JAK1 and JAK2 was assayed (see Materials and methods) in whole cell extracts from the indicated parental, mutant and transfected cell lines. IFN-γ pre-treatment was for 15 min at 37°C with 1000 IU/ml. Extracts were immunoprecipitated with polyclonal antisera to JAK1 or JAK2 and probed after SDS–PAGE (7%) analysis with a mix (see Materials and methods) of anti-phosphotyrosine antibodies (upper panels) and, after stripping, antisera to JAK1 or JAK2 (lower panels).

and gene expression, but substantially inhibits the development of an antiviral state in response to IFN-γ. A model for the IFN-γ response involving at least two JAK1-dependent phosphorylation signals is indicated.

Results

Kinase-negative mutants of JAK1 and JAK2

The mutant human cell line U4A which lacks JAK1 is defective in the response to the IFNs and shows a much reduced response to interleukin (IL)-6 (Müller *et al.*, 1993a; Guschin *et al.*, 1995). Expression of wild-type JAK1 in the U4A cells restores these responses (Müller *et al.*, 1993a; Guschin *et al.*, 1995), thus establishing a central role for JAK1 in signalling by these ligands. To determine whether this role is dependent on the protein tyrosine kinase activity of JAK1, two kinase-negative mutants of JAK1 were assayed in the JAK1-negative U4A cells. In the first mutant (JAK1.KE) a lysine in motif II of the kinase domain (Hanks *et al.*, 1988) was mutated to glutamic acid, and in the second (JAK1.SFG) the aspartic acid in the highly conserved DFG motif VII (Hanks *et al.*, 1988) of the kinase domain was changed to a serine. These mutant proteins were first shown to be without detectable protein tyrosine kinase activity. The JAK1.KE and JAK1.SFG mutants were inactive in *in vitro* protein kinase assays (e.g. Figure 1A) and did not sustain IFN-α/β-inducible gene expression (Figures 1B and 2D) when overexpressed (Figure 1A, lower panel) in U4A cells. The JAK1.KE mutant was also shown to be inactive when expressed in a baculovirus system (F.W.Quelle, B.Witthuhn and J.N.Ihle, unpublished data) and functions as a dominant-negative inhibitor of the IFN-α/β and IL-6 responses (Figure 7 and our unpublished data).

For comparison, the effects of the corresponding kinase-negative mutant of JAK2, JAK2.KE, were also assayed in the JAK2 kinase-deficient γ2A and wild-type cells. This mutant is inactive in the baculovirus system (F.W.Quelle, B.Witthuhn and J.N.Ihle, unpublished data) and unable to sustain IFN-γ-inducible gene expression (Figure 1C). It functions as a dominant-negative inhibitor of the IFN-γ and IL-6 responses (Guschin *et al.*, 1995; N.C.Rogers, J.Briscoe and I.M.Kerr, unpublished).

Kinase-negative mutants of JAK1 sustain IFN-γ-inducible gene expression

The induction of a number of mRNAs in response to IFN-γ in wild-type (2fTGH), mutant U4A cells and U4A cells expressing either wild-type JAK1, or JAK1.KE or JAK1.SFG was assayed by RNase protection (Figure 2A–D). Similar results were observed using populations of transfected cells or individual clones expressing the JAK1 mutants. Included are the results for mRNAs for which the IFN-γ response is primary (not requiring protein synthesis, e.g. IRF-1, Figure 2A), those for which the response is secondary (e.g. invariant chain) and those for which the initial primary response is enhanced by a protein synthesis-dependent secondary phase (e.g. 9-27, STAT1).

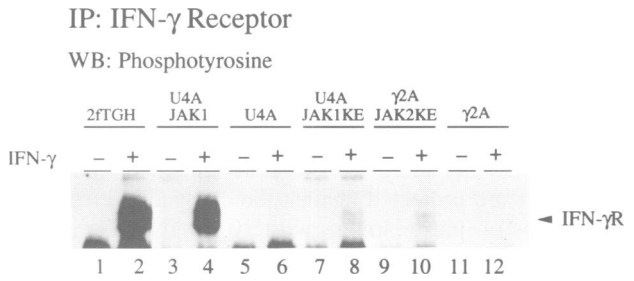

Fig. 4. Kinase-negative JAK1 and JAK2 can each sustain low level tyrosine phosphorylation of the IFN-γ receptor α chain. The IFN-γ receptor α chain immunoprecipitated from each of the indicated cell lines was probed after SDS–PAGE (7%) with a mixture of anti-phosphotyrosine antibodies (see Materials and methods). Pre-treatment with IFN-γ, as indicated, was with 1000 IU/ml for 5 min at 37°C. The antibody to the IFN-γ receptor α chain is not suitable for Western transfer analysis of protein levels. Similar results to those presented were, however, obtained in multiple assays of this type.

Mutant U4A cells are defective in the response of all genes tested (Müller *et al.*, 1993a and Figure 2A, lanes 4–6). The responses are restored fully by wild-type JAK1 and substantially by the kinase-negative JAK1.KE mutant (Figure 2A, lanes 7–9 and 10–12, respectively). In a more extensive quantitative analysis, the levels of induced mRNAs sustained in the U4A/JAK1.KE cells varied from 30 to 90% of maximum wild-type levels over the period from 24 to 65 h of exposure to 1000 IU/ml of IFN-γ (Figure 2B and C). A similar quantitative analysis of the dose response of these mRNAs to 5, 50, 500 and 5000 IU/ml of IFN-γ suggested that the absence of enzymically active JAK1 becomes limiting at higher IFN-γ concentrations. In U4A/JAK1.KE cells the maximum response (30–90% of the maximum wild-type levels) was obtained at 50 IU/ml, whereas in wild-type cells the response did not plateau until 500 IU/ml (data not presented). The kinase-negative JAK1.SFG mutant also restored IFN-γ- but not IFN-α-inducible gene expression (Figure 2D, lanes 12 and 11, respectively) to levels comparable with those observed with JAK1.KE. For subsequent experiments, only the results for the JAK1.KE mutant are shown; similar results were, however, obtained with the JAK1.SFG mutant. Cell surface expression of class I HLAs in response to IFN-α and -γ and of class II HLAs in response to IFN-γ is also defective in U4A cells. As expected, in U4A/JAK1.KE cells, inducible expression is not restored in response to IFN-α but is restored substantially with IFN-γ (J.Briscoe, data not shown). It appears, therefore, that in contrast to a kinase-negative mutant of JAK2 (e.g. Figure 1C), kinase-negative JAK1 mutants can sustain substantial IFN-γ-inducible gene expression (Figure 2). Interesting differences in the ability of these two types of mutant to support different steps in the JAK/STAT pathway in response to IFN-γ were revealed by a more detailed analysis.

JAK1 and 2 phosphorylation in the IFN-γ response

Both JAK1 and JAK2 are phosphorylated and activated in response to IFN-γ. In mutant cell lines which lack either JAK1 or JAK2, the remaining kinase is not phosphorylated/activated (Müller *et al.*, 1993a and Figure 3, lanes 3 and 4). This interdependence suggests a possible structural as well as enzymic requirement for these proteins. Consistent with a structural role for the kinase-negative JAK1, there is substantial restoration of JAK2 phosphorylation/activation (Figure 3A, lane 8) and of inducible phosphorylation of the transfected JAK1.KE (Figure 3B, lane 8)—presumably by JAK2—in U4A/JAK1.KE cells. This becomes particularly obvious for JAK1 when one takes into account the higher constitutive levels of tyrosine phosphorylation observed in the wild-type and U4A/JAK1 cells compared with the U4A/JAK1.KE cells (Figure 3B, lanes 1, 5 and 7). In contrast, in γ2A/JAK2.KE cells there is no inducible phosphorylation of either JAK1 or JAK2 in response to IFN-γ (Figure 3C and D, lanes 7 and 8). It can be concluded that JAK1 protein is required for the correct assembly of the receptor and, more importantly, the data suggest that the initial events following ligand binding and receptor oligomerization are the phosphorylation of JAK2 and JAK1 by JAK2, with no requirement for JAK1 kinase activity.

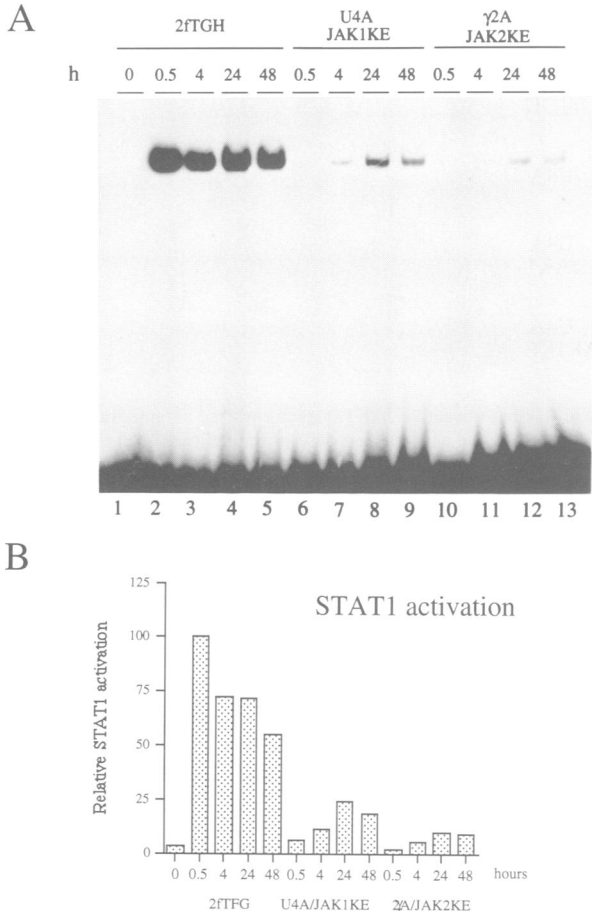

Fig. 5. Kinase-negative JAK1 and JAK2 can each sustain STAT1 activation. (**A**) The kinetics of STAT1 activation in response to IFN-γ (1000 IU/ml) for the indicated cell lines was monitored by EMSA of whole cell extracts with an hSIE probe (see Materials and methods). (**B**) Quantitation by PhosphorImager of the data in (A) relative to an arbitrary value of 100 for the 30 min time point for the parental (2fTGH) cell line. Similar results were obtained in several assays of this type.

Phosphorylation of the IFNγR α subunit

Tyrosine phosphorylation of the IFNγR α subunit probably plays a central role in IFN-γ signalling by recruiting STAT1 to the receptor complex (Greenlund *et al.*, 1994).

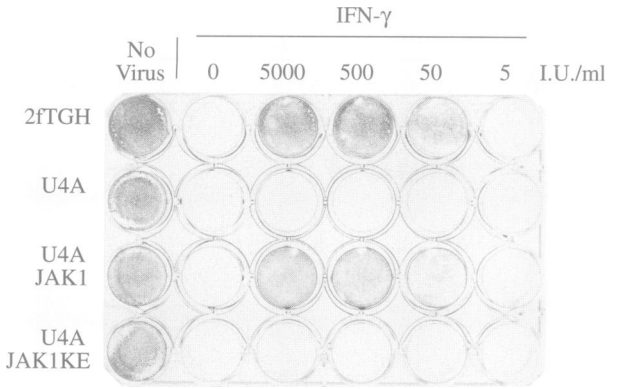

Fig. 6. A kinase-negative JAK1 does not restore an antiviral response to EMC virus. The antiviral response of the indicated cell lines was monitored by cytopathic assay. Cells were treated for 24 h with 5, 50, 500 or 5000 IU/ml of IFN-γ, infected with EMC virus at 0.3 p.f.u./cell, incubated at 37°C and fixed and stained 24 h post-infection.

In U4A and γ2A cells which lack JAK1 and JAK2, respectively, phosphorylation of the IFNγR α subunit is not detected (Figure 4, lanes 6 and 12). It is restored by wild-type JAK1 or 2 in complemented cells (Figure 4; D.Guschin, N.C.Rogers, J.Briscoe and I.M.Kerr, unpublished data). It is also restored in U4A/JAK1.KE cells but only very inefficiently (to ~10% of wild-type levels, Figure 4, lane 8). Accordingly, it would appear that, despite substantial activation in these cells (Figure 3A), JAK2 cannot efficiently phosphorylate the receptor even in the presence of the kinase-negative JAK1.KE. Surprisingly, phosphorylation of the receptor subunit is also partially restored in γ2A/JAK2.KE cells (Figure 4, lane 10), despite the absence of detectable activation of JAK1 (Figure 3D, lane 8). Ligand-mediated dimerization/oligomerization of the receptor presumably permits low but significant phosphorylation of the receptor by basal JAK1 enzyme activity. Taken together, these results indicate that JAK2 activation does not require active JAK1, that JAK1 activation does require active JAK2 and, most probably, that phosphorylation of the IFNγR α subunit receptor can be mediated efficiently only by JAK1.

STAT1 activation in the IFN-γ response

In wild-type 2fTGH cells, STAT1 is activated rapidly in response to the IFNs (Watling *et al.*, 1993). In the case of IFN-γ, this response can be sustained for at least 48 h (Figure 5, lanes 1–5). No such response is observed in JAK1-deficient U4A and JAK2-deficient γ2A cells (Müller *et al.*, 1993a; Watling *et al.*, 1993). Like receptor subunit phosphorylation (above), STAT1 activation, although delayed, is partially restored in both U4A/JAK1.KE and γ2A/JAK2.KE cells (to ~20 and 10% of wild-type levels, respectively, Figure 5A and B). Interestingly, this is accompanied by substantial restoration of IFN-γ-inducible gene expression in U4A/JAK1.KE (Figure 2A–C) but not γ2A/JAK2.KE cells (Figure 1C). The reason for the apparent inability of the low levels of STAT1 to support IFN-γ-inducible gene expression in the γ2A/JAK2.KE cells is not known (see Discussion). It can be concluded, however, that kinase-negative JAK1 can sustain substantial IFN-γ-inducible gene expression despite low level receptor and reduced STAT1 phosphorylation. Despite this, kinase-negative JAK1 does not sustain an antiviral response to encephalomyocarditis (EMC) virus or Semliki forest virus (SFV) in the U4A/JAK1.KE cells.

Kinase-negative JAK1 mutants do not sustain an antiviral response

Wild-type 2fTGH and U4A cells complemented with wild-type JAK1 are protected against the cytopathic effects of EMC virus on pre-treatment with 10–50 IU/ml of IFN-γ (Figure 6). No protection was observed for U4A or U4A/JAK1.KE cells on treatment with up to 5000 IU/ml (Figure 6) or even 50 000 IU/ml of IFN-γ. Similar results were obtained with SFV (data not presented). In alternative virus yield assays, the wild-type 2fTGH and JAK1-complemented U4A cells showed an ~30-fold reduction in EMC virus yield upon pre-treatment with 1000 IU/ml of IFN-γ; no reduction was observed upon pre-treatment of U4A or U4A/JAK1.KE cells. Results of this type were reproducibly obtained in multiple independent assays. Thus, over the period from 24 to 65 h of exposure to IFN-γ, U4A/JAK1.KE cells sustain ~20% of maximum wild-type levels of STAT1 (Figure 5) and 30–90% of maximum wild-type levels of the IFN-γ-inducible mRNAs assayed (Figure 2). Despite this, when assayed during this period, no antiviral state was observed. There are a number of possible explanations for this (see Discussion). Amongst these is a requirement for a relatively high (threshold) level of active STAT1 for the induction of one or more inducible genes essential to the antiviral response. Alternatively, a second JAK1-dependent signal in addition to the activation of STAT1 may be required for the antiviral response to EMC virus and SFV. Evidence in favour of the latter possibility was provided by the differential effects of the JAK1.KE mutant protein on IFN-γ-inducible gene expression and the antiviral response when it was expressed in wild-type cells.

A kinase-negative JAK1 inhibits the antiviral response but is without effect on activation of the JAK/STAT1 pathway or IFN-γ-inducible gene expression in wild-type cells

Overexpression of the JAK1.KE mutant (e.g. Figure 7B, lanes 7 and 8, lower panel) in wild-type 2C4 cells inhibits STAT1 activation in response to IFN-α and IL-6 but not IFN-γ (Figure 7A, lanes 5–8) and inducible gene expression is substantially inhibited (N.C.Rogers, B.A.Witthuhn, J.Briscoe, D.Watling, J.N.Ihle and I.M.Kerr, in preparation). (2C4 cells, the parental line for the IFN-γ series mutants, carry the 9-27CD2 rather than the 6-16gpt marker, but are otherwise identical to the 2fTGH cells used above, Watling *et al.*, 1993.) The JAK1.KE mutant is, however, without detectable inhibitory effect on receptor phosphorylation, JAK/STAT phosphorylation/activation (Figure 7B–D) or gene expression (Figure 8) in response to IFN-γ (Figures 7B–D and 8). Quantitation of the electrophoretic mobility shift assays (EMSAs) for STAT1 (Figure 7C and D) and of the RNase protections for IFN-γ-inducible mRNA levels from 24 to 72 h of exposure to 1000 IU/ml of IFN-γ (e.g. Figure 8A and B) failed to reveal any consistent difference between the data for the 2C4 and 2C4/JAK1.KE cells. The early and more transient induction of IRF-1 mRNA was similarly unaffected. Similarly, while inhibiting the IFN-α response of the class I HLAs in the 2C4/JAK1.KE cells compared with 2C4

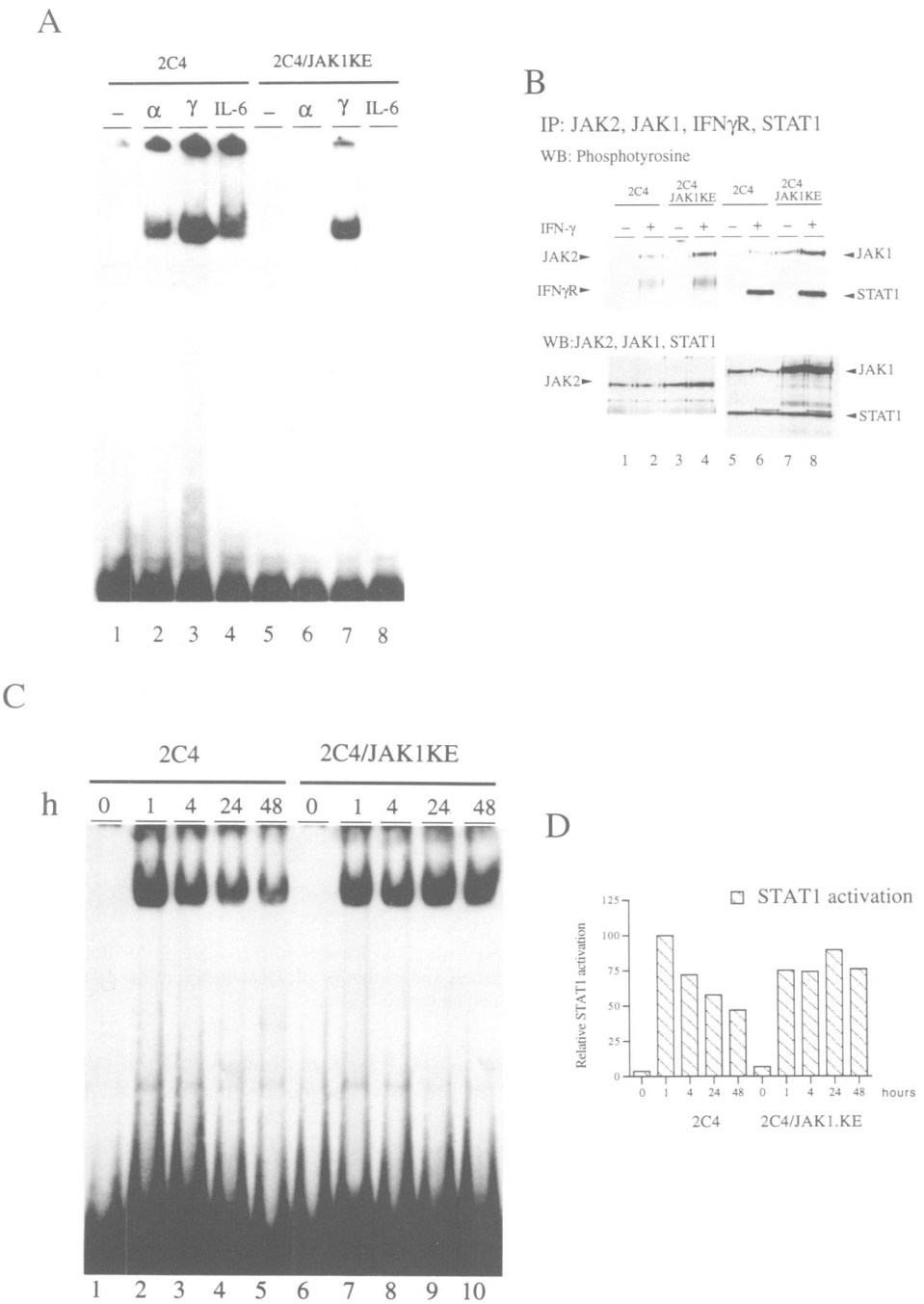

Fig. 7. The IFN-γ-mediated JAK/STAT1 pathway in wild-type cells overexpressing a kinase-negative JAK1. (**A**) The kinase-negative JAK1 inhibits STAT1 activation by IL-6 and IFN-α but not by IFN-γ. EMSAs of whole cell extracts from the indicated cell lines were with an hSIE probe (see Materials and methods). Treatment of the cells with 1000 IU/ml of IFN-α or -γ or with IL-6 (Materials and methods) was for 15 min at 37°C. (**B**) The kinase-negative JAK1 is without detectable effect on tyrosine phosphorylation of the IFNγR α subunit, JAK1 and 2 and STAT1. The components of interest were immunoprecipitated from the indicated whole cell extracts analysed by SDS–PAGE (7%), transferred and probed with a mix of anti-phosphotyrosine antibodies (upper panel) and, after stripping, with anti-JAK or STAT1 antibodies (lower panel). Data for parental (2C4) cells and a clone of 2C4 cells expressing the JAK1.KE mutant are presented. Treatment of the cells with 1000 IU/ml of IFN-γ, where indicated, was for 15 min at 37°C. (**C** and **D**) The kinase-negative JAK1 is without inhibitory effect on the kinetics of STAT1 activation. (**C**) EMSAs of whole cell extracts from the above cells with or without treatment with 1000 IU/ml of IFN-γ for 1, 4, 24 and 72 h was with an hSIE probe (see Materials and methods). (**D**) Quantitation of the STAT1 complex in (C) by PhosphorImager analysis.

cells, the JAK1.KE mutant was without effect on expression of class I and II and CD2 antigen (from the transfected 9-27-CD2 marker construct) when compared with 2C4 cells over the period of maximum expression from 48 to 72 h of exposure to 1000 IU/ml of IFN-γ (N.C.Rogers, data not shown). In contrast, the 2C4/JAK1.KE cells, but not cells transfected with wild-type JAK1, consistently showed a reduced antiviral response to IFN-γ compared with 2C4 cells, assayed both by reduced protection from cytopathic effect and by reduced inhibition of virus yield (>10-fold) for both EMC virus and SFV (e.g. Figure 9A and B). As this inhibition of the antiviral response (assayed

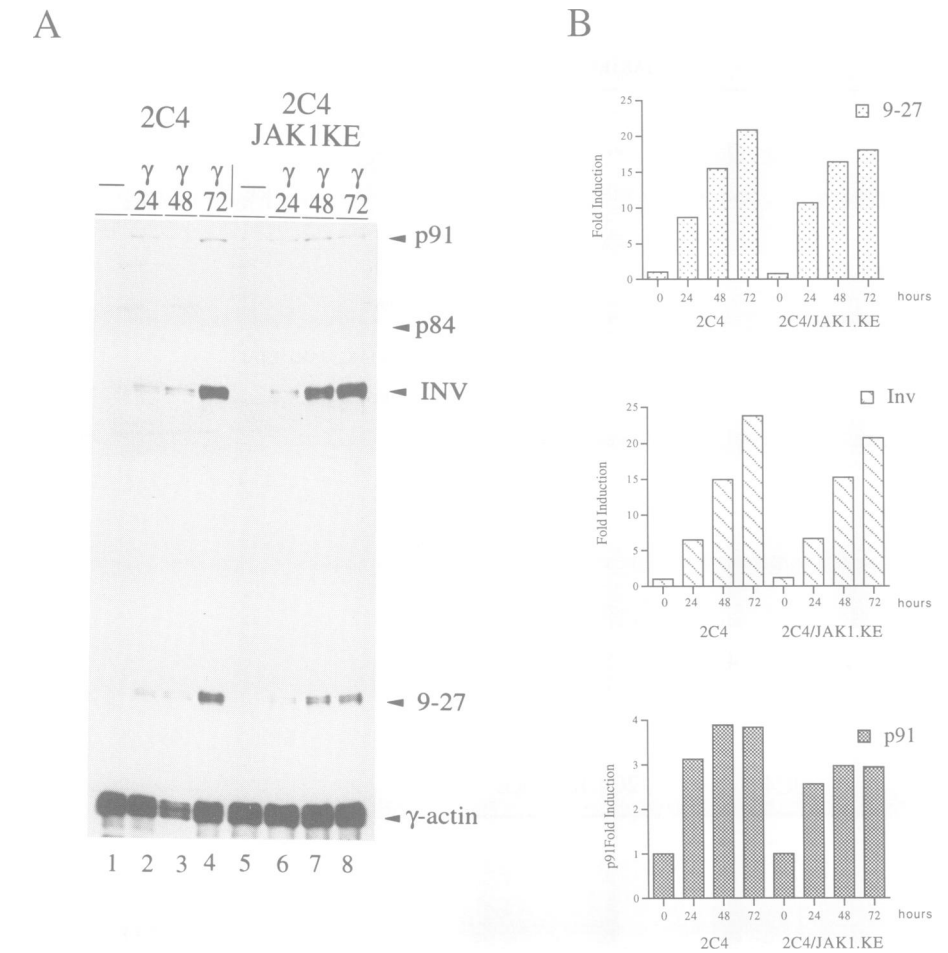

Fig. 8. A kinase-negative JAK1 does not inhibit IFN-γ-inducible gene expression. (**A**) Kinetics of IFN-γ-inducible mRNA synthesis in 2C4 and 2C4/JAK1.KE cells was assayed by RNase protection (see Figure 1 and Materials and methods). (**B**) Quantitation of the data in (A) by PhosphorImager analysis. Fold induction was calculated after correction for the γ-actin loading control.

on virus replication during the period 18–48 h after the initiation of IFN treatment) occurs in the absence of detectable inhibition of the JAK/STAT1 pathway (Figure 7), it presumably reflects the disruption by excess JAK1.KE protein of an interaction of JAK1 with an additional substrate and/or a component of an additional signalling pathway. Similar results were obtained with three independent clones of 2C4/JAK1.KE cells.

Discussion

Two kinase-negative mutants of JAK1 (JAK1.KE and JAK1.SFG) sustain neither IFN-α nor IL-6 responses but support substantial IFN-γ-inducible gene expression in cells lacking endogenous JAK1 (Figures 1 and 2). Consistent with this, in transfected wild-type cells, kinase-negative JAK1.KE has a dominant-negative effect on the IFN-α and IL-6 (Figure 7A; N.C.Rogers, J.Briscoe and I.M.Kerr, unpublished) but not the IFN-γ (Figures 7 and 8) responses. In IFN-γ signalling, it appears that JAK2 can phosphorylate/activate all of the components of the JAK/STAT1 signal transduction pathway in the absence of enzymatically active JAK1, albeit inefficiently in some cases (Figures 3–5). JAK1 protein is, however, required for JAK2 activation (Figure 3A). This indicates a structural role for JAK1 in the IFN-γ response, despite the fact that for IFN-γ, in contrast to IFNs-α/β, ligand binding is not reduced in the absence of JAK1 (Müller *et al.*, 1993a).

The JAK/STAT pathway in the presence of kinase-negative JAK1

Enzymically active JAK1 is not required for the phosphorylation/activation of JAK2 (Figure 3A, lane 8). Activated JAK2 cannot, however, phosphorylate the IFNγR α subunit efficiently (Figure 4, lane 8), this function presumably normally being carried out by JAK1. Using a different approach, Sakatsume *et al.* (1995) recently have also concluded that phosphorylation of the IFNγR α subunit is likely to be by JAK1. Whether the reduction in JAK2 phosphorylation observed in the absence of enzymatically active JAK1 (Figure 3A, lane 8) reflects a decrease in the phosphorylation levels of all potentially phosphorylated tyrosines or whether JAK1 kinase activity is required to phosphorylate specific JAK2 tyrosines is not known. Although an IFN-γ response is seen in the absence of JAK1 enzyme activity, the activation of STAT1 is less efficient than in wild-type cells expressing JAK1 (Figure 5). Activation of STAT1 is likely to be dependent on SH2 group-mediated recruitment to the α subunit of the IFN-γ receptor phosphorylated on Tyr440 (Greenlund *et al.*, 1994). The lower level of STAT activation could, therefore, reflect either decreased STAT1 phosphorylation

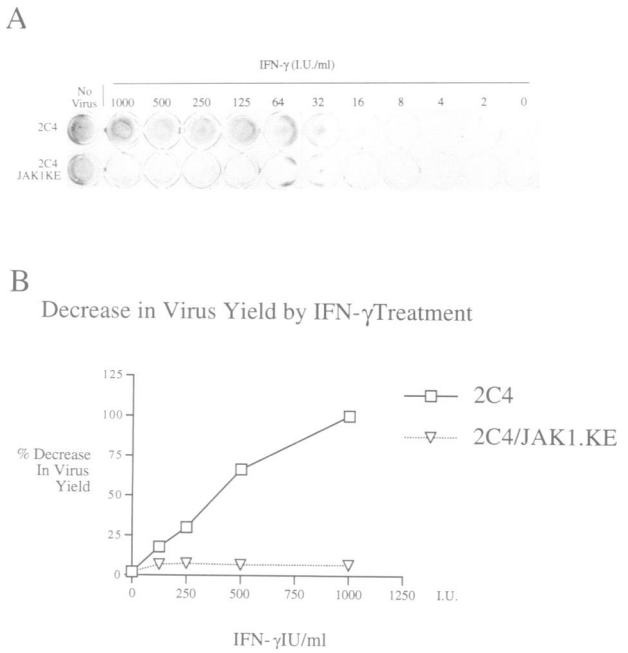

Fig. 9. A kinase-negative JAK1 can inhibit the antiviral response to IFN-γ. (**A**) Cytopathic assay. 2C4 and 2C4/JAK1.KE cells, with or without treatment for 24 h with 2–1000 IU/ml of IFN-γ, were infected with EMC virus at 0.3 p.f.u./cell, incubated at 37°C and fixed and stained 24 h post-infection. (**B**) Virus yield assay. 2C4 and 2C4/JAK1.KE cells, with or without treatment for 24 h with the indicated concentrations of IFN-γ, were infected as above for 24 h with EMC virus and the virus yields compared by serial dilution on 2C4 cells. The graph shows the IFN-γ-mediated inhibition of virus yield relative to the maximum observed with 1000 IU/ml in the wild-type, 2C4, cells taken as 100%. It represents the average of six experiments in all of which a >10-fold difference in the results for the 2C4 and 2C4/JAK1.KE cells was observed. In the absence of IFN, the virus yields from the 2C4 and 2C4/JAK1.KE cells were essentially identical. Similar results were obtained with three independently isolated clones of 2C4/JAK1.KE cells

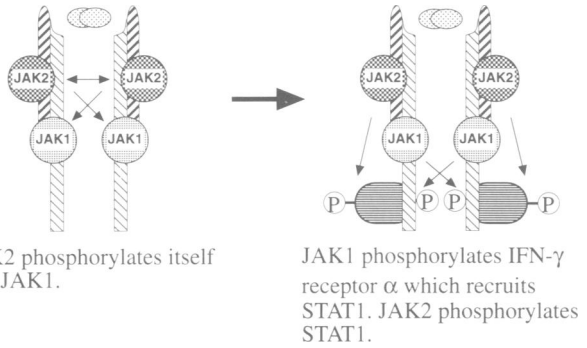

Fig. 10. Model for the activation of the JAK/STAT1 pathway by IFN-γ. A description of the model is given in the text.

and/or recruitment. In the absence of enzymatically active JAK1, phosphorylation of the IFNγR α subunit is much reduced (Figure 4, lane 8), despite substantial phosphorylation/activation of JAK2 (Figure 3A, lane 8). Accordingly, recruitment of STAT1 is probably impaired.

The JAK/STAT pathway in the presence of kinase-negative JAK2

The data for the IFN-γ response, obtained with the kinase-negative JAK2, are consistent with a primary role for JAK2 in initiating/triggering the response through JAK activation, but also with a possible requirement for JAK2 in the downstream activation of STAT1. In γ2A cells expressing a kinase-negative JAK2, no IFN-γ-induced gene expression (Figure 1C, lanes 11–12) or phosphorylation of JAK1 are detected (Figures 3D, lane 8; N.C.Rogers, B.A.Witthuhn, J.Briscoe, D.Watling, J.N.Ihle and I.M.Kerr, manuscript in preparation). Enzymatically active JAK2 is, therefore, required for JAK1 and JAK2 phosphorylation. Despite the absence of detectable activation of JAK1 in the γ2A cells expressing the kinase-negative JAK2 (Figure 3D, lane 8), there is a low but detectable level of phosphorylation of the IFNγR α subunit (Figure 4, lane 10). This may reflect receptor phosphorylation by basal JAK1 kinase activity upon receptor dimerization/oligomerization in response to IFN-γ in these cells. Interestingly, the level of receptor phosphorylation observed in the absence of enzymatically active JAK1 (U4A/JAK1.KE cells) and JAK2 (γ2A/JAK2.KE cells) are quantitatively comparable (Figure 4, lanes 8 and 10). In the former there is significant (~20% of wild-type) activation of STAT1 (Figure 5, lanes 6–9) and substantial IFN-γ-inducible mRNA synthesis (Figure 2A–C, lanes 10–12), whereas in the latter, despite apparently significant although reduced induction of STAT1 DNA binding activity (~10% of wild-type, Figure 5, lanes 10–13), there is no detectable IFN-γ-inducible mRNA synthesis (Figure 1C, lanes 10–12; N.C.Rogers, J.Briscoe and I.M.Kerr, unpublished). It would appear, therefore, that enzymatically active JAK2 may be required for fully functional activation of STAT1 in the IFNγR–JAK complex. This is in contrast to the situation with JAK1, JAK2 and Tyk2 expressed in a baculovirus system where each activates STAT1 with comparable efficiency (F.W.Quelle, B.A.Witthuhn and J.N.Ihle, in preparation). Whether phosphorylation of the IFNγR α subunit is at the same site(s) in the U4A/JAK1.KE and γ2A/JAK2.KE cells and activation of STAT1 is indeed mediated by JAK2 in the normal IFN-γ response remain, of course, to be established. Recent data indicate that, although activation of the STATs is triggered by tyrosine phosphorylation, STAT activity with respect to gene induction is subject to modulation by phosphorylation on one or more serines (Boulton *et al.*, 1995; Eilers *et al.*, 1995; Lutticken *et al.*, 1995; Wen *et al.*, 1995). The level of activation of STAT1 observed in the γ2A/JAK2.KE cells is sufficiently low, however, to preclude direct analysis for a defect in such phosphorylation.

The JAK/STAT pathway in response to IFN-γ

The data suggest the following model for the IFN-γ signal transduction pathway (Figure 10). On ligand binding and receptor dimerization/oligomerization, JAK2 phosphorylates itself and JAK1, increasing both kinase activities. JAK1 phosphorylates the IFNγR α subunit which, in turn, leads to the recruitment of STAT1, its phosphorylation, possibly by JAK2, release and dimerization to form an active transcription factor. In this model, JAK2 kinase activity is required predominantly for initiating signalling and possibly for the phosphorylation of STAT1, whereas JAK1 is required for the IFNγR α subunit phosphorylation and recruitment of STAT1.

Requirements for the antiviral response

The above model represents only the simplest interpretation of the data, and the failure of the JAK1 mutants to sustain an antiviral response to IFN-γ raises the possibility of an additional layer of complexity. Which of the multiple antiviral mechanisms (Lengyel, 1982; Staeheli, 1990) operates in the IFN-γ-mediated inhibition of EMC virus and SFV replication in the cells used here is not known. For example, 2–5A synthetase mRNA is poorly induced in response to IFN-γ in wild-type cells (e.g. Figure 2B) and the maximum inhibition of EMC virus yield was substantially less for IFN-γ (30- to 100-fold) than IFN-α (~10^3 fold), emphasizing that different subsets of mechanisms may be activated by the two types of IFN in these cells. The important point here, however, is not the nature of the antiviral state, but the possible requirement for a JAK1-dependent signal in addition to STAT1 activation for the development of a full IFN-γ-mediated biological response. That said, an antiviral state is not observed in the absence of STAT1 or STAT1 activation in U1A, U3A, U4A, γ1A and γ2A cells (Pellegrini *et al.*, 1989; John *et al.*, 1991; Velazquez *et al.*, 1992; N.C.Rogers, J.Briscoe, D.Watling and I.M.Kerr, in preparation). STAT1 activation is, therefore, absolutely required for the antiviral state in response to both type I and type II IFNs. The results here suggest that it may not be sufficient. The data for U4A/JAK1.KE cells do not exclude a requirement for a high (threshold) level of STAT1 activation for the induction of one or more IFN-γ-inducible genes essential for the antiviral response. The inhibition of the antiviral response in wild-type cells by the kinase-negative JAK1 (Figure 9) without any detectable inhibition of the 'classical' JAK/STAT1 pathway (Figures 7 and 8) argues against this hypothesis and for a requirement for an additional JAK1-dependent signal. The alternative possibility that the antiviral state is exquisitely sensitive to any reduction in the JAK/STAT1 pathway can essentially be excluded by the fact that, despite significant JAK/STAT inhibitory activity, on introduction into wild-type cells the kinase-negative JAK2 is less effective than the kinase-negative JAK1 in the inhibition of the antiviral state (data not shown). Soh *et al.* (1994) have, however, also argued for complexity in the IFN-γ response from work showing a differential inducibility of the HLAs and antiviral states for EMC virus and vesicular stomatitis virus in response to human IFN-γ in hamster cells carrying different human chromosome fragments and IFNγR subunits. In addition, it is intriguing that tyrosine phosphorylation of the kinase-negative JAK1 in U4A/JAK1.KE cells is low (Figure 3B, lane 7) compared with that of endogenous JAK1 in wild-type cells (lane 1) or transfected JAK1 in the U4A/JAK1 cells (lane 5). This could reflect decreased tyrosine phosphorylation of a site(s) on JAK1 essential for the docking of a protein(s) required for induction of the antiviral state. Whether this is the case and whether the additional signal suggested here operates through a unique modification of STAT1, the activation of a novel STAT or an alternative transcription factor, or even through the enzymic modification of pre-existing 'antiviral proteins' remains to be established, as does the role for JAK1 in mediating such a signal, either directly or through other kinases or adaptor molecules.

Materials and methods

Cell culture, DNA transfections and selections

Parental and mutant cells were derived as described previously (Pellegrini *et al.*, 1989; McKendry *et al.*, 1991; Watling *et al.*, 1993). Cells were grown in Dulbecco's modification of Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum and 5 μM L-glutamine. Hygromycin-resistant cells were maintained in medium containing 250 μg/ml hygromycin, G418-resistant ($G418^R$) cells in 400 μg/ml G418 and puromycin-resistant cells in 0.5 μg/ml puromycin. IFN-α was a highly purified mixture of human subspecies [Wellferon, 1.5×10^8 IU/mg protein (Allen *et al.*, 1982), provided by Wellcome Research Laboratories, Beckenham, Kent, UK]. Recombinant IFN-γ (4×10^7 IU/mg protein) was supplied by Dr G.Adolf, Ernst Boehringer Institut für Arzneimittelforschung, Vienna, Austria. IL-6 treatments were carried out using a mixture of IL-6 (400 ng/ml) and soluble IL-6 receptor α chain (500 ng/ml).

Plasmids and DNA transfections

The murine and human JAK1 and murine JAK2 expression constructs have been described previously (Müller *et al.*, 1993a; Silvennoinen *et al.*, 1993). Kinase-negative versions were generated by mutating the lysine (amino acid number 833) to glutamate in murine JAK1 (JAK1.KE), the aspartate (aa 1020) to serine in the human JAK1 (JAK1.SFG) and the lysine (aa 882) to glutamate in the murine JAK2 (JAK2.KE). Cells stably expressing these constructs were obtained by co-transfection with the drug resistance plasmids pRc/CMV (Invitrogen), pSV2.hyg (Promega) and pBABE.puro (a gift of H.Land) for G418, hygromycin and puromycin selections respectively. DNA transfections using calcium phosphate–DNA precipitates were performed according to standard protocols (Ausubel *et al.*, 1992).

Antibodies

Rabbit polyclonal antisera were raised against the JH2 domain of JAK1 (amino acids 565–866, Wilks *et al.*, 1991) and the JH1 domain of JAK2 (A.Ziemiecki, unpublished). The polyclonal antibodies to STAT1 were as previously described (Fu, 1992; Schindler *et al.*, 1992a,b). The IFNγR α subunit antibody was a mouse monoclonal from Genzyme. A mixture of 4G10 (UBI) and PY20 (ICN) anti-phosphotyrosine monoclonal antibodies were used throughout. Peroxidase-conjugated rabbit anti-mouse and goat anti-rabbit secondary antibodies were from Amersham.

Immunoprecipitation, SDS–PAGE and Western blotting

Immunoprecipitations were carried as described previously (Guschin *et al.*, 1995). Briefly, cells were lysed in ice-cold lysis buffer [0.5% NP-40 or Triton X-100, 10% glycerol, 50 mM Tris–HCl pH 8.0, 200 mM NaCl, 0.1 mM EDTA, 1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 3 μg/ml aprotinin, 1 μg/ml leupeptin] (Schindler *et al.*, 1992b). Nuclei were removed by centrifugation and supernatants pre-cleared with pre-immune serum and protein A–Sepharose for 1 h. Appropriate antibodies and protein A–Sepharose were added and incubated for 1–18 h at 4°C. The immunoprecipitates were washed in ice-cold cell lysis buffer and in phosphate-buffered saline. Proteins were separated on 7% polyacrylamide–SDS gels (Laemmli, 1970) and transferred electrophoretically to Immobilon™ PVDF (Millipore) membranes. Membranes were blocked with 5% BSA (fraction V) in TBST (10 mM Tris–HCl pH 7.4, 75 mM NaCl, 1 mM EDTA, 0.1% Tween 20) with 1 mM sodium orthovanadate for 1 h and subsequently incubated with relevant primary antibody for 30 min, washed in TBST and incubated for 30 min with peroxidase-conjugated secondary antibody. The membranes were washed in TBST and exposed to enhanced chemiluminescence (ECL) (Amersham Life Science) followed by fluorography (Kodak AR) or 3,3′-diaminobenzidine tetrahydrochloride (DAB) detection. After anti-phosphotyrosine immunoblotting, membranes were stripped in 0.1 M glycine pH 2.5 for 1 h, neutralized in 1 M Tris–HCl pH 8.0 and incubated with appropriate primary and secondary antibodies.

In vitro kinase assays

JAK1 was immunoprecipitated as described above, washed once in lysis buffer and twice in 50 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1 mM Na_3VO_4, 10 mM HEPES pH 7.4 and *in vitro* kinase assays performed in the same buffer by incubation at room temperature for 30 min in the presence of 0.25 mCi/ml [γ-^{32}P]ATP. Complexes were washed and proteins eluted in sample buffer and separated by SDS–PAGE (7%). ^{32}P-Labelled proteins were detected by autoradiography.

Electrophoretic mobility shift assays

Whole cell extracts were prepared by lysis of cells in 0.5% NP-40, 10% glycerol, 50 mM Tris–HCl pH 8.0, 200 mM NaCl, 0.1 mM EDTA, 1 mM sodium orthovanadate, 0.5 mM PMSF, 1 mM DTT, 3 μg/ml aprotinin, 1 μg/ml leupeptin. The sequence of the oligonucleotide probe used corresponded to the high-affinity SIE (hSIE) of the c-*fos* gene (SIEM67) GTCGACATTTCCCGTAAATC (Sadowski *et al.*, 1993). Probes were end-labelled with [γ-^{32}P]ATP and aliquots equivalent to ~30 000 c.p.m. used per reaction. Binding reactions were performed in a total volume of 20 μl, in 10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 5% glycerol, 1 mg/ml BSA, 0.125 mg/ml $pd(N)_5$, 0.25 mg/ml tRNA, 2% Ficoll. Extracts were pre-incubated for 10 min at room temperature with 150 μg/ml poly(dI–dC·dI–dC) prior to incubation with probe for an additional 15 min at room temperature. Complexes were separated on 6% non-denaturing acrylamide gels in 0.5% TBE, and detected by autoradiography.

RNase protection assay

Total cellular RNA was prepared from monolayer cells by NP-40 lysis and phenol–chloroform extraction (Porter *et al.*, 1988). RNase protections (Melton *et al.*, 1984) were performed using probes (Müller *et al.*, 1993b) synthesized from SP6/T7 transcription vectors. Probes were labelled with [^{32}P]UTP to a specific activity of $2–5\times10^8$ c.p.m. per μg of input DNA. Aliquots equivalent to $\sim1–3\times10^5$ c.p.m. of each probe and 10 μg of cytoplasmic RNA were used in each assay.

Antiviral assays

Cells were seeded at a density of 5×10^4/ml, treated with IFN-γ at 2–50 000 IU/ml for 24–65 h, infected with SFV or EMC virus at 0.3–0.5 p.f.u./cell and, at 24–30 h post-infection, fixed and stained with Giemsa to assay for cytopathic effects of the virus or freeze–thawed and the supernatants assayed for virus yield by serial dilution on control cells. The dilutions at which a 50% cytopathic effect was observed were used to calculate the viral titre by comparison with EMC virus of known titre diluted in parallel.

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