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Activation of JAK kinases and STAT proteins by interleukin-2 and interferon α, but not the T cell antigen receptor, in human T lymphocytes

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The activation of Janus protein tyrosine kinases (JAKs) and signal transducer and activator of transcription (STAT) proteins by interleukin (IL)-2, the T cell antigen receptor (TCR) and interferon (IFN) α was explored in human peripheral blood-derived T cells and the leukemic T cell line Kit225. An IL-2-induced increase in JAK1 and JAK3, but not JAK2 or Tyk2, tyrosine phosphorylation was observed. In contrast, no induction of tyrosine phosphorylation of JAKs was detected upon stimulation of the TCR. IFNα induced the tyrosine phosphorylation of JAK1 and Tyk2, but not JAK2 or JAK3. IFNα activated STAT1, STAT2 and STAT3 in T cells, but no detectable activation of these STATs was induced by IL-2. However, IL-2 regulates the DNA binding and tyrosine phosphorylation of two STAT-like protein complexes which do not include STAT1, STAT2 or STAT3. STAT4 is not activated by IL-2. The activation of STAT5 cannot be excluded, so the IL-2-activated complexes most probably include at least one novel STAT. No STAT activity was detected in TCR-stimulated lymphocytes, indicating that the JAK/STAT pathway defined in this study constitutes an IL-2R-mediated signaling event which is not shared by the TCR. Finally, in other cell types the correlation between JAK1 activation and the induction of STAT1 has suggested that JAK1 may activate STAT1. The observation that IL-2 and IFNα activate JAK1 to a comparable degree, but only IFNα activates STAT1, indicates that JAK1 activation is not the only determining factor for STAT1 activation. Moreover, the data show that JAK1 stimulation is also not sufficient for STAT3 activation.
Key words: interferon α/interleukin-2/JAKs/STATs/T cell antigen receptor

Introduction

Interleukin (IL)-2 is a pivotal mediator of an immune response as this cytokine induces the proliferation and functional differentiation of T lymphocytes, B cells and natural killer (NK) cells (Smith, 1988). IL-2 is secreted by antigen-activated T lymphocytes and mediates its effects through interaction with a specific high-affinity receptor (IL-2R) comprising three subunits, α, β and γ (Takeshita *et al.*, 1992). The process of T cell activation and growth is initiated by the interaction of antigen with the T cell antigen receptor (TCR), which triggers the G_0-G_1 transition of the cell cycle and induction of IL-2 secretion and IL-2R expression. It is the subsequent IL-2−IL-2R interaction which ultimately drives G_1-S phase progression, T cell clonal expansion and functional differentiation (Cantrell and Smith, 1984; Smith, 1988).

IL-2-mediated events are signaled through the IL-2R β and γ chains (Hatakeyama *et al.*, 1989; Asao *et al.*, 1993) which are members of a hematopoietic growth factor receptor family that includes the receptors for erythropoietin (EPO), growth hormone (GH), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3–7 (Bazan, 1990; Cosman *et al.*, 1990). Within this hematopoietic receptor group, subclasses have been distinguished based upon shared components. Thus, IL-3, IL-5 and GM-CSF utilize a common β chain (Miyajima *et al.*, 1993), while the IL-6, IL-11, ciliary neurotrophic factor, leukemia inhibitory factor and oncostatin M receptors share the gp130 protein initially described as the signal-transducing component of the IL-6R (Kishimoto *et al.*, 1994). Similarly, the IL-2Rβ chain is shared with IL-15 (Bamford *et al.*, 1994; Grabstein *et al.*, 1994), and the IL-2Rγ chain functions as a subunit of the receptors for IL-4, IL-7 and IL-15 (Kondo *et al.*, 1993; Noguchi *et al.*, 1993; Russell *et al.*, 1993; Giri *et al.*, 1994).

Previously characterized biochemical events triggered by the IL-2R include the induction of tyrosine phosphorylation of cellular proteins, notably the src family kinase $p56^{lck}$ (Hatakeyama *et al.*, 1991; Horak *et al.*, 1991). IL-2 also regulates the activity of the GTP binding protein $p21^{ras}$ (Satoh *et al.*, 1991; Graves *et al.*, 1992), the serine/threonine-specific kinase Raf-1 (Turner *et al.*, 1991; Zmuidzinas *et al.*, 1991) and phosphatidylinositol-3-kinase (Augustine *et al.*, 1991; Merida *et al.*, 1991; Remillard *et al.*, 1991). These events may be important for IL-2R-mediated signal transduction but, because they are also induced by the TCR (Weiss and Littman, 1994), they do not explain the distinct patterns of gene expression regulated by the TCR and IL-2R (Stern and Smith, 1986; Beadling *et al.*, 1993). Accordingly, it is expected that TCR- and IL-2R-specific biochemical signaling pathways must exist. One well-documented TCR-mediated signaling pathway that is not shared by the IL-2R involves the activation of phosphatidylinositol hydrolysis, intracellular calcium mobilization and protein kinase C activation (Mills *et al.*, 1991). However, no IL-2-specific signal transduction counterpart has yet been defined.

Recently, a signal transduction pathway which involves

Janus kinases (JAKs) and signal transducer and activator of transcription (STAT) proteins has been found to be regulated by a number of the hematopoietic cytokine receptors (reviewed in Darnell *et al.*, 1994; Ihle *et al.*, 1994). The JAK family, which includes as members Tyk2, JAK1, JAK2 and JAK3, are ~130 kDa non-receptor tyrosine kinases which are distinguished by the presence of two C-terminal kinase-related domains and the absence of a src homology 2 (SH2) domain (Krolewski *et al.*, 1990; Wilks *et al.*, 1991; Silvennoinen *et al.*, 1993c; Ihle *et al.*, 1994; Johnston *et al.*, 1994; Kawamura *et al.*, 1994; Witthuhn *et al.*, 1994). The kinases have been characterized extensively in the signaling pathways activated by type I and II interferons (IFNs). IFNγ activates JAK1 and JAK2, while IFNα/β activates JAK1 and Tyk2 (Velazquez *et al.*, 1992; Muller *et al.*, 1993a; Shuai *et al.*, 1993; Silvennoinen *et al.*, 1993a; Watling *et al.*, 1993). It is likely that the specificity of downstream signaling events induced by each receptor will be influenced by the pattern of JAK kinases activated.

One key group of downstream effectors of the JAKs are the STAT proteins. The STATs are cytosolic SH2 domain-containing proteins, which upon tyrosine phosphorylation form SH2 domain-mediated homo- and heterodimeric complexes. These complexes then migrate to the nucleus where they interact with specific DNA sequences to effect gene expression (reviewed in Pellegrini and Schindler, 1993; Shuai, 1994). To date, five members of this family have been identified (Fu *et al.*, 1992; Schindler *et al.*, 1992a; Akira *et al.*, 1994; Wakao *et al.*, 1994; Zhong *et al.*, 1994a,b). STAT1 and STAT2 were originally characterized in the IFNα signaling pathway as components of IFN-stimulated gene factor 3 (ISGF-3; Fu *et al.*, 1990). In response to IFNα, STAT1, which may be expressed either in a 91 or alternatively spliced 84 kDa form lacking 38 C-terminal amino acids, and the 113 kDa STAT2 become tyrosine phosphorylated. A STAT1–STAT2 complex is then generated, which interacts with an additional 48 kDa protein designated ISGF-3γ and binds to IFN-stimulatable response elements (ISREs) upstream of IFNα-inducible genes (Kessler *et al.*, 1990; Fu *et al.*, 1992; Schindler *et al.*, 1992a,b; Muller *et al.*, 1993b). IFNγ induces tyrosine phosphorylation of STAT1, but not STAT2, thereby generating specific DNA binding complexes comprising STAT1 homodimers which interact with IFNγ activation sequences (GAS; Schindler *et al.*, 1992b; Shuai *et al.*, 1992; Muller *et al.*, 1993b).

The first links between the JAK/STAT pathway and the hematopoietic cytokine receptor family were provided by studies demonstrating the activation of JAK2 by EPO (Witthuhn *et al.*, 1993), GH (Argetsinger *et al.*, 1993) and IL-3 (Silvennoinen *et al.*, 1993c). It has also been shown that G-CSF activates JAK1 (Nicholson *et al.*, 1994) and that IL-6 can activate JAK1, JAK2 and/or Tyk2, depending on the cell type examined (Lutticken *et al.*, 1994; Stahl *et al.*, 1994). With regard to STATs, cytokine-responsive proteins have been identified which appear to be related to STAT1 based upon sequence similarity, immunological cross reactivity and/or related DNA binding specificity (Bonni *et al.*, 1993; Kotanides and Reich, 1993; Larner *et al.*, 1993; Ruff-Jamison *et al.*, 1993; Sadowski *et al.*, 1993; Silvennoinen *et al.*, 1993b; Akira *et al.*, 1994; Feldman *et al.*, 1994; Finbloom *et al.*, 1994; Lamb *et al.*, 1994; Meyer *et al.*, 1994; Schindler *et al.*, 1994; Zhong *et al.*, 1994a,b). STAT3 has been cloned by virtue of induction by IL-6 as well as homology to STAT1 (Akira *et al.*, 1994; Zhong *et al.*, 1994a,b); STAT4 was similarly isolated by low-stringency screening of a mouse thymus cDNA library with a probe comprising the SH2 domain of STAT1 (Zhong *et al.*, 1994b). In the context of the IL-2, IL-4 and IL-7 receptor group, IL-4-induced STAT-like proteins have been described which are inducibly tyrosine phosphorylated and apparently distinct from STAT1 or STAT2, although their exact nature remains to be determined (Kotanides and Reich, 1993; Schindler *et al.*, 1994).

Recently, a new JAK family member, designated JAK3, has been shown to be tyrosine phosphorylated and activated in response to IL-2 in T cells and NK cells (Johnston *et al.*, 1994; Witthuhn *et al.*, 1994). However, it is not entirely clear whether the IL-2 receptor is coupled to other JAKs (Witthuhn *et al.*, 1994). It has also not been determined whether there is activation of STATs by IL-2, or whether the activation of a JAK/STAT pathway is a response that is restricted to the IL-2R or a shared response with the TCR. In this study we have investigated the regulation of JAK and STAT proteins in IL-2-stimulated human T lymphocytes. In addition, the involvement of this pathway in TCR-mediated signaling has been assessed, and the IL-2 response compared with the pattern of JAKs and STATs activated by IFNα. Herein we report that in human T lymphocytes, IL-2 stimulates the tyrosine phosphorylation of JAK1 and JAK3, but not JAK2 or Tyk2. DNA binding analyses demonstrate that IL-2 also induces the activation and tyrosine phosphorylation of DNA binding proteins. One of the IL-2-induced DNA binding complexes includes a STAT1-related protein(s), while a second complex does not comprise STAT1, STAT2 or STAT3, but exhibits a DNA binding specificity which suggests that it is a protein complex comprising a member(s) of the STAT family of transcriptional factors. Importantly, neither induction of JAK tyrosine phosphorylation, nor STAT-like DNA binding activity, was observed in response to TCR triggering, indicating that the IL-2-activated JAK/STAT signaling pathway is not shared by the TCR. By comparison, IFNα activated Jak1 and Tyk2, as well as STAT1, STAT2 and STAT3. Finally, while both IL-2 and IFNα activated JAK1 to a comparable degree, the fact that tyrosine phosphorylation of STAT1 and STAT3 was only detected in response to IFNα and not IL-2 indicates that JAK1 activation is not the only factor determining the tyrosine phosphorylation of these STATs.

Results

IL-2 induces tyrosine phosphorylation of JAK1 and JAK3 in human T lymphocytes

In initial experiments, the tyrosine phosphorylation of JAK proteins was monitored in the IL-2-dependent human T cell line Kit225 (Hori *et al.*, 1987). The cells were arrested in a quiescent state by 48 h of growth factor deprivation, and restimulated with 1 nM IL-2 for 15 min. JAKs were then immunoprecipitated with specific antisera, and the phosphorylation levels were determined by Western blotting with anti-phosphotyrosine antibodies. As shown in Figure 1A, IL-2 stimulated an increase in the

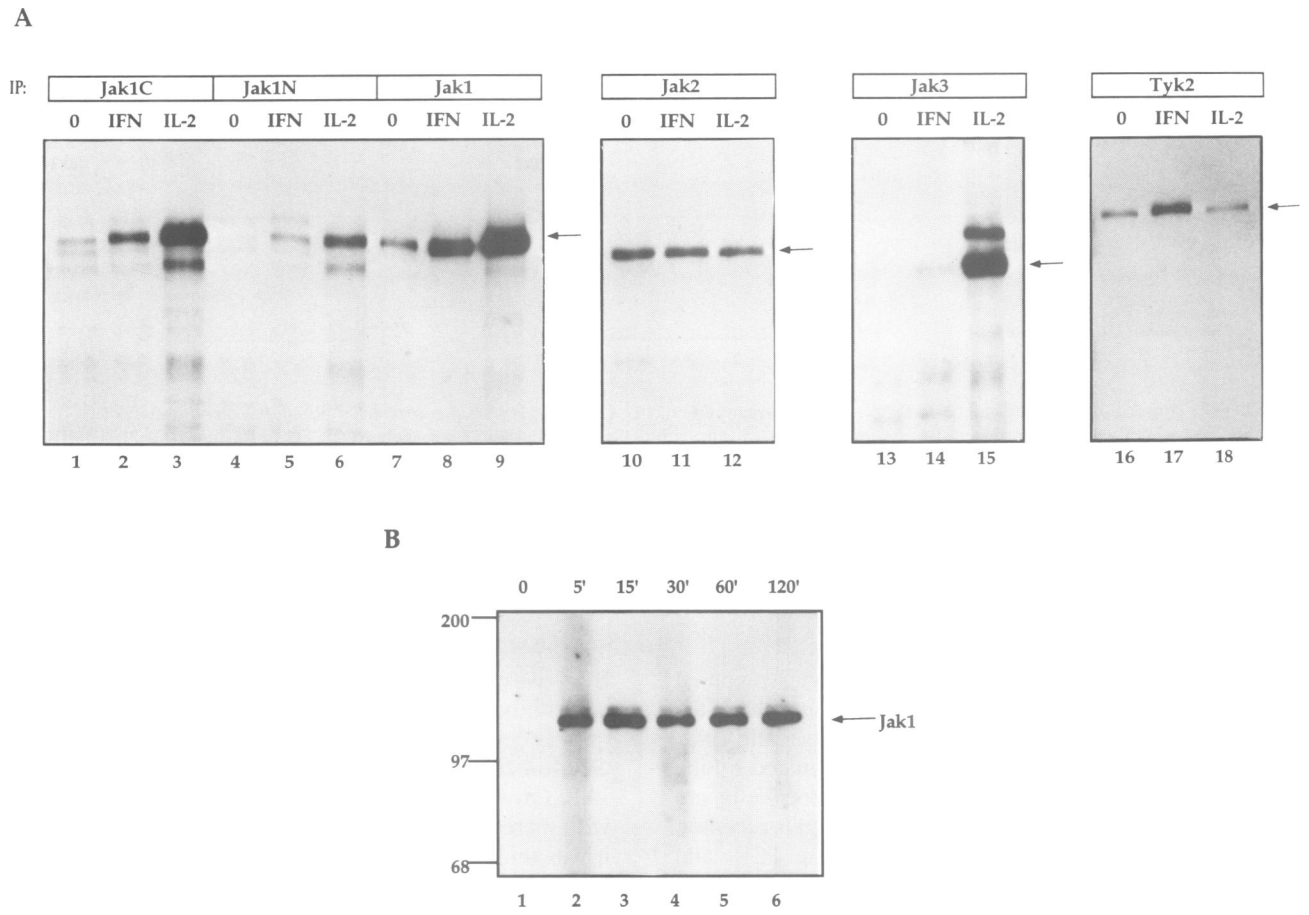


Fig. 1. IL-2- and IFNα-induced JAK tyrosine phosphorylation. (**A**) Kit225 cells were left untreated (0) or stimulated for 15 min with 1000 U/ml IFNα or 1 nM IL-2 for 15 min prior to cell lysis and immunoprecipitation with antibodies specific to JAK1 (lanes 1–9), JAK2 (lanes 10–12), JAK3 (lanes 13–15) or Tyk2 (lanes 16–18). Three distinct antisera were used to immunoprecipitate JAK1, and were raised against C-terminal (JAK1C, lanes 1–3) or N-terminal (JAK1N, lanes 4–6) peptides, or a C-terminal fusion protein (JAK1, lanes 7–9). Immunoprecipitated proteins were separated by SDS−PAGE, and Western blot analysis was performed with anti-phosphotyrosine antibodies. (**B**) Kit225 cells were stimulated for the indicated times with 1 nM IL-2, and anti-phosphotyrosine immunoprecipitations were performed with the mAb FB2, followed by Western blot analysis with antisera raised against a JAK1 C-terminal fusion protein. The positions of the JAK proteins are designated with arrows, and molecular weight standards are indicated in kDa.

level of tyrosine phosphorylation of JAK1. Experiments using three different antisera raised against distinct regions of the JAK1 protein yielded similar results (Figure 1A). In the JAK1 immunoprecipitates from IL-2-activated cells, there was a weak signal from a tyrosine phosphorylated 115 kDa protein that corresponds in size to JAK3. An anti-phosphotyrosine Western blot of JAK3 immunoprecipitates from IL-2-activated cells demonstrated the tyrosine phosphorylation of JAK3 and detected a weak signal from a higher molecular weight protein of the size of JAK1 (Figure 1A). The apparent IL-2 dependence of the additional weak signals is not yet understood, but it seems most likely that they simply reflect a low and variable level of cross-reactivity of the antipeptide antisera. JAK2 and Tyk2 exhibited a basal level of tyrosine phosphorylation in quiescent cells, and no increase in this level was observed upon IL-2 stimulation (Figure 1A). As a comparison, the effect of IFNα on JAK tyrosine phosphorylation was determined. As shown in Figure 1A, 15 min of IFNα treatment of Kit225 cells induced the tyrosine phosphorylation of JAK1 and Tyk2. Further examination of the kinetics of the IL-2-induced JAK1 tyrosine phosphorylation demonstrated that it was induced within 5 min and persisted for at least 2 h (Figure 1B).

JAK tyrosine phosphorylation is not induced by TCR stimulation

Activation of the JAK kinases is not restricted to hematopoietic cytokine receptor signaling, as the tyrosine phosphorylation of JAK1 has been described in response to triggering of the IFN receptors which are only distantly related to the hematopoietic cytokine receptors (Velazquez *et al.*, 1992; Muller *et al.*, 1993a; Shuai *et al.*, 1993; Silvennoinen *et al.*, 1993a; Watling *et al.*, 1993), and also in response to stimulation of the epidermal growth factor receptor (EGFR; Shuai *et al.*, 1993) which is a structurally unrelated protein. Therefore, it was of interest to determine whether JAK phosphorylation in T lymphocytes is driven by TCR triggering in addition to IL-2R stimulation. To this end, JAK phosphorylation was examined in peripheral blood-derived T lymphoblasts. To prepare primary T cell cultures for such studies, peripheral blood lymphocytes were stimulated with phytohemagglutinin (PHA) and IL-2 for 5 days, after which the growth factor was removed

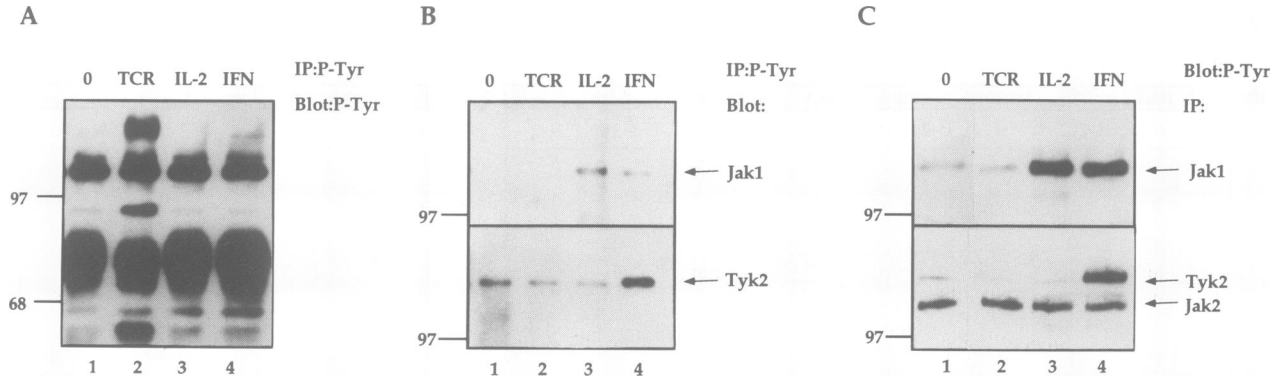


Fig. 2. TCR stimulation does not induce JAK1 tyrosine phosphorylation. (**A**) Cell lysates were prepared from peripheral blood-derived T lymphoblasts which were left untreated (0) or stimulated for 15 min with the monoclonal anti-CD3 antibody UCHT1 (10 μg/ml), IL-2 (1 nM) or IFNα (1000 U/ml). Lysates were subjected to immunoprecipitation with the anti-phosphotyrosine antibody FB2, and immunoprecipitated proteins were separated by SDS−PAGE. Western blot analysis was performed with the anti-phosphotyrosine antibody 4G10. (**B**) Anti-phosphotyrosine immunoprecipitates were prepared from T lymphoblasts treated as in (A), and Western blot analysis was performed with antisera recognizing JAK1 or Tyk2 as indicated. (**C**) Cell lysates prepared from T lymphoblasts treated as in (A) were immunoprecipitated with antisera specific to JAK1, JAK2 or Tyk2, and subjected to Western blot analysis with anti-phosphotyrosine antibodies. Stripping and reprobing of the transfers with appropriate antisera showed for each JAK that equal amounts were present (lanes 1–4). Positions of JAK proteins are designated with arrows, and molecular weight standards indicated in kDa.

for 48 h prior to restimulation. T lymphocytes prepared by this method arrest in a quiescent state, in which the cells express high-affinity IL-2R but do not secrete IL-2. The cells may then be synchronously restimulated by the addition of exogenous IL-2. In addition, the cells are receptive to stimulation via the T cell antigen receptor, and thereby afford the possibility to compare TCR- and IL-2R-driven signaling events (Cantrell and Smith, 1984).

To assess JAK tyrosine phosphorylation, T lymphoblasts were stimulated via the TCR for 15 min with the anti-CD3 mAb UCHT1 or with IL-2. The data in Figure 2A show the characteristic patterns of TCR- and IL-2-induced tyrosine phosphoproteins detected by anti-phosphotyrosine Western blotting of anti-phosphotyrosine immunoprecipitates, confirming that triggering of each receptor is associated with the activation of cellular tyrosine kinases. The JAK phosphorylation states in such TCR- or IL-2-activated lymphoblasts were then monitored by anti-phosphotyrosine immunoprecipitation and Western blotting with antisera raised against each of the JAK proteins. Upon stimulation with IL-2, an induction of JAK1 tyrosine phosphorylation was observed, while Tyk2 exhibited a readily detectable basal level of tyrosine phosphorylation but no IL-2-induced increase in phosphotyrosine content (Figure 2B). In contrast, neither JAK1 nor Tyk2 tyrosine phosphorylation was observed in response to TCR triggering (Figure 2B). The effects of IL-2R and TCR agonists on JAK phosphorylation were confirmed by specific immunoprecipitation of the JAK proteins and subsequent anti-phosphotyrosine Western blotting (Figure 2C). Again, a specific increase in JAK1 tyrosine phosphorylation was observed in IL-2-treated cells, and no JAK1, JAK2 or Tyk2 tyrosine phosphorylation was induced by TCR stimulation. As in the Kit225 cell line, IFNα induced JAK1 and Tyk2 tyrosine phosphorylation in the T lymphoblasts (Figure 2B and C). It was consistently observed that in Kit225 cells and T lymphoblasts there was a high constitutive level of JAK2 tyrosine phosphorylation (Figures 1 and 2). JAK2 is normally tyrosine phosphorylated in response to IFNγ, but no response of either cell type to exogenous IFNγ was observed (data not shown); the basis for the constitutive JAK2 tyrosine phosphorylation is unclear.

Stimulation of JAK-associated kinase activity

JAK tyrosine phosphorylation has not always correlated with increased kinase activity (Witthuhn *et al.*, 1994), and it was thus of interest to determine whether the induction of JAK1 tyrosine phosphorylation observed in response to IL-2 and IFNα was reflected in augmented kinase activity. To address this point, JAK1 immunoprecipitation and *in vitro* kinase assays were performed. These experiments demonstrated that 15 min of IL-2 stimulation of Kit225 cells and T lymphoblasts, which induced JAK1 tyrosine phosphorylation in both cell types, also augmented [^{32}P]ATP incorporation into JAK1 immunoprecipitates *in vitro* (Figure 3). Similarly, IFNα stimulated an increase in the kinase activity of JAK-1 immunoprecipitates in both cell types (Figure 3), and the stimulatory effects of IL-2 and IFNα on JAK1 activity were comparable. JAK3 kinase assays were not possible, as the JAK3 antisera used in immunoprecipitates was raised against a peptide containing the autophosphorylation site and interferes with kinase activity (Witthuhn *et al.*, 1994).

IL-2 and IFNα, but not TCR stimulation, induce DNA binding activity with sis-inducible element (SIE) and FcγR-GAS sequences

As STAT proteins have been shown to act downstream of JAK kinases in regulating gene expression, electrophoretic mobility shift assays (EMSA) were performed with oligonucleotide probes known to bind STATs (Schindler *et al.*, 1992b; Kotanides and Reich, 1993; Larner *et al.*, 1993; Ruff-Jamison *et al.*, 1993; Sadowski *et al.*, 1993; Silvennoinen *et al.*, 1993b; Finbloom *et al.*, 1994; Meyer *et al.*, 1994). The STAT binding oligonucleotides used contain a core sequence with homology to the IFNγ activation sequence, or GAS element, and include the GAS sequence upstream of the IgG Fc receptor I (FcγR-GAS), the high-affinity SIE from the c-*fos* gene (designated SIEM67 in Wagner *et al.*, 1990) and the GAS element of the guanylate

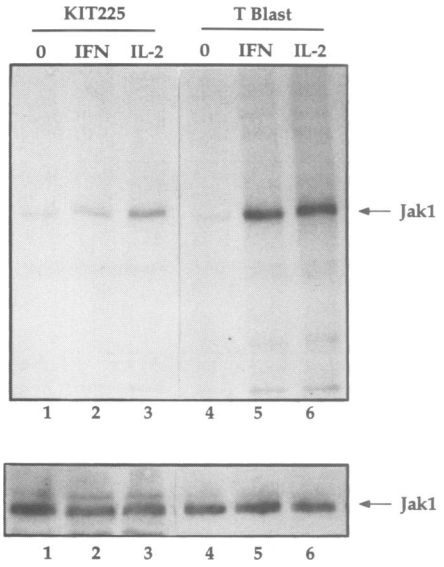


Fig. 3. IL-2 and IFNα induce JAK1-associated kinase activity. Kit225 cells (lanes 1–3) or peripheral blood-derived T lymphoblasts (lanes 4–6) were untreated (0) or stimulated for 15 min with 1000 U/ml IFNα or 1 nM IL-2. JAK1 was immunoprecipitated, and immunoprecipitates were subjected to *in vitro* kinase assay by incubation for 30 min at room temperature in the presence of 0.25 mCi/ml [γ-^{32}P]ATP. Samples were then separated by SDS−PAGE and phosphorylated proteins were detected by autoradiography (top). JAK1 protein levels were determined in parallel by Western blot analysis with JAK1 antisera (bottom).

binding protein (GBP) gene. The ISRE from the IFN-responsive 9-27 gene was also used. As shown in Figure 4A, total cell lysates prepared from IL-2-stimulated Kit225 cells possessed DNA binding activity detected with the SIE and FcγR-GAS probes. The SIE complex resolved predominantly as a single band, while the FcγR-GAS complex resolved as a doublet, with relatively equal intensity of both bands (Figure 4A, lanes 9 and 10). The lower band of this doublet comigrated with the SIE complex. By comparison, total cell extracts from IFNα-treated cells formed complexes with both the SIE and FcγR-GAS probes which were consistently of greater intensity than those activated by IL-2 (Figure 4A, lanes 5 and 6). When these IFNα-induced complexes were resolved separately and subjected to a shorter autoradiographic exposure, they appeared to resolve as single bands (Figure 4A, lanes 13 and 14). In addition, while both IL-2- and IFNα-activated complexes recognize the SIE and FcγR-GAS probes, only IFNα induced proteins which could bind the ISRE and neither IL-2 nor IFNα induced GBP-GAS binding proteins (Figure 4A). The lack of IFNα-activated GBP-GAS binding proteins is probably due to the relatively low affinity of this oligonucleotide for STATs.

Kinetic analyses of the IL-2-activated DNA binding complexes revealed that they were rapidly induced and relatively stable. As shown in Figure 4B, complexes recognizing the SIE and FcγR-GAS probes could be detected within 5 min of IL-2 stimulation of Kit225 cells, and persisted for at least 2 h. To verify that the induction of DNA binding activity was mediated by high-affinity IL-2R stimulation, Kit225 cells were stimulated for 15 min with doses of IL-2 ranging from 1 to 100 pM (Figure 4B). These experiments indicated that the complexes could be induced with 10 pM IL-2, consistent with the 10^{-11} M K_d of the high-affinity IL-2R (Smith, 1988).

The EMSA experiments were extended to primary T cells to assess further the receptor pathways which could trigger the induction of the DNA binding activity. In these experiments, T lymphoblasts were stimulated for 15 min via the TCR or with IL-2, and whole-cell extracts were prepared for use in mobility shift assays. As shown in Figure 5A, lysates from IL-2-stimulated but not unstimulated cells generated complexes with the SIE and FcγR-GAS probes. As observed previously with Kit225 extracts, the SIE complex resolved as a single band, while the FcγR-GAS complex resolved as a doublet of equally intense bands, with the lower band comigrating with the SIE complex. Comparison of the complexes induced by IL-2 in extracts of T lymphoblasts with those of Kit225 cells revealed that the migration patterns of the complexes were similar (Figure 5A). Triggering of the T cell receptor did not induce binding activity detectable with either of the probes (Figure 5A). The signal transduction response of the TCR was confirmed by Western blot analysis of total cell lysates with anti-phosphotyrosine antibodies (Figure 2A). To verify that the lack of detection of TCR-induced DNA binding complexes was not due simply to differences in the kinetics of induction between IL-2R and TCR stimulation, mobility shift experiments were performed with extracts prepared from T lymphoblasts stimulated with either IL-2 or UCHT1 over a time course of 5–60 min. Throughout this time course, only IL-2 induced the SIE and FcγR-GAS DNA binding activity (Figure 5B).

IL-2 induces STAT1-related and distinct DNA binding proteins

To investigate further the IL-2-induced SIE and FcγR-GAS DNA binding complexes, competition studies were performed with a variety of GAS-containing sequences. In these experiments, whole-cell extracts from IL-2-stimulated Kit225 cells were pre-incubated with either a 10- or 100-fold molar excess of unlabeled competitor oligonucleotide prior to incubation with the labeled probe. The competitor oligonucleotide sequences were derived from the Ly6E and GBP enhancers, as well as the ISRE from 9-27. As shown in Figure 6, the IL-2-induced SIE complex was competed effectively by the FcγR-GAS and Ly6E-GAS sequences, but not significantly by GBP-GAS or ISRE. In the case of the FcγR-GAS complexes, both bands of the doublet were competed with the FcγR-GAS oligonucleotide, but only the lower band was significantly competed by the SIE or Ly6E-GAS oligonucleotides. Neither band was competed by GBP-GAS or ISRE oligonucleotides. Therefore, the lower band of the IL-2-induced FcγR-GAS complexes is recognized by proteins cross-reactive with the SIE sequence, while the top band represents an interaction with a distinct protein(s). The IFNα-induced FcγR-GAS and SIE complexes showed an identical competition pattern to the IL-2-induced SIE complex and the lower band of the IL-2-induced FcγR-GAS complexes (data not shown). It was thus not possible to distinguish the SIE complexes formed with IFNα or IL-2 on the basis of differential competition with these oligonucleotides.

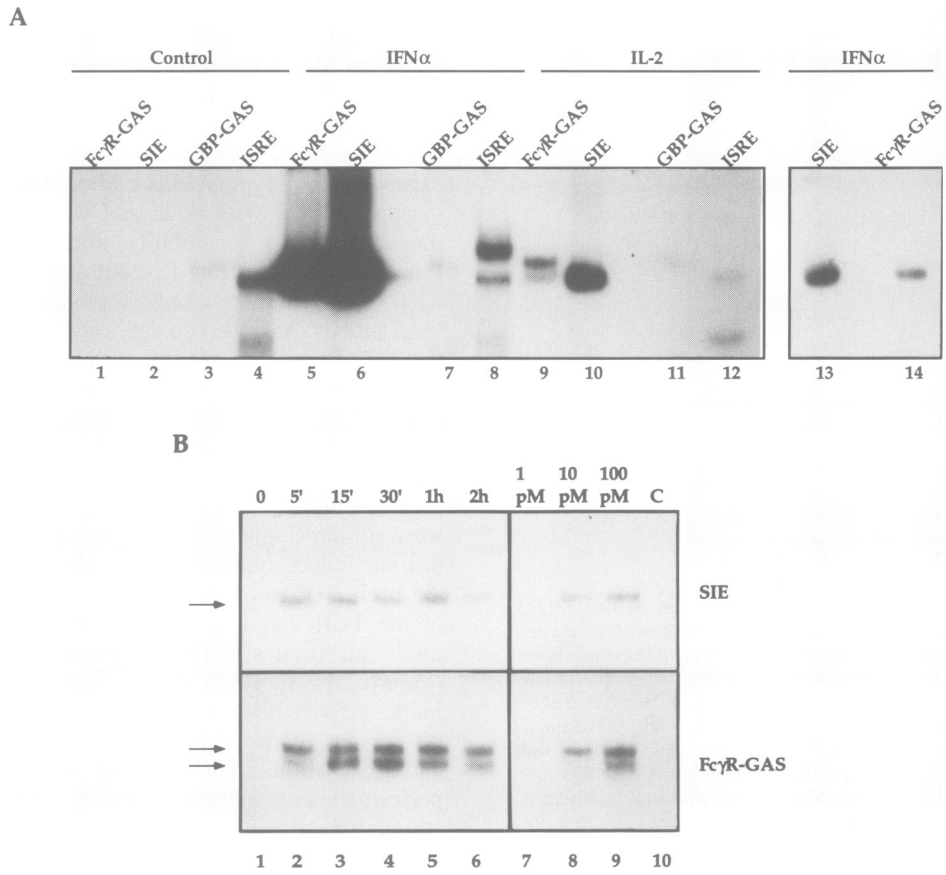


Fig. 4. IL-2- and IFNα-induced DNA binding complexes. (**A**) Kit225 cells were untreated (lanes 1–4) or stimulated with 1000 U/ml IFNα (lanes 5–8) or 1 nM IL-2 (lanes 9–12) for 15 min. Whole-cell lysates were prepared and incubated with ^{32}P-labeled oligonucleotide probes comprising the FcγR-GAS element (lanes 1, 5 and 9), the high-affinity SIE sequence derived from the c-*fos* gene (lanes 2, 6 and 10), GBP-GAS (lanes 3, 7 and 11) or ISRE (lanes 4, 8 and 12). Binding reactions were separated on 6% acrylamide gels in 0.5× TBE, and complexes detected by autoradiography for 24 (lanes 1–12) or 3 h (lanes 13–14). (**B**) Kit225 cells were stimulated for the indicated times with 1 nM IL-2 (lanes 1–6 and 10), or for 15 min with 1–100 pM IL-2 (lanes 7–9). DNA binding reactions were performed with whole-cell lysates, and complexes reacting with the high-affinity SIE (top) or FcγR-GAS (bottom) probes resolved as in (A). A 100-fold molar excess of unlabeled probe was included in a control reaction (C, lane 10). Inducible complexes are indicated by arrows.

As the SIE and FcγR-GAS probes used in the mobility shift assays are known to interact with STAT1 and STAT1-related proteins (Schindler *et al.*, 1992b; Kotanides and Reich, 1993; Larner *et al.*, 1993; Ruff-Jamison *et al.*, 1993; Sadowski *et al.*, 1993; Silvennoinen *et al.*, 1993b; Finbloom *et al.*, 1994; Meyer *et al.*, 1994), supershift experiments were performed with an antibody raised against the C-terminal region of STAT1 to determine whether reactive proteins were present in the IL-2-induced complexes. As shown in Figure 7, the supershift assays demonstrated that the IL-2-induced SIE complex reacted with STAT1 antisera, as detected by the disappearance of the SIE complex when binding reactions were conducted in the presence of the antibody. In the case of the IL-2-induced FcγR-GAS complexes, the lower band of the doublet 'supershifted' in the presence of the STAT1 antisera in an analogous fashion to that seen with the SIE probe. By comparison, the presence of the STAT1 antibody did not alter the apparent mobility of the upper band seen with the FcγR-GAS probe (Figure 7). Neither complex reacted with antisera against STAT2, as the presence of this antisera in the binding reactions did not alter the mobility of any of the complexes (Figure 7). Therefore, the lower band of the IL-2-induced FcγR-GAS-reactive complex contains a STAT1-related protein(s), while the upper complex comprises a distinct protein(s).

The supershift of the IL-2-induced bandshift complexes by STAT1 antisera suggested that either STAT1 or related proteins were activated by IL-2. As activation of STAT1 is dependent upon tyrosine phosphorylation (Shuai *et al.*, 1994), the tyrosine phosphorylation of STAT1 was examined in IL-2-treated T cells. In these experiments, STAT1 was specifically immunoprecipitated from untreated or IL-2-stimulated cells, and Western blot analyses were subsequently performed with anti-phosphotyrosine antibodies. The experiments were performed in both Kit225 and T lymphoblasts with identical results: the data in Figure 8A show that when as a control, Kit225 cells were stimulated with IFNα, there was a clear induction of STAT1 tyrosine phosphorylation. However, no IL-2-induced tyrosine phosphoproteins could be detected in STAT1 immunoprecipitates (Figure 8A). The tyrosine phosphorylation of STAT3 was also investigated. While IFNα induced the tyrosine phosphorylation of STAT3, no such phosphorylation was detected in the immunoprecipitates from IL-2-treated cells (Figure 8B). Even after prolonged exposure of the autoradiographs, no IL-2-induced STAT1 or STAT3 tyrosine phosphorylation was

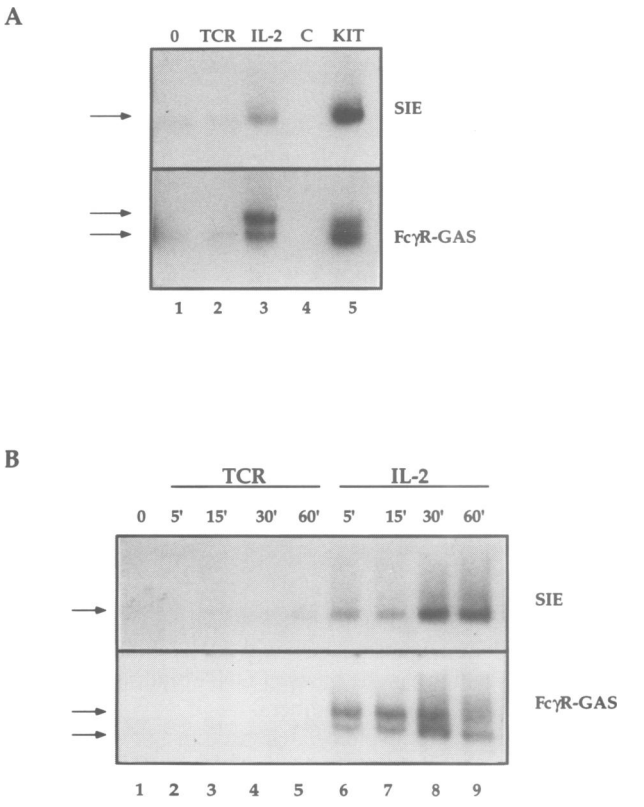
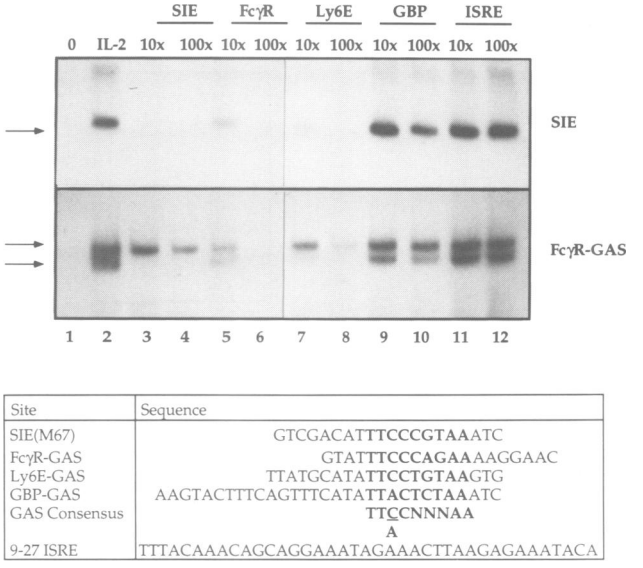


Fig. 5. IL-2, but not TCR, stimulation induces SIE and FcγR-GAS binding activity. (**A**) Peripheral blood-derived T lymphoblasts were left untreated (0, lane 1), or stimulated with the anti-CD3 antibody UCHT1 (10 μg/ml, lane 2) or IL-2 (1 nM, lanes 3 and 4) for 15 min. Whole-cell extracts were prepared and subjected to mobility shift analysis with high-affinity SIE (top) and FcγR-GAS (bottom) probes. A 100-fold molar excess of unlabeled probe was included in control reactions with IL-2-stimulated cell lysates (C, lane 4). Parallel analyses were performed with whole-cell extracts prepared from Kit225 cells stimulated for 15 min with 1 nM IL-2 (KIT, lane 5). Complexes were resolved on 6% acrylamide gels in 0.5× TBE, and detected by autoradiography. (**B**) Whole-cell lysates were prepared from T lymphoblasts left untreated (lane 1) or stimulated for the indicated times with the anti-CD3 antibody UCHT1 (10 μg/ml, lanes 2–6) or with IL-2 (1 nM, lanes 7–11). Mobility shift analyses were performed with high-affinity SIE (top) and FcγR-GAS (bottom) probes as in (A). The positions of inducible complexes are designated by arrows.



Site	Sequence
SIE(M67)	GTCGACATTTCCCGTAAATC
FcγR-GAS	GTATTTCCCAGAAAAGGAAC
Ly6E-GAS	TTATGCATATTCCTGTAAGTG
GBP-GAS	AAGTACTTTCAGTTTCATATTACTCTAAATC
GAS Consensus	TTCCNNNAA
	A
9-27 ISRE	TTTACAAACAGCAGGAAATAGAAACTTAAGAGAAATACA

Fig. 6. Competition of IL-2-induced bandshift complexes. Upper panel: whole-cell extracts were prepared from Kit225 cells left untreated (0, lane 1) or stimulated for 15 min with 1 nM IL-2 (lanes 2–12). Extracts were pre-incubated with a 10- or 100-fold molar excess of unlabeled SIE, FcγR-GAS, Ly6E-GAS, GBP-GAS or ISRE oligonucleotide prior to incubation with ^{32}P-labeled high-affinity SIE or FcγR-GAS probes as indicated. Complexes were resolved on 6% acrylamide gels in 0.5× TBE and detected by autoradiography. Arrows mark the positions of the IL-2-induced complexes. Lower panel: the oligonucleotide sequences utilized are listed, with the GAS consensus in bold face type.

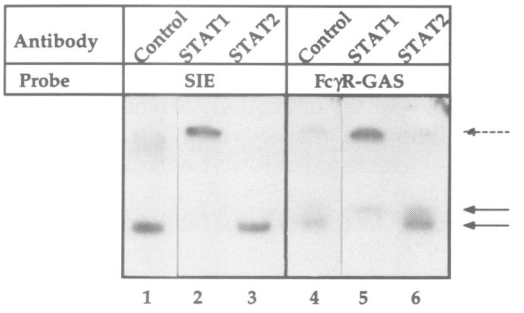


Fig. 7. IL-2-induced DNA binding complexes contain STAT1-related proteins as well as a distinct element(s). Whole-cell extracts were isolated from Kit225 cells which were stimulated for 15 min with 1 nM IL-2. Prior to incubation with ^{32}P-labeled SIE and FcγR-GAS probes, extracts were incubated for 1 h at 4°C with control antisera (lanes 1 and 4), or antisera specific to STAT1 (lanes 2 and 5) or STAT2 (lanes 3 and 6). Complexes were resolved by separation on 6% acrylamide gels in 0.5× TBE and detected by autoradiography. Solid arrows indicate the positions of the native IL-2-induced complexes, and the stippled arrow designates the position of the wells at the top of the gel.

observed. Equivalent amounts of STAT proteins were present in the immunoprecipitates, as detected by Western blotting with the respective antisera (Figure 8A and B). IFNα also induced the tyrosine phosphorylation of STAT2, while IL-2 did not (data not shown).

To characterize further the DNA binding proteins activated by IL-2, a biotinylated FcγR-GAS oligonucleotide was used to affinity purify the proteins from extracts of peripheral blood-derived T lymphoblasts. As shown in Figure 9A (lanes 1–4), stimulation of the cells via the TCR, or with IL-2 or IFNα for 15 min, induced the tyrosine phosphorylation of a number of proteins which could be detected by Western blot analysis of whole-cell extracts, indicating the activation of tyrosine kinases by each stimulus. Similar analysis of whole-cell extracts with STAT1 (Figure 9B, lanes 1–4) and STAT3 (Figure 9C, lanes 1–4) antisera demonstrated the presence of these proteins. DNA binding proteins were isolated from the extracts by affinity purification with an agarose-coupled FcγR-GAS oligonucleotide, and analysed by Western blot with antibodies specific to phosphotyrosine, STAT1 or STAT3. As shown in Figure 9A (lanes 5–8), IL-2 induced the tyrosine phosphorylation of two FcγR-GAS binding proteins of ~93 and ~95 kDa to an equivalent level. By comparison, no induction of tyrosine phosphorylated FcγR-GAS binding proteins was observed upon TCR stimulation, and IFNα predominantly induced the tyrosine phosphorylation of a protein(s) migrating at ~91 kDa.

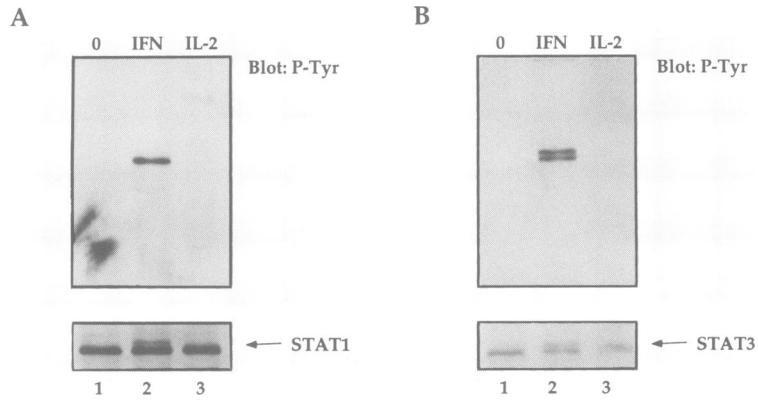


Fig. 8. IFNα but not IL-2 induces STAT1 and STAT3 tyrosine phosphorylation. Kit225 cells were unstimulated (0) or treated for 15 min with 1 nM IL-2 or 1000 U/ml IFNα. Cells were lysed, and (**A**) STAT1 or (**B**) STAT3 was immunoprecipitated with specific antisera. Immunoprecipitates were separated by SDS–PAGE and subjected to Western blot analysis with an anti-phosphotyrosine antibody (top). STAT protein levels were determined by Western blot with the respective STAT antisera (bottom). The basis for the STAT3 doublet is not yet understood, but it has been observed on high-resolution analysis of material from a number of cell types (D.Guschin and I.M.Kerr, unpublished results).

The 89 kDa protein seen in complexes isolated from unstimulated and activated cells binds non-specifically with agarose beads alone, whereas the IL-2- and IFNα-induced proteins were only coprecipitated with agarose-coupled FcγR-GAS oligonucleotides (data not shown). These data show that IL-2 regulates the tyrosine phosphorylation of DNA binding proteins which, on the basis of their DNA binding specificity, are members of the STAT family. When the FcγR-GAS binding proteins were subjected to Western blot analysis with STAT1 antisera, it was found that IFNα strongly induced the binding of a STAT1-reactive protein to the FcγR-GAS oligonucleotide, whereas IL-2 did not (Figure 9B, lanes 5–8). In addition, Western blot analysis of FcγR-GAS binding proteins with STAT3 antisera demonstrated that IFNα activated STAT3, while IL-2-activated proteins were not reactive with STAT3 antisera (Figure 9C, lanes 5–8). Similar partial purification and analysis of factors using a biotinylated high-affinity SIE oligonucleotide demonstrated the presence of tyrosine phosphorylated STAT1 and STAT3 in IFNα- but not IL-2-activated cells (data not shown).

Discussion

The present data have established that the IL-2R activates a JAK/STAT pathway which is not shared with the TCR. Thus, IL-2 induces the tyrosine phosphorylation of JAK1 and JAK3, but not the related kinases JAK2 or Tyk2, while TCR stimulation does not induce tyrosine phosphorylation of any of the JAKs analysed. The data also demonstrate that IL-2, but not TCR, stimulation induced a DNA binding complex that could bind the high-affinity SIE sequence from the human c-*fos* gene, and two protein complexes that recognize the FcγR-GAS element. One of the protein complexes detected with the FcγR-GAS oligonucleotide was indistinguishable from the IL-2-induced SIE complex, in that the interaction with the FcγR-GAS probe was competed by the SIE oligonucleotide, and the complex was reactive with STAT1 antisera in supershift assays. The second IL-2-induced complex interacting with the FcγR-GAS probe was clearly distinct from the SIE binding complex in terms of the lack of reactivity with either an SIE oligonucleotide or with STAT1 antisera. Thus, IL-2 activates at least two DNA binding protein complexes which, on the basis of DNA binding specificity, comprise STAT family members.

Two tyrosine phosphorylated proteins of ~93 and 95 kDa were affinity purified with an FcγR-GAS oligonucleotide from lysates of IL-2-stimulated, but not quiescent or TCR-activated, T cells. These IL-2-induced tyrosine phosphorylated DNA binding proteins did not apparently react with STAT1 antisera in Western blot analysis, consistent with the lack of detectable tyrosine phosphorylation of STAT1 in STAT1 immunoprecipitates isolated from IL-2-stimulated cells. In contrast, tyrosine phosphorylated STAT1 was readily detected in IFNα-activated cells, and was affinity purified with the FcγR-GAS oligonucleotide from IFNα-treated cell extracts. One of the IL-2-induced DNA binding complexes detected in the EMSA was immunoreactive with a STAT1 antisera by supershift analysis. This result is apparently in discrepancy with the failure to detect tyrosine phosphorylation of STAT1 in IL-2-treated cells. This dichotomy could reflect the fact that the STAT1 antisera cross-reacts with an IL-2-activated STAT1-related protein with a low affinity that precludes efficient immunoprecipitation or detection by Western blot, but allows reaction in EMSA supershifts. Future experiments must determine the relationship of the tyrosine phosphorylated DNA binding proteins detected by affinity purification to the DNA binding complexes detected by EMSA.

The IL-2-activated FcγR-GAS binding factors apparently do not include STAT3, as the IL-2-induced proteins were not reactive with STAT3 antisera on Western blots. These results are consistent with the lack of tyrosine phosphorylation of STAT3 in immunoprecipitates from IL-2-stimulated cells. The IL-2-activated factors do not include STAT4, as previous studies have demonstrated that STAT4 is not regulated by this cytokine (Yamamoto *et al.*, 1994). The recently cloned ovine mammary gland factor (MGF) binds a consensus sequence of TTC-NNNGAA (Wakao *et al.*, 1994), with the G essential for MGF binding (Schmitt-Ney *et al.*, 1992). While the FcγR-GAS sequence is consistent with the MGF consensus, the

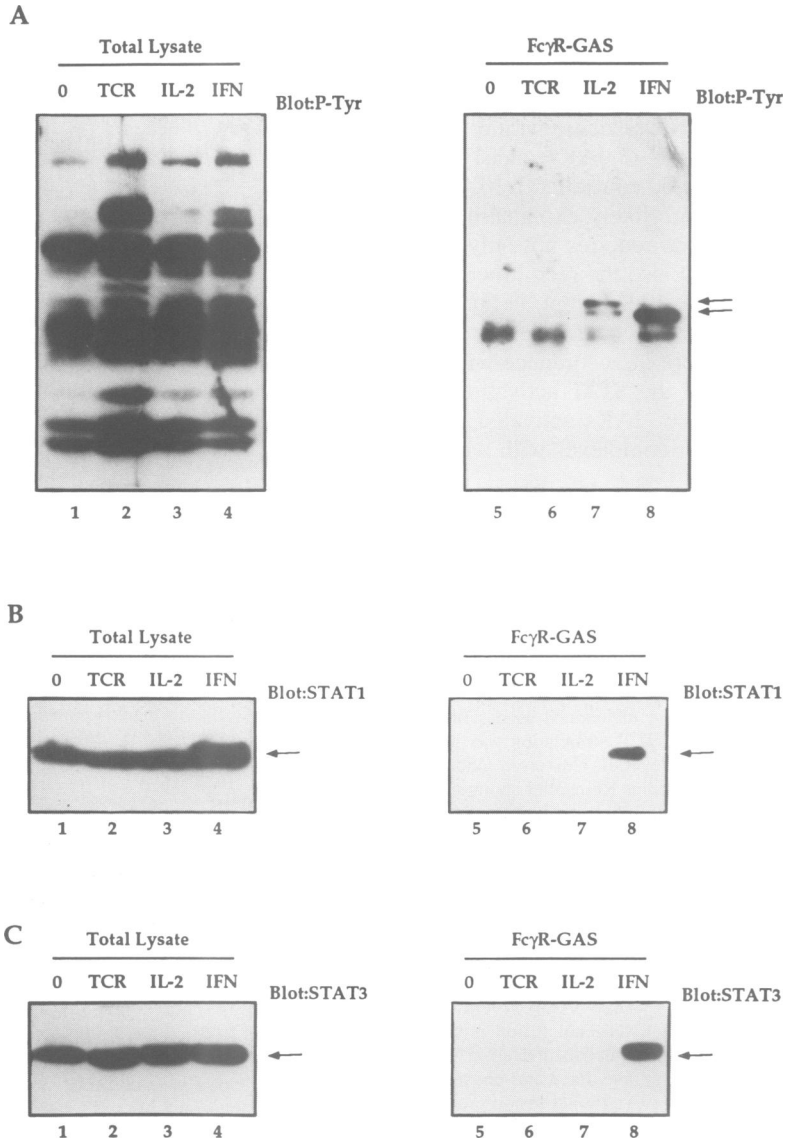


Fig. 9. IL-2 and IFNα, but not TCR, stimulation activate FcγR-GAS binding proteins which are tyrosine phosphorylated. Peripheral blood-derived T lymphoblasts were left untreated (lanes 1 and 5) or treated for 15 min with 10 μg/ml anti-CD3 antibody UCHT1 (lanes 2 and 6), 1 nM IL-2 (lanes 3 and 7) or 1000 U/ml IFNα (lanes 4 and 8). Whole-cell extracts were prepared and aliquots separated by SDS−PAGE (lanes 1–4). These were subjected to Western blot analysis with antibodies against (**A**) phosphotyrosine, (**B**) STAT1 or (**C**) STAT3. DNA binding proteins were affinity purified from the remainder of the extracts with a biotinylated FcγR-GAS oligonucleotide (lanes 5–8), and isolated proteins subjected to Western blot analysis with antibodies specific to (A) phosphotyrosine, (B) STAT1 or (C) STAT3. On high-resolution analysis, STAT3 has been observed to be a doublet.

SIE oligonucleotide used in the present study contains a T in this position. Therefore, it is possible that the IL-2-activated FcγR-GAS binding protein which does not bind the SIE could be the human homolog of MGF. Finally, IL-4-activated STATs have been described (Kotanides and Reich, 1993; Schindler *et al.*, 1994), and the relationship between these factors and IL-2-regulated STATs is currently under investigation. Preliminary results indicate that the IL-2- and IL-4-induced STATs are distinct (C.Schindler, personal communication).

Importantly, neither JAK tyrosine phosphorylation nor activation of the IL-2-responsive STAT proteins was observed in response to TCR stimulation. Although receptors which are not members of the hematopoietic family have been shown to activate JAKs and STAT proteins, the TCR did not activate those investigated in the present study. This finding is of particular relevance, as the TCR and IL-2R act at distinct stages of the T cell cycle and trigger discrete cellular responses (Cantrell and Smith, 1984; Stern and Smith, 1986). Genes induced specifically by the TCR or IL-2R have been identified (Stern and Smith, 1986; Beadling *et al.*, 1993), yet no IL-2-restricted signaling pathways have been characterized previously which might account for this difference. The JAK/STAT pathway thus provides the first clue as to the mechanisms of differential gene regulation effected by these two receptors.

Based on studies of EGF and IFN signaling, it has been suggested that JAK1 is the kinase responsible for STAT1 tyrosine phosphorylation (Shuai *et al.*, 1993). However, although IL-2 induced the tyrosine phosphorylation and activation of JAK1 to as great an extent as IFNα, there

was no detectable IL-2-induced tyrosine phosphorylation of STAT1. One explanation for this difference could be that STAT1 tyrosine phosphorylation requires JAK1 and an additional determining effect of a second JAK family member. The specificity of the IFNα response would thus be dictated by the combined actions of JAK1 and Tyk2, whereas the IL-2 response would be controlled by JAK1 in conjunction with JAK3. A more likely explanation is that STAT tyrosine phosphorylation requires not only the activation of the JAK kinases, but also the recruitment of the STAT proteins to the receptor–JAK complex (Fu and Zhang, 1993; Greenlund *et al.*, 1994; Lutticken *et al.*, 1994). The data reported herein are in accordance with a model in which the specificity of the STAT activation is defined not only by the patterns of JAKs activated, but also by the context of the receptor complexes with which the JAKs are associated.

Materials and methods

Cell culture

Kit225 cells (Hori *et al.*, 1987) were maintained in RPMI 1640 with 10% fetal calf serum and 1 nM recombinant IL-2 (Eurocetus). The cells were arrested in a quiescent state by washing three times in PBS, and replacing in RPMI 1640 with 10% serum in the absence of IL-2 for 48 h. Primary human T cells were isolated and maintained as described previously (Cantrell and Smith, 1984), and TCR stimulation was performed with the anti-CD3 mAb UCHT1 (P.Beverley, University College London, UK). IFNα was obtained from Wellcome Research Laboratories (Beckenham, UK).

Immunoprecipitation and Western blot

Cells were lysed at ~40×10^6/ml in 50 mM HEPES, pH 7.4, 150 mM NaCl, 0.5% NP-40, 10 mM NaF, 10 mM iodoacetamide, 1 mM Na_3VO_4, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml chymostatin. Immunoprecipitations of pre-cleared lysates were performed for 1–2 h at 4°C with antibodies directed against the following: JAK1 C-terminal peptide (Upstate Biotechnology Inc.), JAK1 N-terminal peptide (Silvennoinen *et al.*, 1993c), JAK1 C-terminal fusion protein (Muller *et al.*, 1993a), JAK2 (A.Ziemiecki, unpublished data), JAK3 (Witthuhn *et al.*, 1994), Tyk2 (Upstate Biotechnology Inc.), anti-phosphotyrosine (monoclonal FB2, 10 μg/ml), STAT1 C-terminal fusion protein (Schindler *et al.*, 1992a), STAT2 (Fu *et al.*, 1992) and STAT3 (Zhong *et al.*, 1994b). Immunoprecipitated proteins were separated by SDS–PAGE and transferred to PVDF membranes. Western blots were performed with antibodies against the following: phosphotyrosine (4G10, Upstate Biotechnology; PY20, ICN Biomedicals Inc.), JAK1 fusion protein (Muller *et al.*, 1993a), JAK2 (Upstate Biotechnology, Inc.), Tyk2 (Upstate Biotechnology Inc.), STAT1 (Schindler *et al.*, 1992a), STAT2 (Fu *et al.*, 1992) and STAT3 (Zhong *et al.*, 1994b). Immunoreactive bands were visualized with the epichemiluminescence Western blotting system (Amersham).

In vitro kinase assay

JAK1 was immunoprecipitated with protein A–Sepharose-coupled antisera raised against a JAK1 C-terminal fusion protein (Muller *et al.*, 1993a). Precipitated complexes were washed 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 followed by separation by SDS–PAGE. ^{32}P-labeled proteins were detected by autoradiography.

EMSA and affinity purification of DNA binding proteins

Whole-cell extracts were prepared by lysis of ~40×10^6 cells/ml in a lysis buffer comprising 50 mM Tris–Cl, pH 8.0, 0.5% NP-40, 150 mM NaCl, 10% glycerol, 0.1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_3, 1 mM PMSF, 1 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml chymostatin. The oligonucleotide sequences used were derived from the high-affinity SIE of the c-*fos* gene (SIEM67) GTCGACATTTCCCGTAAATC; FcγR-GAS, GTATTTCCCAGAAAAGGAAC; Ly6E-GAS, TTATGCATATTCCTGTAAGTG; GBP-GAS, AAGTACTTTCAGTTTCATATTACTCTAAATC; and 9-27 ISRE, TTTACAAACAGCAGGAAATAGAAACTTAAGAGAAATACA. Probes were end-labeled with [γ-^{32}P]ATP and ~30 000 c.p.m. used per reaction (~2 ng double-stranded DNA). 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, with extracts from ~1.5×10^5 cell equivalents. 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. For competition assays, unlabeled oligonucleotides were pre-incubated with cell extracts for 10 min at room temperature prior to probe addition, and for supershifts the pre-incubation was for 1 h at 4°C with control antisera (KSTIC), antisera against a STAT1 C-terminal fusion protein, or against STAT2. DNA binding proteins were isolated from extracts of ~20×10^6 cells in the above buffer by incubation at 4°C for 1 h with 75 μg/ml poly(dI–dC) and 1 μg double-stranded 5′-biotinylated FcγR-GAS oligonucleotide coupled to Streptavidin agarose. Complexes were washed twice in lysis buffer, eluted in sample buffer and separated by SDS–PAGE. Bound proteins were detected by Western blot analysis as described above.

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