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A major role for the protein tyrosine kinase JAK1 in the JAK/STAT signal transduction pathway in response to interleukin-6

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The protein tyrosine kinases JAK1, JAK2 and Tyk2 and STATs (signal transducers and activators of transcription) 1 and 3 are activated in response to interleukin-6 (IL-6) in human fibrosarcoma cells. In mutant cells lacking JAK1, JAK2 or Tyk2, the absence of one kinase does not prevent activation of the others; activation does not, therefore, involve a sequential three-kinase cascade. In the absence of JAK1, the phosphorylation of the gp130 subunit of the IL-6 receptor and the activation of STATs 1 and 3 are greatly reduced. JAK1 is also necessary for the induction of IRF1 mRNA, thus establishing a requirement for the JAK/STAT pathway in the IL-6 response. JAK2 and Tyk2 although activated cannot, in the absence of JAK1, efficiently mediate activation of STATs 1 and 3. A kinase-negative mutant of JAK2 can, however, inhibit such activation, and ancillary roles for JAK2 and Tyk2 are not excluded. A major role for JAK1 and the non-equivalence of JAK 1 and JAK2 in the IL-6 response pathway are, nevertheless, clearly established for these cells.

Key words: cytokines/JAKs/mutants/signal transduction/STATs

Introduction

The JAK (Janus kinase) family of protein tyrosine kinases and the STATs (signal transducers and activators of transcription) constitute a novel type of signal transduction pathway activated in response to a wide variety of polypeptide ligands. Activation of the STATs by the JAKs occurs in receptor complexes at the cell membrane (reviewed in Darnell *et al.*, 1994). The JAK family of non-receptor protein tyrosine kinases has four known members: JAK1 (Wilks *et al.*, 1991), 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 in mass and has a C-terminal protein tyrosine kinase domain, an adjacent kinase-related domain and five further domains with amino acid similarity between members of the family extending towards the N-terminus (Harpur *et al.*, 1992). Different cytokines activate different JAKs and STATs. JAK activation on receptor dimerization in response to ligand involves auto- or cross-phosphorylation and leads to phosphorylation of the receptors and to the recruitment/activation of subsets of STATs, presumably through their SH2 domains. STATs 1–6 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). Each contains an SH2 and putative SH3 domain. Activation of STATs 1 and 2 in response to the interferons (IFNs) involves the phosphorylation of a conserved tyrosine just C-terminal to the SH2 domain (Schindler *et al.*, 1992b; Shuai *et al.*, 1993; Improta *et al.*, 1994). Similar activation of the other STATs is likely with subsequent homo- or heterodimerization (Shuai *et al.*, 1994) yielding, with or without additional polypeptides, a spectrum of transcription factors with differing affinities for different DNA response elements.

Interleukin-6 (IL-6) is a major cytokine required for B- and T-cell differentiation and the acute phase response (reviewed in Heinrich *et al.*, 1990; Kishimoto *et al.*, 1994). It mediates the induction of acute phase proteins and a number of immediate early response genes, including junB, ICAM-1 and IRF1 (Lord *et al.*, 1991; Nakajima and Wall, 1991; Yuan *et al.*, 1994). The IL-6 receptor comprises a gp80 ligand binding α subunit and a signal transduction β subunit, gp130, which is shared with the receptors for LIF, OSM, CNTF and IL-11 (Kishimoto *et al.*, 1994). IL-6 receptor binding induces gp130 homodimerization, while LIF, OSM and CNTF induce gp130 heterodimerization with a second β chain LIFRβ. In response to ligand, these receptors activate a number of signalling pathways, including those which utilize JAKs and STATs (reviewed in Mackiewicz *et al.*, 1992; Kishimoto *et al.*, 1994; Rosejohn and Heinrich, 1994). More specifically, IL-6 activates JAK1, JAK2 and Tyk2 (Lutticken *et al.*, 1994; Stahl *et al.*, 1994), and STAT1 and STAT3 (initially characterized as the acute phase response factor APRF; Wegenka *et al.*, 1993; Yuan *et al.*, 1994). STATs 1 and 3 recognize a family of DNA response elements (Yuan *et al.*, 1994) termed the GAS (gamma activatable sequence) elements. Different homo- and heterodimeric STATs have very different affinities for different GAS elements. Activation of a particular gene will therefore depend, in part at least, on the activation of the appropriate combination of STATs.

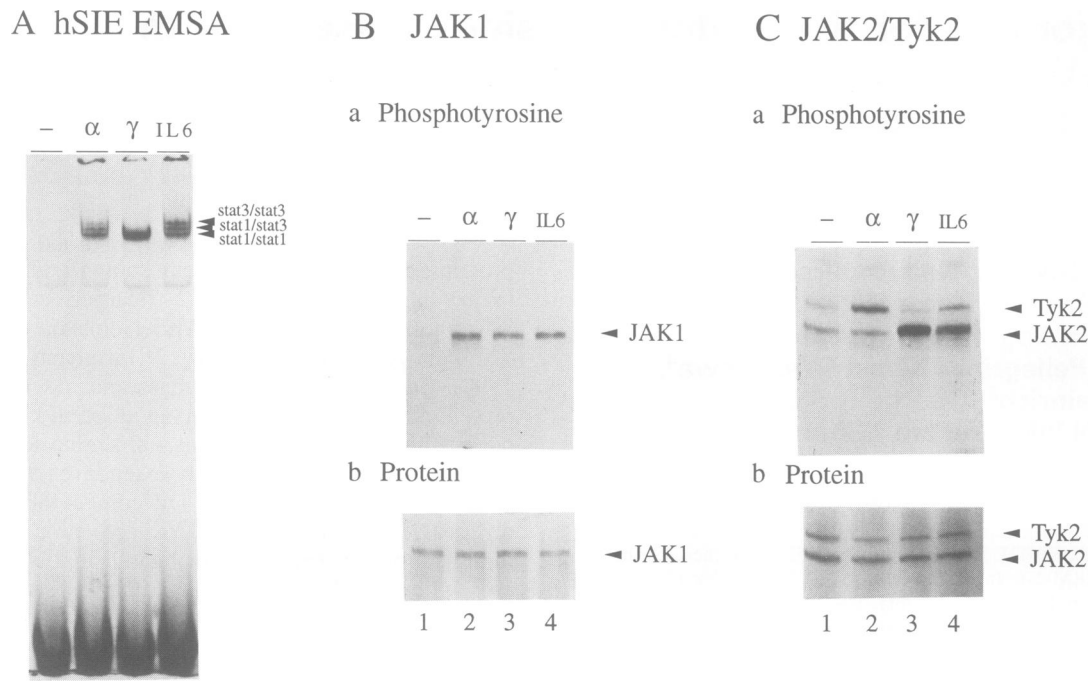

Fig. 1. IL-6 response in the human fibrosarcoma cell line 2fTGH. (**A**) IFN-α, IFN-γ and IL-6-induced GAS binding activity. EMSA using whole-cell extracts from 2fTGH with or without treatment for 15 min at 37°C incubated with a 20 bp oligodeoxynucleotide probe containing the hSIE (Materials and methods). DNA–protein complexes were separated by PAGE (6%). (**B** and **C**) IFN- and IL-6-induced phosphorylation of JAK1, JAK2 and Tyk2. Whole-cell extracts from 2fTGH with or without treatment for 15 min at 37°C were immunoprecipitated with rabbit polyclonal antisera to (B) JAK1 and (C) JAK2 and Tyk2, and probed after SDS–PAGE (7%) analysis with (a) a mixture of anti-phosphotyrosine antibodies and after stripping (b) antisera to JAK1 or Tyk2 and JAK2.

The IL-6 gp130 receptor subunit appears unusual in mediating activation of JAK1, JAK2 and Tyk2 compared with other cytokine receptor subunits which, to the extent that they have been characterized, each appear to interact primarily with only one JAK family member. It is not yet known whether the JAKs directly phosphorylate the STATs in mammalian cells. However, transfected JAK1, JAK2 and Tyk2 expressed in a baculovirus system each activate STAT1 by phosphorylation on Y^{701} with comparable efficiency (F.Quelle, B.Witthuhn and J.N.Ihle, in preparation) and interaction between STAT SH2 groups and phosphorylated receptor subunits probably plays a significant role in STAT selection (Greenlund *et al.*, 1994; Hou *et al.*, 1994). Against this background, it seemed *a priori* possible that JAK1, JAK2 and Tyk2 might be essentially interchangeable in the IL-6 system. To investigate this, use was made of mutant human HT1080 cells lacking JAK1, JAK2, Tyk2 or STAT1. The mutant cells were those previously isolated and characterized in the analysis of the IFN response pathways (Pellegrini *et al.*, 1989; McKendry *et al.*, 1991; Watling *et al.*, 1993) where they revealed both a requirement for, and an interdependence of, JAK1 and Tyk2 in the IFN-α, and of JAK1 and JAK2 in the IFN-γ responses (Müller *et al.*, 1993a; Watling *et al.*, 1993). The cells express the gp130 subunit of the IL-6 receptor and respond to IL-6 in the presence of the sIL-6R. Accordingly, here they were used, together with a dominant negative mutant of JAK2, to analyse the roles of the individual JAKs and STAT1 in the JAK/STAT pathway(s) in response to IL-6.

Results

The JAK/STAT pathway in response to IL-6 in human fibrosarcoma cells

JAK1, JAK2, Tyk2 and STATs 1 and 3 are all activated in response to IL-6 in rat liver and a variety of cell lines (Wegenka *et al.*, 1993; Lutticken *et al.*, 1994). The same is true for the wild-type HT1080 human fibrosarcoma-derived cell lines used here (Figure 1). These cells express the gp130, but not the gp80 IL-6 receptor subunits (F.Horn, unpublished). Treatment was, therefore, routinely with a combination of IL-6 and sIL-6R which elicits activation of the JAKs and STATs to levels comparable (with the possible exception of Tyk2) with those observed in response to one or other of the IFNs (Figure 1). In electrophoretic mobility shift assays (EMSAs) for STAT activity, three complexes are routinely observed in response to IL-6 (Figure 1A). These correspond to the STAT1 and STAT3 homodimers which migrate fastest and slowest, respectively, and to the intermediate STAT1/3 heterodimer (Figure 1A). The identity of these complexes was confirmed by appropriate supershifts in the presence of antibodies specific for STAT1 or 3 (data not presented). Although significantly higher levels of IL-6 (in combination with sIL-6R) are required to elicit an optimal response, activation of the JAKs and STATs in the HT1080-derived cell lines occurs to comparable levels and with essentially identical kinetics compared with typical IL-6-responsive HepG2 liver cells (J.Briscoe, D.Guschin and I.M.Kerr, unpublished).

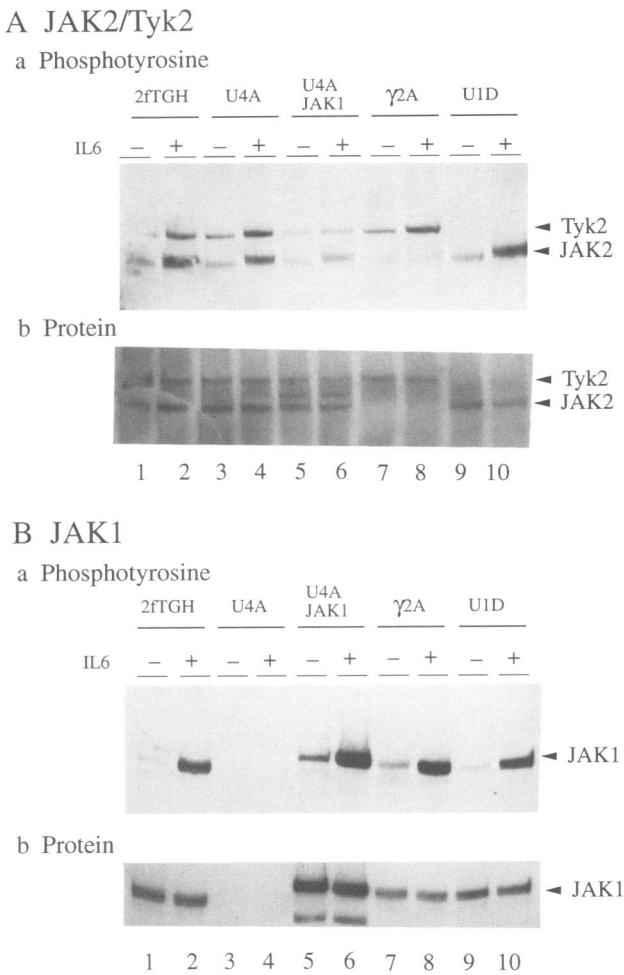

Fig. 2. IL-6-induced tyrosine phosphorylation of JAK1, JAK2 and Tyk2 in parental and mutant cell lines. Whole-cell extracts from the indicated cell lines, with or without treatment for 15 min at 37°C with IL-6 and sIL-6R (Materials and methods), were immunoprecipitated with rabbit polyclonal antisera to (**A**) Tyk2 and JAK2 and (**B**) JAK1, probed after SDS−PAGE (7%) analysis with (a) a mixture of anti-phosphotyrosine antibodies and after stripping (b) antisera to Tyk2 and JAK2 or JAK1.

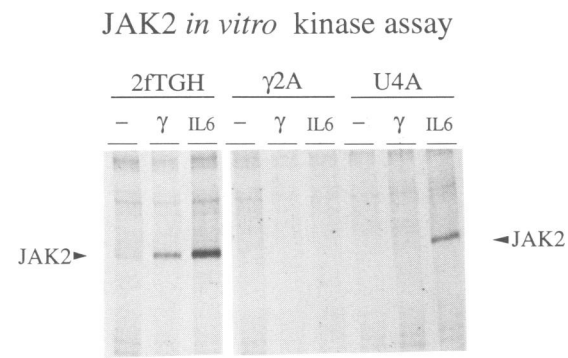

Fig. 3. *In vitro* kinase assays. IFN-γ- and IL-6-dependent kinase activity of immunoprecipitated JAK2. Treatment of 2C4, γ2A and U4A cells was with 1000 IU/ml of IFN-γ or IL-6 and sIL-6R for 15 min at 37°C. Incorporation of ^{33}P from [γ-^{33}P]ATP was assayed by SDS−PAGE (7%) (Materials and methods).

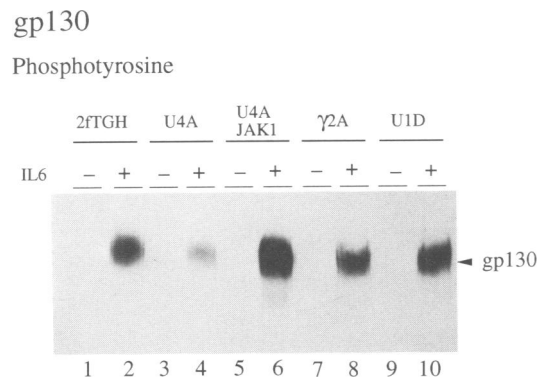

Fig. 4. IL-6-induced tyrosine phosphorylation of gp130 in parental and mutant cell lines. Whole-cell lysates from the indicated cell lines with or without treatment for 15 min at 37°C with IL-6 and sIL-6R (Materials and methods) were immunoprecipitated with a mouse monoclonal antibody to gp130 and after SDS−PAGE (7%) probed with a mixture of anti-phosphotyrosine antibodies. The antibody to gp130 is not suitable for Western analyses excluding parallel controls for protein levels. Similar results, however, were obtained in a number of independent experiments.

The absence of one JAK family member does not prevent activation of the others by IL-6

The HT1080-derived 2fTGH and 2C4 parental and mutant U1D, U3A and U4A cell lines which lack Tyk2, STAT1 and JAK1, respectively, have been described previously (Pellegrini *et al.*, 1989; McKendry *et al.*, 1991; Müller *et al.*, 1993a). Mutant γ2A cells were derived from 2C4s in parallel with the γ1A mutants (Watling *et al.*, 1993); they lack JAK2 mRNA and protein, and will be described in detail elsewhere (N.Rogers, D.Watling and I.M.Kerr, in preparation). JAK1, JAK2 and Tyk2 are rapidly phosphorylated on tyrosine in response to IL-6 in the parental cell lines (e.g. lane 2, Figure 2A and B). The phosphorylation of Tyk2 was, however, routinely the least obvious (cf. Figure 1C). Preferential activation of two of the three kinases has previously been noted for several cell lines and may reflect the dimeric nature of the activated receptor (Stahl *et al.*, 1994). In mutant U4A(JAK1$^-$), γ2A(JAK2$^-$) and U1D(Tyk2$^-$) cells, there is normal tyrosine phosphorylation of JAK2 and Tyk2 (lane 4), JAK1 and Tyk2 (lanes 8), and JAK1 and JAK2 (lanes 10), respectively (Figure 2A and B). The absence of one kinase does not, therefore, prevent activation of the other two. Overexpression of transfected JAK1 in U4A/JAK1 transfectants, however, in addition to restoring the JAK1 response (lane 6, Figure 2B) also inhibited the phosphorylation of JAK2 and Tyk2 (lane 6, Figure 2A), consistent with competition between the JAKs for interaction with the receptor (Discussion).

A priori correct assembly of receptor complexes and activation of the JAKs could be STAT dependent. There is, however, no such dependence on STAT1 at least. Phosphorylation of the JAKs in response to IL-6 is normal in the complete absence of STAT1 in mutant U3A cells (data not presented).

Activation of JAK2 by IL-6 in mutant U4A cells lacking JAK1 was confirmed by *in vitro* kinase assay in which autophosphorylation of immunoprecipitated JAKs is observed on incubation with [γ-^{33}P]ATP (Figure 3). In contrast, JAK2 was not activated by IFN-γ in these cells. This latter reflects the interdependence of the JAK1 and JAK2 kinases in the IFN-γ response pathway, in which

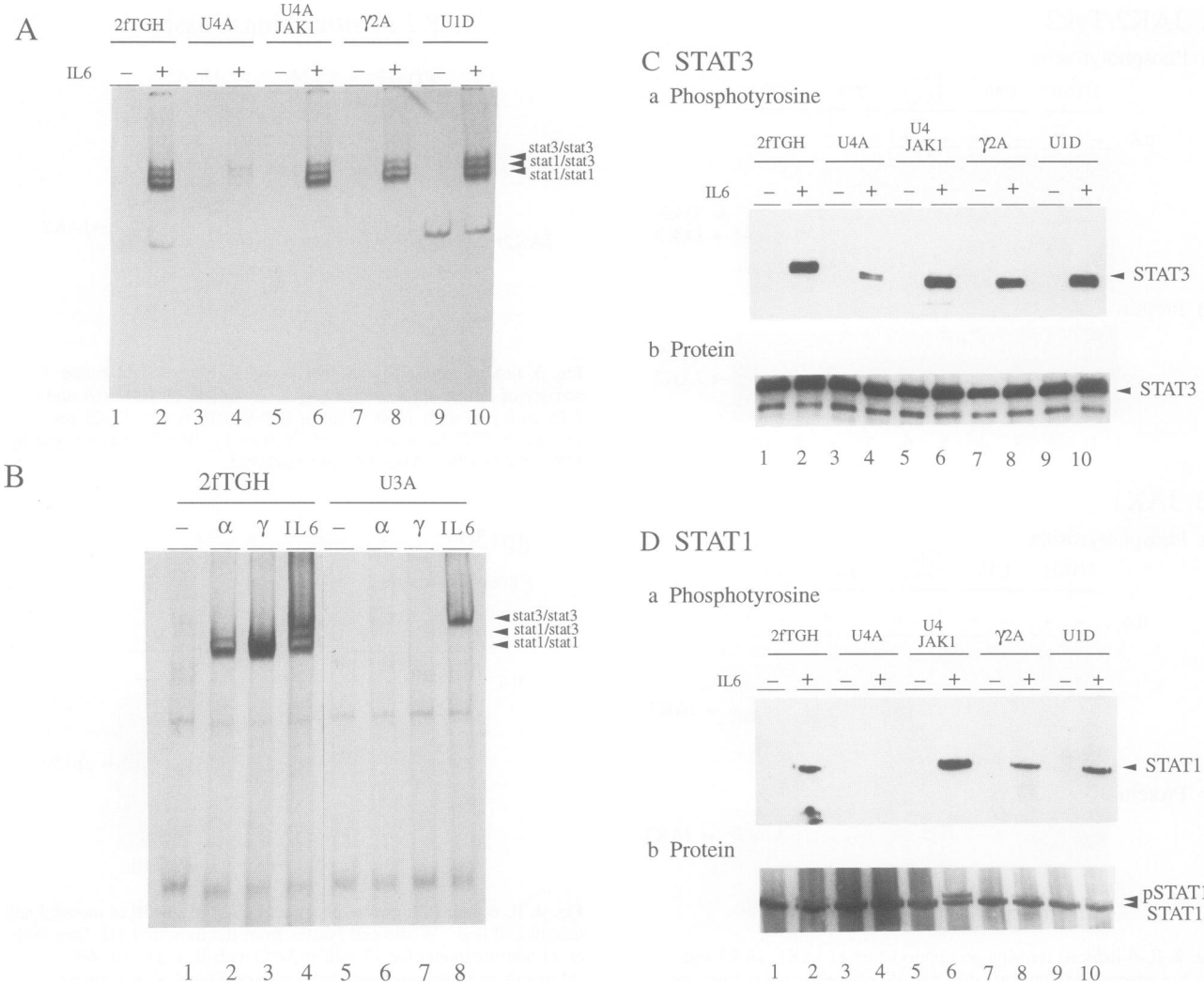

Fig. 5. IL-6-induced phosphorylation of STAT1 and STAT3. (**A** and **B**) EMSAs with whole-cell extracts from IL-6-treated parental and mutant cell lines using an hSIE probe (Materials and methods). DNA–protein complexes were separated by PAGE (6%). For high-resolution autoradiography, the probe was labelled with [γ-^{33}P]ATP (Materials and methods). (**C** and **D**) Phosphorylation of STAT1 and STAT3. Whole-cell extracts were immunoprecipitated with rabbit polyclonal antisera to (C) STAT3 and (D) STAT1. After SDS–PAGE (7%) analysis, they were probed with (a) a mixture of anti-phosphotyrosine antibodies and after stripping (b) antisera to STAT3 (C) or STAT1 (D). (A–D) Treatment of cells with IL-6 and sIL-6R (Materials and methods) was for 15 min at 37°C. The doublet observed for STAT3 is thought to reflect different constitutive levels of serine/threonine phosphorylation.

the absence of one kinase prevents the activation of the other (Müller *et al.*, 1993a).

JAK1 plays a major role in the phosphorylation of the gp130 subunit of the IL-6 receptor

The JAKs are constitutively associated with the IL-6 gp130 receptor subunit, which is known to be phosphorylated in response to IL-6 (Lutticken *et al.*, 1994; Stahl *et al.*, 1994). Consistent with this, this subunit was rapidly phosphorylated in response to IL-6 in the wild-type 2fTGH cells (lane 2, Figure 4A). Such phosphorylation was greatly reduced in the absence of JAK1 (U4A cells, lane 4), but was unimpaired in the absence of JAK2 or Tyk2 (lanes 8 and 10, Figure 4A). It appears, therefore, that JAK1 plays a major role in mediating the phosphorylation of the gp130 receptor subunit in response to IL-6.

STAT activation and induction of IRF1 mRNA by IL-6 are dependent predominantly on JAK1

STAT activation in the wild-type and mutant cells was monitored by EMSAs (Figure 5A and B) and tyrosine phosphorylation (Figure 5C and D) with essentially identical results. The activation of both STAT1 and STAT3 is profoundly inhibited in the absence of JAK1 in U4A cells (lane 4, Figure 5A, C and D) despite good activation of JAK2 in these cells (Figure 3). The absence of JAK2 or Tyk2 was, however, without significant effect on the activation of STATs 1 or 3 (e.g. γ2A and U1D cells; lanes 8 and 10, Figure 5A). The absence of STAT1 in U3A cells (Müller *et al.*, 1993b) was without effect on the activation of STAT3 (Figure 5B).

The induction of IRF1 mRNA in response to IL-6 in wild-type and mutant cells was assayed by RNase protection (Figure 6). The induction is a primary response which

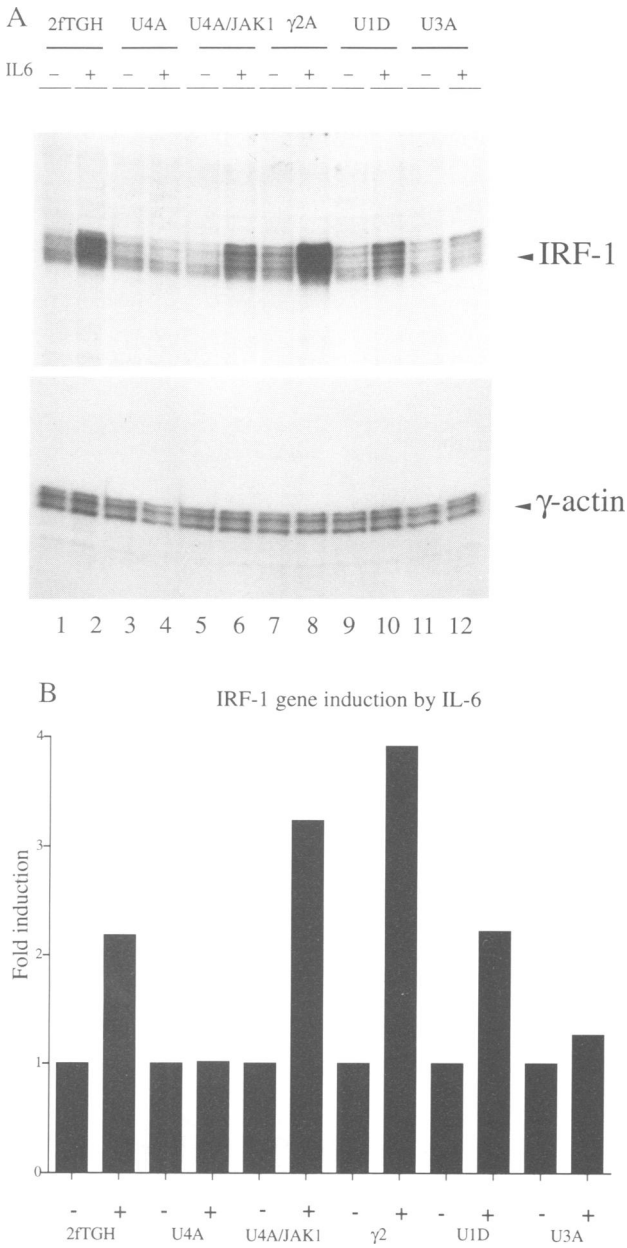

Fig. 6. Analysis of IL-6-dependent expression of IRF1 in parental and mutant cell lines by RNase protection. (**A**) 10 μg aliquots of cytoplasmic RNA from the indicated cell lines treated where indicated for 1 h with IL-6 and sIL-6R or untreated were analysed with a probe for IRF1 and for γ-actin (Materials and methods). (**B**) Quantitation: average of data from three analyses of the type shown in (A) obtained by scanning the gels with a PhosphorImager. All values were corrected versus the actin control.

is first clearly detectable after ~30 min and peaks within 1–2 h (J.Briscoe, data not shown). There is no such induction in mutant U4A cells (lane 4, Figure 6A and B). Induction of IRF1 mRNA is, therefore, dependent on JAK1. It is unaffected by the absence of Tyk2 (lane 10). It was consistently enhanced in the γ2A cells which lack JAK2 (lane 8); the basis for this is not yet certain. It can be concluded that in the context of the IL-6 gp130 receptor complex JAK1, but not JAK2 or Tyk2, can efficiently mediate the activation of STATs 1 and 3, and the induction of IRF1 mRNA.

Both STAT1 and STAT3 can form EMSA complexes with the IRF1 GAS element (Harroch *et al.*, 1994; Yuan *et al.*, 1994). Activation of either might, therefore, be expected to mediate the induction of IRF1 mRNA. Despite activation of STAT3 to wild-type levels (Figure 5B), the induction of IRF1 mRNA in response to IL-6 is much reduced in the absence of STAT1 in mutant U3A cells (lane 12, Figure 6A and B). The induction of IRF1 mRNA in this system, therefore, appears largely dependent on JAK1 and STAT1.

A kinase-negative mutant of JAK2 can function as a dominant negative inhibitor of the IL-6 response

A kinase-negative mutant of JAK2 (JAK2KE) was made in an appropriate expression construct by replacing the highly conserved lysine (K) in motif II of the tyrosine kinase domain with glutamic acid (E). The mutant, in contrast to wild-type JAK2, is inactive when overexpressed in a baculovirus system and is without inducible *in vitro* kinase activity when expressed in the γ2A(JAK2$^-$) human cells. Nor does it complement the IFN-γ response in these cells (a detailed characterization of the mutant kinase will be published elsewhere; B.Witthuhn, N.Rogers, I.M.Kerr and J.N.Ihle). γ2A cells, which lack JAK2, were stably transfected either with wild-type or mutant JAK2. Individual clones expressing high levels (Figure 7B, lanes 7–12) of the transfected wild-type or mutant JAK2 (γ2A/JAK2 and γ2A/JAK2KE) were selected and analysed for JAK/STAT activation, the phosphorylation of gp130, and the induction of IRF1 mRNA in response to IL-6 and IFN-γ. A dominant negative effect of the kinase-negative mutant was observed in all cases.

Activation of the JAKs and STATs was monitored by tyrosine phosphorylation (Figure 7A and B). STAT activation was also assayed by EMSA using a GAS probe with essentially identical results (data not shown). As expected, activation of STAT1 by IFN-γ was high in the parental 2C4 cells, absent in γ2A cells and restored by the wild-type but not the kinase-negative mutant of JAK2 (lanes 2, 5, 8 and 11, Figure 7A), consistent with a lack of kinase activity for the mutant JAK2KE. As the IL-6 response through JAK1 is retained in the JAK2-negative γ2A cells, it was possible to assay for a dominant negative effect of the JAK2KE mutant on the IL-6 response in these cells. Consistent with such an effect, the activations of JAK1, STAT1 and STAT3 were all substantially inhibited in the γ2a cells overexpressing the mutant JAK2KE (lane 12, Figure 7A and B) compared with the untransfected γ2A cells or the wild-type 2C4 cells (lanes 6 and 3, respectively, Figure 7A and B). An inhibition of the phosphorylation of the gp130 subunit of the IL-6 receptor was also observed in the mutant JAK2KE-expressing cells (lane 9, Figure 8A). The dominant negative inhibitory effect of the kinase-negative mutant is not restricted to γ2A cells, or to the IL-6 response. It is also seen, for example, on overexpression of the mutant JAK2KE in the parental 2C4 cells and in response to IFN-γ (Figure 7C). The phosphorylation of JAK2 and STAT1 in reponse to IFN-γ or IL-6 is much reduced in the presence of the overexpressed mutant JAK2KE in these cells (Figure 7C), as is the phosphorylation of JAK1, Tyk2 and STAT3, and the induction of IRF1 mRNA (data not presented).

Interestingly, overexpression of wild-type JAK2, in

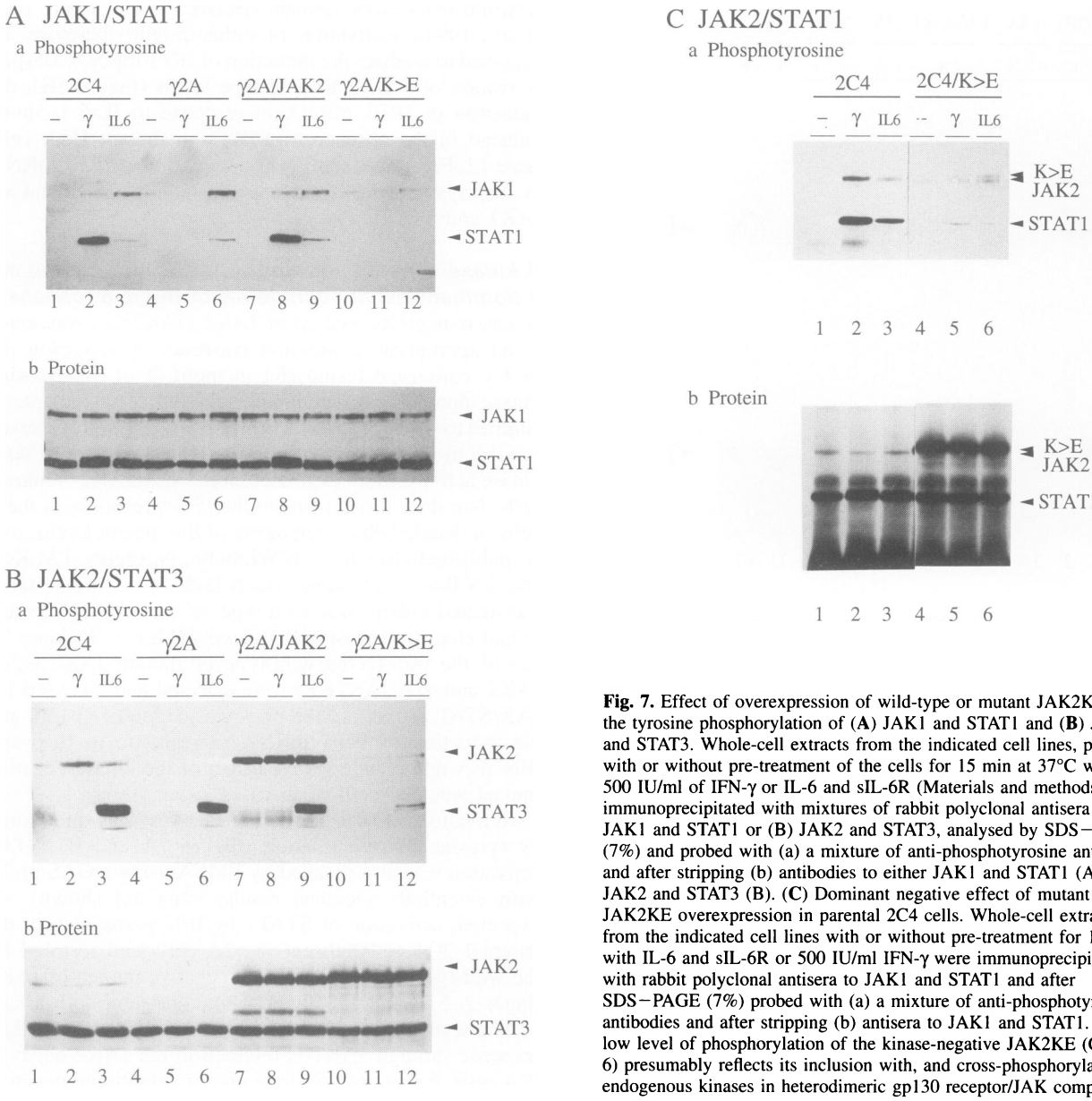

Fig. 7. Effect of overexpression of wild-type or mutant JAK2KE on the tyrosine phosphorylation of (**A**) JAK1 and STAT1 and (**B**) JAK2 and STAT3. Whole-cell extracts from the indicated cell lines, prepared with or without pre-treatment of the cells for 15 min at 37°C with 500 IU/ml of IFN-γ or IL-6 and sIL-6R (Materials and methods), were immunoprecipitated with mixtures of rabbit polyclonal antisera to (A) JAK1 and STAT1 or (B) JAK2 and STAT3, analysed by SDS–PAGE (7%) and probed with (a) a mixture of anti-phosphotyrosine antibodies and after stripping (b) antibodies to either JAK1 and STAT1 (A) or JAK2 and STAT3 (B). (**C**) Dominant negative effect of mutant JAK2KE overexpression in parental 2C4 cells. Whole-cell extracts from the indicated cell lines with or without pre-treatment for 15 min with IL-6 and sIL-6R or 500 IU/ml IFN-γ were immunoprecipitated with rabbit polyclonal antisera to JAK1 and STAT1 and after SDS–PAGE (7%) probed with (a) a mixture of anti-phosphotyrosine antibodies and after stripping (b) antisera to JAK1 and STAT1. The low level of phosphorylation of the kinase-negative JAK2KE (C, lane 6) presumably reflects its inclusion with, and cross-phosphorylation by, endogenous kinases in heterodimeric gp130 receptor/JAK complexes.

contrast to kinase-negative JAK2, was without inhibitory effect on STAT1 and 3 activation (lane 9, Figure 7A and B) or on gp130 phosphorylation (lane 6, Figure 8A) in response to IL-6 in γ2A cells. In these circumstances, therefore, enzymically active but not mutant JAK2 can sustain a response. Even overexpressed JAK2 cannot, however, sustain a response in the absence of JAK1 (Figure 8B and C). This suggests that JAK2 may be able to activate JAK1 and/or, for example, that the kinase-negative JAK2KE mutant may sequester JAK1 in inactive receptor complexes (Discussion).

Discussion

The tyrosine kinases JAK1, JAK2 and Tyk2 are constitutively associated with the gp130 IL-6 receptor subunit (Lutticken *et al.*, 1994; Stahl *et al.*, 1994) and are activated on receptor dimerization in response to IL-6 (Figure 1). The absence of one kinase does not prevent the activation of the others (Figures 2 and 3). Activation does not, therefore, involve an obligatory three-kinase cascade. Of the three kinases, only the absence of JAK1 has a significant inhibitory effect on gp130 phosphorylation, the activation of STATs 1 and 3, and the induction of IRF1 mRNA in these cells (Figures 4, 5 and 6). A kinase-negative mutant of JAK1 does not restore the response (data not presented). Active JAK1, not just the physical presence of JAK1, is therefore required. In each of the available mutants, however, two of the three kinases are activated (Figure 2) and interchangeability of function in a dimeric receptor cannot be excluded. It remains, therefore, to be established whether JAK1 alone is sufficient to confer a response. Indeed, a number of models can be invoked. One such model is that JAK1 associates with gp130 and triggers downstream events, but requires either JAK2 or Tyk2 for efficient activation on ligand-induced dimerization of the receptor complex. *A priori*, JAK2 and Tyk2 could associate with the same or a distinct region of the receptor from JAK1 or even with JAK1. Consistent with the above model, when overexpressed in the presence

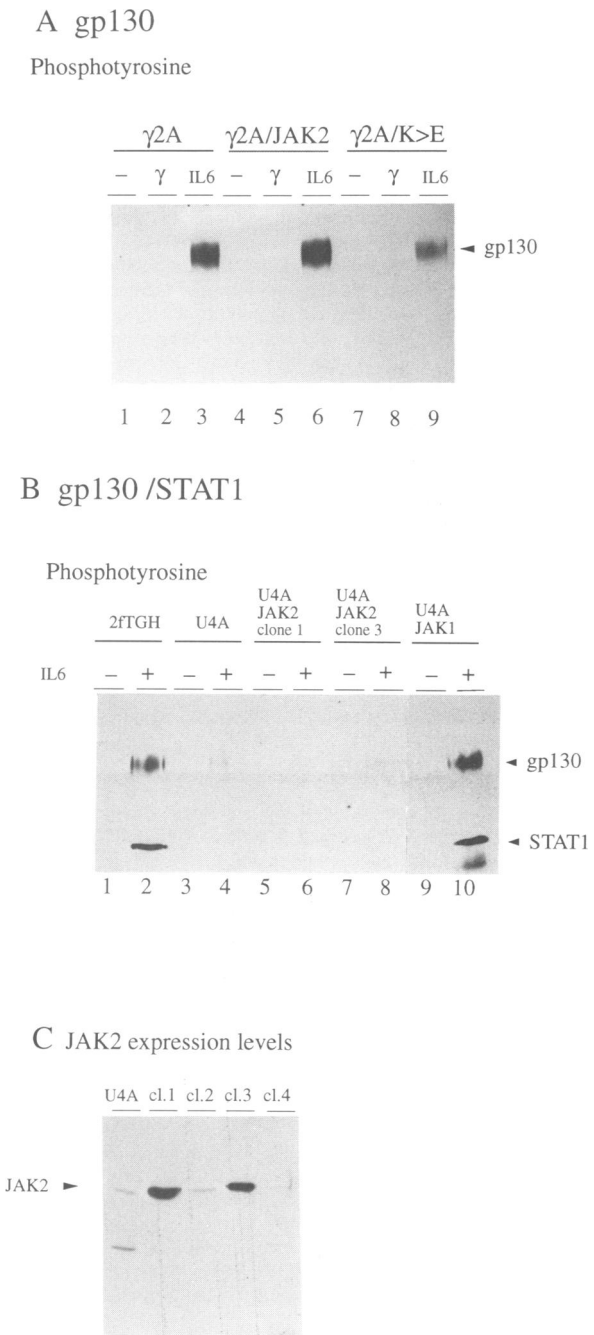

Fig. 8. Wild-type but not mutant JAK2 sustains an IL-6 response in the presence (**A**) but not the absence (**B**) of JAK1. Whole-cell lysates from the indicated cell lines with or without treatment for 15 min at 37°C with IL-6 and sIL-6R (Materials and methods) were immunoprecipitated with monoclonal antibody to gp130 together in (B) with rabbit polyclonal antiserum to STAT1 and, after SDS–PAGE (7%), probed with a mixture of anti-phosphotyrosine antibodies. The levels of JAK2 protein overexpression in U4A cells (B) analysed by Western transfer (**C**) were comparable with those for JAK2 and mutant JAK2KE in the γ2A cells in (A) (Figure 7B, lanes 7–12).

but not the absence of JAK1, wild-type, but not kinase-negative, JAK2 can sustain essentially wild-type levels of gp130 phosphorylation and activation of the STATs (Figures 8A and B and 6A and B). This suggests that JAK2 must be enzymically active to sustain the response and that although unable to replace JAK1, it may be able to act through it. This emphasizes the possibility, at least, of a role for JAK2 in normal JAK1 activation. Any tendency of the mutant kinase to sequester the gp130 receptor subunit and/or JAK1 in inactive complexes would, however, contribute to the differential response observed with the active and inactive enzymes. Indeed such a tendency, if large, could, combined with an effectively neutral role for JAK2 in these assays, suffice to explain the differential response to the wild-type and mutant kinases. In addition, on overexpression of JAK1 the activation of JAK2 and Tyk2 is substantially inhibited (e.g. Figure 2A, lane 6) without any inhibitory effect on the activation of JAK1 and the STATs (Figures 2B and 4), while the phosphorylation of gp130 and the induction of IRF1 are, if anything, increased (lanes 6, Figures 7 and 5B). Taken together, therefore, these data suggest that JAK2 may be able to activate JAK1, but leave open whether it normally does so and, if so, whether it (interchangeably with Tyk2) is essential for JAK1 activation or is simply an alternative to (intermolecular) autophosphorylation by JAK1. Similarly, it remains to be established how the residual phosphorylation of gp130 (lane 4, Figure 4) and activation of STAT3, in particular (lane 4, Figure 5C), observed in the absence of JAK1, are mediated. The most striking aspect of the results, however, is the inhibitory effect of the absence of JAK1, but not of Tyk2 or JAK2, on the phosphorylation of gp130, the activation of STATs 1 and 3, and the induction of IRF1 mRNA. Overall, the results establish the non-equivalence of JAK1 and JAK2, and a major role for JAK1 in the IL-6 response pathway in these cells.

It will be intriguing to determine if JAK1 plays the same central role in the LIF or CNTF responses, or whether the absence of JAK2 or Tyk2 more profoundly affects signal transduction through the gp130/LIFβ heterodimeric receptors. For the class I cytokine receptors, a membrane-proximal region known as box 1/box 2 (Murakami *et al.*, 1991) appears essential for interaction with JAKs1 and 2 (Witthuhn *et al.*, 1993; Quelle *et al.*, 1994). For the IFN receptors and the JAKs, the domain requirements are not known. It will be important to establish how the receptors discriminate between the JAKs and whether the interaction can be modulated by adaptors.

Here it is established for the first time directly, rather than by inference, that the JAK/STAT pathway is essential for an aspect of the IL-6 response—the induction of IRF1 mRNA (Figure 6). In addition, the major role of JAK1 in the phosphorylation of gp130 in response to IL-6 (Figure 4) suggests that the JAKs may play a more general role in the IL-6 response. This, however, remains to be established. The dominant inhibitory effect of the JAK2KE mutant is probably based on competition for the receptor. It may, therefore, extend to all JAK-mediated aspects of the IL-6 response and have an analytical advantage in this regard over a defect in any one kinase. Although the JAK2KE-mediated inhibitions reported here are not complete, they are substantial and, if reproducible in other cell types, could prove sufficient to determine the importance of JAK-dependent pathway(s) in different aspects of the pleiotropic IL-6 response. In more general terms, the JAK2KE mutant also inhibits the IFN-γ response (Figure 7) and another kinase-negative mutant of JAK2 has independently been shown to inhibit the erythropoietin response (Zhuang *et al.*, 1994). Such kinase-negative

JAK2 mutants may, therefore, prove effective and of wider application in the inhibition of additional JAK- or JAK2-dependent pathways. Meanwhile, the JAK-negative mutant cell lines will continue to provide a suitable background for further structure–function analysis of the interaction of the JAKs and STATs with the IL-6 gp130 and related receptor subunits.

Material and methods

Cell culture, DNA transfection and selections

Parental and mutant cells were derived as described previously (Pellegrini *et al.*, 1989; McKendry *et al.*, 1991; Watling *et al.*, 1993). All cells were grown in Dulbecco's modification of Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum and 5 mM L-glutamine. 2fTGH cells and derived mutant cell lines were maintained in hygromycin (250 μg/ml). 2C4 and γ2A cells were maintained in medium containing 700 μg/ml G418. IFNs were used at 500 or 1000 IU/ml for the times stated. 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 sIL-6R α chain (500 ng/ml).

Antibodies

Rabbit polyclonal antisera were raised against the JH2 domain of JAK1 (Wilks *et al.*, 1991), the JH1 and JH6 domains of JAK2 (A.Ziemiecki, unpublished) and amino acids 289–450 of Tyk2 (Barbieri *et al.*, 1994). Polyclonal STAT1 and STAT3 specific antibodies have been described previously (Fu *et al.*, 1992; Schindler *et al.*, 1992a,b; Zhong *et al.*, 1994). The preparation of the gp130 monoclonal antibodies will be described separately (K.Yasukawa). A mixture of 4G10 (UBI) and PY20 (ICN) anti-phosphotyrosine monoclonal antibodies was used throughout. Peroxidase-conjugated rabbit anti-mouse or goat anti-rabbit (Amersham) were used as secondary antibodies.

Immunoprecipitation, SDS–PAGE and Western blotting

Cells ($5–7\times10^6$) were harvested and lysed for 20 min in 1 ml 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 phenylmethylsulphonyl fluoride, 1 mM dithiothreitol, 3 mg/ml aprotinin, 1 mg/ml leupeptin] (Schindler *et al.*, 1992b). Nuclei were removed by centrifugation for 5 min at 4°C in a microfuge. The supernatant was pre-cleared with pre-immune serum and protein A–Sepharose (40 μl of 50% slurry equilibrated in cell lysis buffer) for 1 h. Appropriate antibodies and protein A–Sepharose were added and incubated for 1–2 h at 4°C. The immunoprecipitates were washed twice in ice-cold cell lysis buffer and once in phosphate-buffered saline supplemented with 0.1 mM Na_3VO_4. Bound proteins were eluted in reducing SDS loading buffer and separated on a 7% polyacrylamide–SDS gel (Laemmli, 1970). Proteins were transferred electrophoretically to Immobilon™ PVDF (Millipore) membranes. Membranes were blocked with 5% bovine serum albumin (BSA) (Fraction V) in TBST [10 mM Tris–HCl (pH 7.4), 75 mM NaCl, 1 mM EDTA, 0.1% Tween 20] for at least 1 h at 4°C, incubated with the relevant primary antibody for 1 h, washed in TBST, incubated for 1 h with peroxidase-conjugated secondary antibody and re-washed in TBST. Detection was by enhanced chemiluminescence (ECL, Amersham Life Science) and fluorography (Fuji RX) or 3,3′-diaminobenzidine tetrahydrochloride (DAB) staining. 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

JAK proteins were immunoprecipitated as described above, washed twice in lysis buffer and once in 50 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1 mMNa_3VO_4, 10 mM HEPES (pH 7.4) and incubated in the same buffer at room temperature for 30 min in the presence 0.25 mCi/ml [γ-^{33}P]ATP. Complexes were washed and proteins dissolved in sample buffer, followed by separation by SDS–PAGE. ^{33}P-labelled proteins were detected by autoradiography.

Electrophoretic mobility shift assays

Whole-cell extracts were prepared by lysis of cells in 0.5% NP40, 10% glycerol, 50 mM Tris–HCl (pH 8.0), 200 mM NaCl, 0.1 mM EDTA, 1 mM sodium orthovanadate, 0.5 mM phenylmethylsulphonyl fluoride, 1 mM dithiothreitol, 3 mg/ml aprotinin, 1 mg/ml leupeptin. The oligonucleotide sequence used was derived from the high-affinity SIE of the c-*fos* gene (SIEM67), GTCGACATTTCCCGTAAATC. Probes were end-labelled with either [γ-^{32}P]ATP or [γ-^{33}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) 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 NP40 lysis and phenol–chloroform extraction (Porter *et al.*, 1988). RNase protections (Melton *et al.*, 1984) were performed using probes synthesized from SP6/T7 transcription vectors. Probes were labelled with [^{32}P]UTP to a sp. act. of $2–5\times10^8$ c.p.m./μg of input DNA. Aliquots equivalent to ~$1–3\times10^5$ c.p.m. of each probe and 10 μg of cytoplasmic RNA were used in each assay. The IRF1 probe was kindly provided by S.Goodbourn and has been described previously (Müller *et al.*, 1993b); the actin probe was a cDNA fragment of human γ-actin which yields a 130 bp fragment on protection (Enoch *et al.*, 1986).

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