The EMBO Journal vol.14 no.6 pp.1166–1175, 1995

Interleukin-3, granulocyte-macrophage colony stimulating factor and interleukin-5 transduce signals through two STAT5 homologs

Alice L.-F.Mui[1], Hiroshi Wakao, Anne-Marie O'Farrell, Nobuyuki Harada and Atsushi Miyajima

Department of Cell Biology, DNAX Research Institute of Molecular and Cellular Biology, 901 California Avenue, Palo Alto, CA 94304-1104, USA

[1]Corresponding author

Communicated by P.Berg

Interleukin-3 (IL-3) is an important regulator of hemopoiesis and considerable effort has been directed towards the study of its mechanism of signal transduction. In this paper, we describe the first molecular identification of a STAT transcription factor that is activated by IL-3. STATs exist in a cytoplasmic, transcriptionally inactive form which, in response to extracellular signals, become tyrosine phosphorylated and translocate to the nucleus where they bind to specific DNA elements. Several of these DNA elements were found which bind proteins in an IL-3-responsive manner. Analysis of these bandshift complexes with available antibodies to the known STATs suggests that IL-3 activates the DNA-binding ability of STAT5, a protein which was originally characterized as a prolactin-responsive transcription factor in sheep. IL-5 and granulocyte-macrophage colony stimulating factor (GM-CSF), which share a common signaling receptor subunit with IL-3, also activate STAT5. Unexpectedly, two murine STAT5 homologs, 96% identical to each other at the amino acid level, were isolated and IL-3-dependent GAS binding could be reconstituted in COS cells transfected with IL-3 receptor and either STAT5 cDNA. In IL-3-dependent hemopoietic cells, both forms of STAT5 are expressed and activated in response to IL-3.

Key words: hemopoietic receptor/JAK kinase/signal transduction/tyrosine phosphorylation

Introduction

Hemopoiesis is tightly regulated, in part, by soluble factors called cytokines (Arai *et al.*, 1990). Among these, interleukin-3 (IL-3) was one of the earliest characterized because of its profound effects on cells at multiple stages of hemopoietic development (Schrader, 1986). IL-3 not only supports the growth of both pluripotent stem cells and the more differentiated, committed progenitors, but also stimulates the functional activity of some fully differentiated cells. Two other cytokines, granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-5 (IL-5), are more restricted in action and possess subsets of these IL-3 activites (Arai *et al.*, 1990). Cloning of the receptors for IL-3, GM-CSF and IL-5 has provided a molecular basis for the observed similarity in action between these cytokines (Miyajima *et al.*, 1992). The receptor for each cytokine consists of a ligand-specific α subunit and a common β subunit which is shared by the three cytokines. Both subunits belong to the class I cytokine receptor superfamily and both are required to transduce a signal across the membrane; however, the β subunit, because of its considerably larger cytoplasmic domain, is believed to perform the greater role in signal transduction (Sakamaki *et al.*, 1992; Sato *et al.*, 1993). In mice, the situation is complicated by the existence of two different β subunits (Miyajima *et al.*, 1992). In addition to β_c (originally called AIC2B; Gorman *et al.*, 1990), the murine counterpart of the human common β subunit, a second β subunit specific for IL-3 exists (Itoh *et al.*, 1990). This alternate β subunit, $\beta_{IL\text{-}3}$ (or AIC2A), associates only with IL-3Rα to generate a high-affinity receptor. No functional difference has been found between the two different forms of the high-affinity murine IL-3 receptor (Hara and Miyajima, 1992).

Although the IL-3R does not possess intrinsic tyrosine kinase activity, one of the earliest events to occur after IL-3 binding to its receptor is induction of protein tyrosine phosphorylation (Koyasu *et al.*, 1987; Sorensen *et al.*, 1989; Isfort and Ihle, 1990; Linnekin *et al.*, 1992). Many cellular proteins, including the receptor itself (Isfort *et al.*, 1988; Sorensen *et al.*, 1989; Duronio *et al.*, 1992; Sakamaki *et al.*, 1992), become tyrosine phosphorylated in response to IL-3, and much effort has been directed towards identifying both these tyrosine-phosphoryated substrates and the kinase(s) responsible. Two Src family kinases, Lyn and Fyn, have been implicated in IL-3 action in certain cell types (Torigoe *et al.*, 1992; Minami *et al.*, 1993). However, recently a non-Src type kinase, JAK2, has been shown to be activated by IL-3 (Silvennoinen *et al.*, 1993) and GM-CSF (Quelle *et al.*, 1994).

JAK2 belongs to a family which includes JAK1, JAK3 and TYK2 (Ihle *et al.*, 1994). These kinases are involved in the signaling pathways of many cytokine receptors investigated: indeed, JAK1 or JAK2 have been reported to directly associate with several cytokine receptors including the receptors for GM-CSF (Quelle *et al.*, 1994), erythropoietin (Witthuhn *et al.*, 1993), interleukin-6 (Stahl *et al.*, 1994), G-CSF (Nicholson *et al.*, 1994) and growth hormone (Artgetsinger *et al.*, 1993). More importantly, from studies of the interferon system, the JAK kinases have been shown to function upstream of transcription factors called STATs (Signal Transducers and Activators of Transcription) (Darnell *et al.*, 1994). STAT proteins exist in latent cytoplasmic forms which, upon ligand stimulation, become tyrosine phosphorylated, undergo dimerization and translocate to the nucleus where they bind to DNA sequences, most of which are related to the

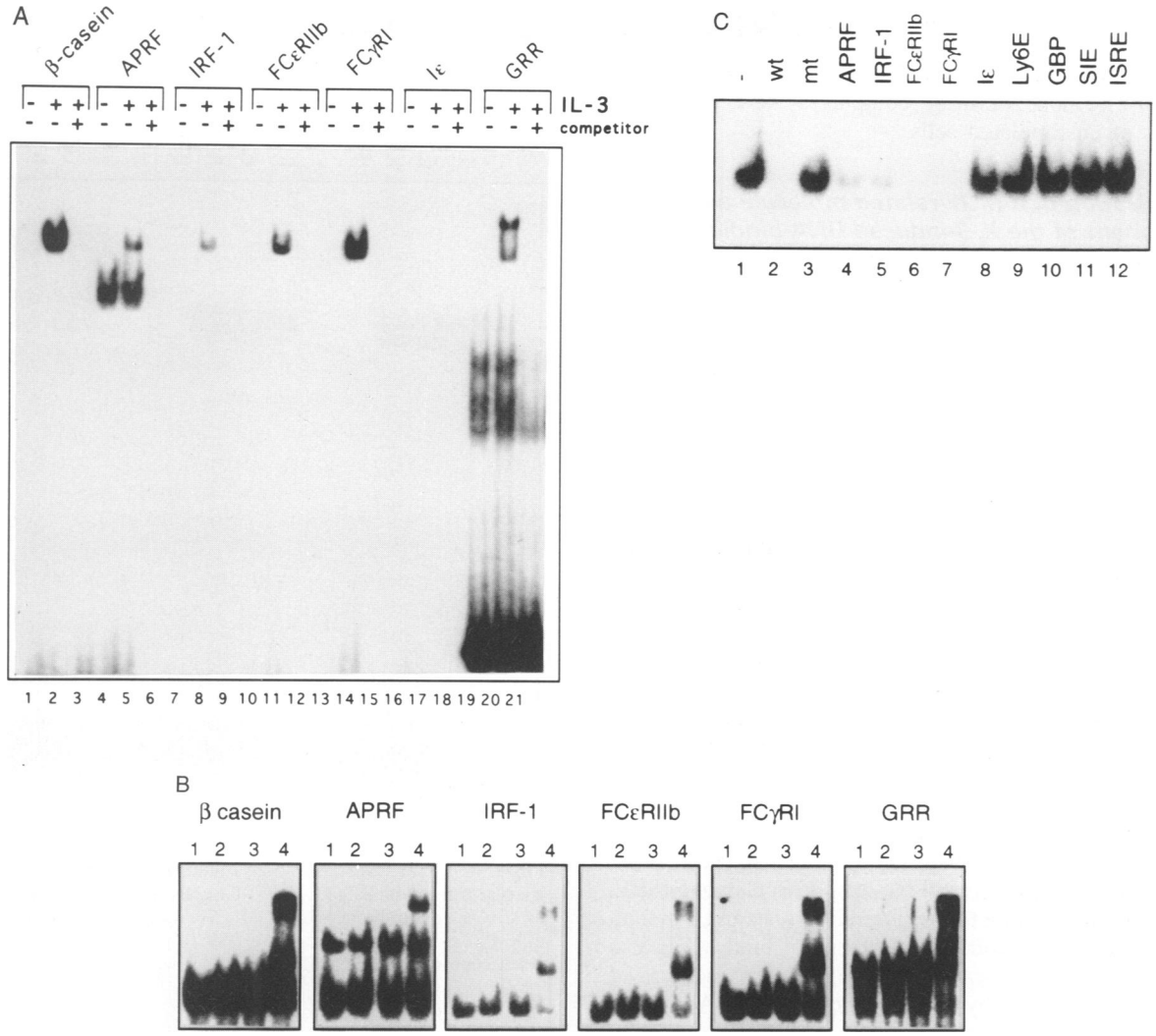

Fig. 1. (**A**) IL-3-induced GAS-related probe-binding activity. Nuclear extracts were prepared from OTT-1 cells stimulated with IL-3 or control buffer for 10 min at 37°C. These extracts were then subjected to EMSA analysis with various labeled GAS-related probes in the presence and absence of a 100-fold molar excess of unlabeled probe. (**B**) Antibodies to MGF react with the IL-3-induced GAS-related probe-binding activity. Extracts from IL-3-stimulated cells were prepared as described in (A). For each probe, no antibodies were added to the gel shift reaction for lane 1. The following antibodies were added to the reactions in the other lanes: lane 2, anti-STAT1; lane 3, control IgY; lane 4, anti-MGF IgY. (**C**) Ability of other GAS-related sequences to compete with the β-casein GAS probe. Nuclear extracts from IL-3-stimulated cells were incubated with labeled β-casein probe in the absence (control) or presence of a 100-fold molar excess of various unlabeled GAS-related oligonucleotides.

gamma interferon (IFNγ) activated site (GAS), a regulatory element in the promoter of IFNγ-inducible genes (Darnell *et al.*, 1994). The STAT family presently includes six members, each of which functions in specific cytokine systems (Darnell *et al.*, 1994; Hou *et al.*, 1994). For example, while IFNα activates STAT1 and STAT2 (Fu *et al.*, 1992; Schindler *et al.*, 1992b), IL-6 utilizes STAT3 (Akira *et al.*, 1994; Zhong *et al.*, 1994b).

An IL-3-responsive STAT has not yet been identified; however, since IL-3 utilizes a JAK family member it is reasonable to presume that a STAT protein also functions in IL-3 signaling. In this paper, we report that IL-3 activates STAT molecules which are related to a prolactin-responsive factor that was first described in lactating sheep mammary gland (Wakao *et al.*, 1992, 1994). This factor, originally called Mammary Gland Factor (MGF), is now referred to as STAT5 (Gouilleux *et al.*, 1994). GM-CSF and IL-5, which share a common signaling subunit with IL-3, stimulate a STAT response similar to IL-3. Unexpectedly, however, during isolation of the mouse counterpart of MGF (sheep STAT5) we found two highly homologous genes which we have designated STAT5A and STAT5B.

Results

Identification of an IL-3-responsive GAS-related sequence

As an initial step in defining an IL-3-responsive STAT, several GAS-related sequences were assessed for their ability to undergo IL-3-dependent shifts in an electrophoretic mobility shift assay (EMSA). As shown in Figure 1, nuclear extracts from OTT-1 cells, a murine hemopoietic cell line (Hawley *et al.*, 1991), treated for 10 min with IL-3, contained proteins that bound to the β-casein, APRE, IRF-1, FcεRIIb, FcγRI and GRR, but not Iε GAS-related sequences (sequences are defined in Materials and methods). These gel shift bands are not observed in the

presence of an excess of unlabeled probe and with the exception of the two lower bands detected with the APRE (Figure 1A, lanes 4–6) and GRR probe (Figure 1A, lanes19–21), none of these complexes were present in extracts of unstimulated cells.

Identification of a MGF-related molecule as a component of the IL-3-induced DNA-binding activity

The composition of the IL-3-induced DNA−protein complexes was analyzed using available antibodies to STAT1 and MGF (STAT5). As shown in Figure 1B, antibodies to STAT1 had no effect on the IL-3-induced bands (lane 2). Antibodies to MGF, on the other hand, supershifted a significant fraction of each of the IL-3-stimulated complexes (lane 4) while control IgY did not (lane 3). This result suggests that IL-3 activates a molecule immunologically related to MGF. At the time these studies were performed, antibodies to STAT2, 3, 4 and 6 were not yet available, but subsequent experiments with these antibodies confirmed a lack of reactivity with these IL-3-induced complexes (data not shown).

Another criterion with which to evaluate the relatedness of the IL-3-induced DNA-binding activity to the MGF originally characterized in sheep is to compare the ability of different GAS-related sequences to compete with the β-casein probe for binding. Consistent with the data shown in Figure 1, the APRE, Fcε, Fcγ and IRF-1 GAS oligonucleotides competed with β-casein probe for binding (Figure 1C). Several other GAS-related sequences, Iε, Ly6E, GBP, SIE and the IFNα response element ISRE, did not inhibit binding to the β-casein probe, and a mutant β-casein oligonucleotide which does not bind MGF also had no effect on β-casein binding. This spectrum of reactivity displayed by the IL-3-induced DNA-binding activity completely mirrors the pattern exhibited by prolactin-stimulated MGF (H.Wakao, N.Harada, T.Kitamura, A.Mui and A.Miyajima, submitted).

Since the IL-3 receptor shares a signaling component with the receptors for GM-CSF and IL-5 (Miyajima *et al.*, 1992), a MGF-related molecule may also be implicated in GM-CSF and IL-5 action. As shown in Figure 2, GM-CSF and IL-5 similarly stimulate the formation of β-casein probe binding activity and this activity could also be supershifted with antibodies to MGF.

IL-3 stimulates tyrosine phosphorylation and nuclear translocation of a MGF-related molecule

A requisite step in the activation of STAT proteins is phosphorylation on tyrosyl residues (Schindler *et al.*, 1992a; Shuai *et al.*, 1992). To further characterize the involvement of a MGF-related molecule in IL-3 signal transduction, proteins were immunoprecipitated with anti-MGF antibodies from IL-3-stimulated cells and subjected to Western blotting analysis with the anti-phosphotyrosine antibody 4G10. As shown in Figure 3, a 90 kDa tyrosine-phosphorylated band could be immunoprecipitated from IL-3-stimulated cells with anti-MGF (lanes 6 and 8), but not control antibody (lanes 2 and 4). Interestingly, phosphorylation of a MGF-related molecule could also be observed in cells which had been cooled to and stimulated at 4°C (lane 6). A similar 90 kDa tyrosine-phosphorylated

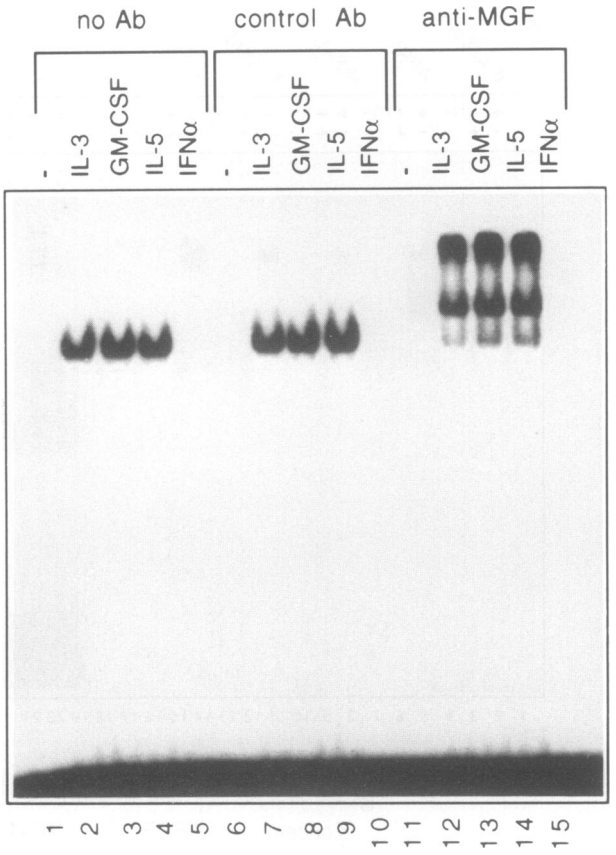

Fig. 2. IL-3, GM-CSF and IL-5 all stimulate β-casein probe binding. Nuclear extracts prepared from OTT-1 cells stimulated for 10 min at 37°C with control buffer, IL-3, GM-CSF, IL-5 or IFNα were subjected to EMSA using labeled β-casein probe. To a separate set of reactions, either control IgY or anti-MGF antibodies were added.

band could be immunoprecipitated from GM-CSF- and IL-5-treated cells (Figure 3B).

Subsequent to tyrosine phosphorylation, STATs dimerize and translocate to the nucleus (Shuai *et al.*, 1993, 1994). Thus, the presence of a MGF-related molecule in the nuclear fraction from cells stimulated with IL-3, GM-CSF or IL-5 was tested by immunoblotting this fraction with anti-MGF antibody. The polyclonal anti-MGF antibody recognizes two bands of ~90 kDa (Figure 3C, lanes 2, 3 and 4). The upper band is non-specific since it is detected even when Western analysis is performed with the anti-MGF antibody in the presence of the immunizing peptide (data not shown). Furthermore, the appearance of this upper band is not dependent on cytokine stimulation; it is observed in all lanes including that of the unstimulated sample (Figure 3C, lane 1). After IL-3, GM-CSF or IL-5 treatment, the lower anti-MGF reactive band could clearly be detected in the nuclear fraction (Figure 3C, lanes 2, 3 and 4). The presence of this lower band can be competed away by the immunizing peptide (data not shown).

Isolation of the cDNA encoding murine STAT5

A cDNA library from an IL-3-dependent cell line, MC/9, was screened with the ovine STAT5 (MGF) cDNA to isolate the murine counterpart. Two different but highly homologous murine clones, designated STAT5A and STAT5B (Figure 4), were isolated. These two molecules

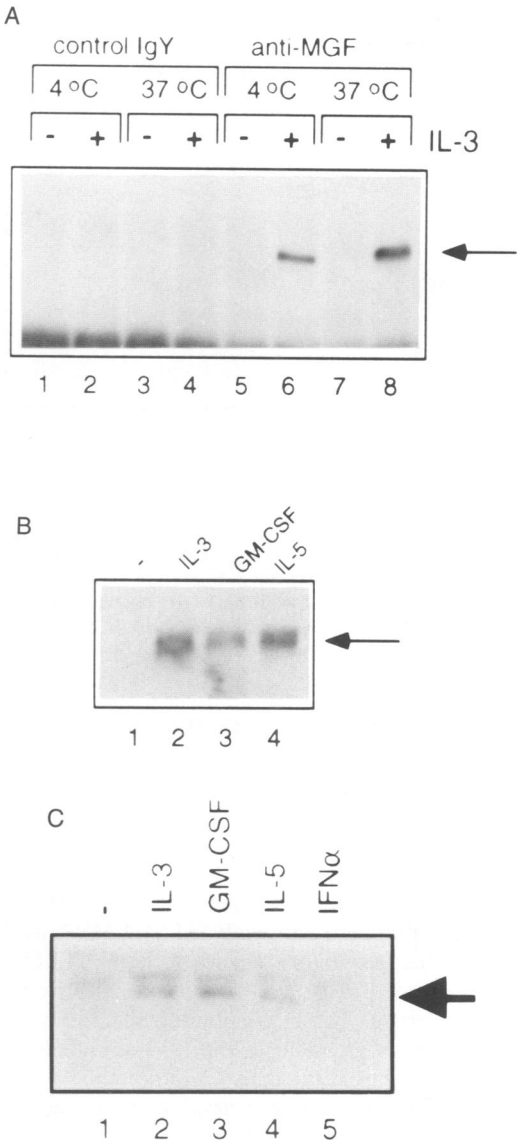

Fig. 3. (**A**) Tyrosine phosphorylation of a MGF-related molecule. Cell lysates from OTT-1 cells stimulated either at 4°C (lanes1, 2, 5, 6) or 37°C (3, 4, 7, 8) with control buffer (lanes 1, 3, 5, 7) or IL-3 (lanes 2, 4, 6, 8) were immunoprecipitated with control IgY (lanes 1–4) or anti-MGF (5–8). The immunoprecipitates were then subjected to Western analysis with anti-phosphotyrosine antibody 4G10. In (**B**), cell lysates from OTT-1 cells treated with control (lane 1), IL-3 (lane 2), GM-CSF (lane 3) or IL-5 (lane 4) were immunoprecipitated and analyzed as in (A). (**C**) Nuclear translocation of a MGF-related molecule. The nuclear fractions of IL-3 (lane 2), GM-CSF (lane 3), IL-5 (lane 4), IFNα (lane 5) or control treated (lane 1) OTT-1 cells were separated by SDS−PAGE and subjected to Western analysis with anti-MGF antibody. The arrow indicates the position of specific, anti-MGF-reactive band.

```
STAT5A   1 MAGWIQAQQLQGDALRQMQVLYGQHFPIEVRHYLAQWIESQPWDAIDLDNPQDRGQATQL
STAT5B     ..M...........H...A.............S......A..S.......ENIK....
MGF        ..............................................................A.V...

STAT5A  61 LEGLVQELQKKAEHQVGEDGFLLKIKLGHYA-TQLQNTYDRCPMELVRCIRHILYNEQRL
STAT5B     ................................-....S.......................
MGF        ..............................VHVSSRTRTTAA.WSWL..............

STAT5A 120 VREANNCSSPAGVLVDAMSQKHLQINQRFEELRLITQDTENELKKLQQTQEYFIIQYQES
STAT5B     .....G.....S.A.............T.....................................
MGF        ....T.GN.S..I..............T......V.....................................

STAT5A 180 LRIQAQFAQLGQLNPQERMSRETALQQKQVSLETWLQREAQTLQQYRVELAEKHQKTLQL
STAT5B     .....................................................................
MGF        ..........A.......L..............A.....................................

STAT5A 240 LRKQQTIILDDELIQWKRRQQLAGNGGPPEGSLDVLQSWCEKLAEIIWQNRQQIRRAEHL
STAT5B     .....................................................................
MGF        ...................HDWR.MEA..R-.....................................

STAT5A 300 CQQLPIPGPVEEMLAEVNATITDIISALVTSTFIIEKQPPQVLKTQTKFAATVRLLVGGK
STAT5B     .....................................................................
MGF        .....................................................................

STAT5A 360 LNVHMNPPQVKATIISEQQAKSLLKNENTRNECSGEILNNCCVMEYHQATGTLSAHFRNM
STAT5B     ...............................DY....................................
MGF        ..................................................R..................

STAT5A 420 SLKRIKRADRRGAESVTEEKFTVLFESQFSVGSNELVFQVKTLSLPVVVIVHGSQDHNAT
STAT5B     .......S..............I..D......G.....................N...
MGF        .....................................................................

STAT5A 480 ATVLWDNAFAEPGRVPFAVPDKVLWPQLCEALNMKFKAEVQSNRGLTKENLVFLAQKLFN
STAT5B     .....................................................................
MGF        ..................................................L..................

STAT5A 540 ISSNHLEDYNSMSVSWSQFNRENLPGWNYTFWQWFDGVMEVLKKHHKPHWNDGAILGFVN
STAT5B     ..........................R.....................L..............
MGF        N..S......G.....................................D..................

STAT5A 600 KQQAHDLLINKPDGTFLLRFSDSEIGGITIAWKFDSPDRNLWNLKPFTTRDFSIRSLADR
STAT5B     ....................................QE.MF...M.....................
MGF        .....................................................G..............

STAT5A 660 LGDLNYLIYVFPDRPKDEVFAKYYTPV-----LAKAVDGYVKPQIKQVVPEFVNASTD-A
STAT5B     ..................YS......PCEPAT...A..............A.....-.
MGF        ..................S......-----.....................S..A.S.

STAT5A 715 GASATYMDQAPSPVVCPQPHYNMYPPNPDPVLDQDGEFDLDESMDVARHVEELLRRPMDS
STAT5B     .SG..............A..........S...T..D...EDT.....R.....G.....
MGF        .S...........A...........Q..............T..............-NG

STAT5A 775 LDARLS-PPAGLFTSARSSLS
STAT5B     QWIPHAQS------------
MGF        QSGP..P.......P..G...
```

Fig. 4. Alignment of the predicted peptide sequences of STAT5A, STAT5B and MGF (sheep STAT5). The MGF peptide sequence shown is based on a corrected nucleotide sequence. Non-identical amino acids are indicated.

are ~80% identical both to each other and to the sheep cDNA. At the amino acid level, the homology is much higher: the predicted murine proteins are 96% identical and both share >90% identity to the sheep sequence. This degree of conservation between mouse and sheep is substantially greater than the similarity between either human and mouse STAT1s (60%) or STAT2s (61%) (Zhong *et al.*, 1994a); the murine STAT5s are also more related to the sheep homolog than they are to the other four murine STATs. STAT5A and STAT5B differ mainly at the C-terminus. While the predicted peptide sequence of STAT5 is the same length as the corrected sequence for MGF, STAT5B is shorter by seven amino acids.

Tissue and cellular expression of STAT5A and STAT5B

To examine the tissue and cell expression pattern of the two molecules, S1 nuclease protection analysis was performed by using the STAT5A plasmid as a probe. STAT5A RNA protects a 1000 bp fragment (Figure 5A, lane 2). The homology between STAT5A and STAT5B is sufficiently high, in the region from which this S1 probe is derived, such that STAT5B RNA is able to protect a 800 bp fragment (Figure 5A, lane 3). A survey of tissue and cellular RNA showed that both molecules are ubiquitously expressed with the highest levels in thymus (Figure 5B) and T cells (Figure 5C). Interestingly, with the exception of brain tissue (Figure 5B, lane 2) and phorbol ester- and calcium ionophore-activated D10 cells (a type I helper T-cell clone; Figure 5C, lane 13), the STAT5A and STAT5B appear to be co-ordinately expressed. Interestingly, neither STAT5A or STAT5B expression is restricted to IL-3/GM-CSF/IL-5-dependent cells.

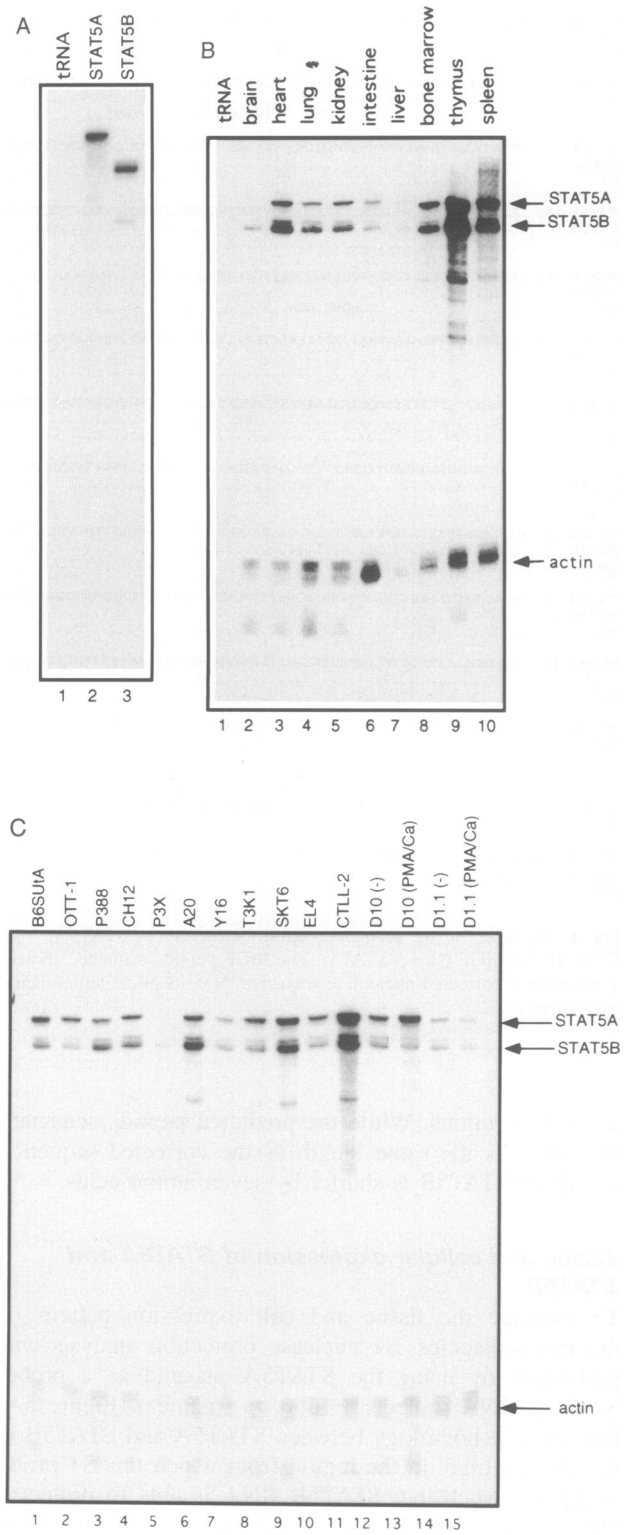

Fig. 5. Tissue and cellular distribution of STAT5A and STAT5B. (**A**) RNA was prepared from COS cells transfected with STAT5A (lane 1) or STAT5B (lane 2) and analyzed by S1 nuclease protection assay using a STAT5A probe. This probe was then used to survey the tissue (**B**) and cellular (**C**) distribution of STAT5A and STAT5B. The lineage of the various cell types used in (C) are: B6SUtA$_1$ and OTT-1, myeloid; P388, macrophage; CH12, P3X, A20 and Y16, B-cell; T3K1 and SKT6, erythroleukemic; EL4, CTLL-2, and D10 and D1.1, T-cell. For lanes 13 and 15, RNA was prepared from D10 (Th1 type) or D1.1 (Th2 type) cells treated for 3 h with phorbol myristic acid and calcium ionophore.

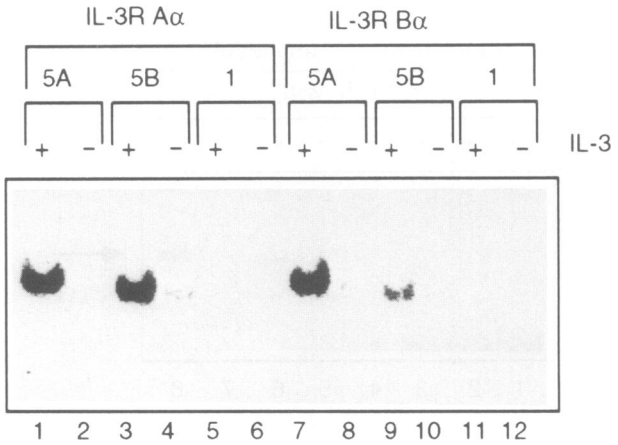

Fig. 6. Reconstitution of STAT5 activation in COS cells. COS cells were transfected with the appropriate cDNAs to generate both forms of the mouse IL-3R: Aα (IL-3Rα and AIC2A, lanes 1, 2, 7, 8) or Bα (IL-3Rα and AIC2B, lanes 3, 4, 9, 10). These cells were also co-transfected with either STAT1 (lanes 5, 6, 11, 12), STAT5A (lanes 1, 2, 7, 8) or STAT5B (lanes 3, 4, 9, 10) cDNAs. Two days after transfection, the cells were stimulated, or not, with IL-3 and cell extracts were analyzed for β-casein probe binding.

Reconstitution of STAT5 activation in COS and OTT-1 cells

The function of the cloned murine STAT5s was assessed by expressing them in combination with either form of the murine IL-3R (i.e. IL-3Rα and AIC2A or IL-3Rα and AIC2B; Hara and Miyajima, 1992) in COS cells, an SV40-transformed monkey epithelial cell line. Whole-cell extracts were prepared and analyzed by EMSA using the β-casein probe. As Figure 6 shows, both STAT5A and STAT5B, but not STAT1, can respond to IL-3 by binding the β-casein probe. The DNA-binding specificity of both cloned STAT5s is identical to the IL-3-stimulated DNA-binding activity in OTT-1 cells (data not shown).

DNA binding in a gel shift assay does not necessarily imply transactivation ability. In order to evaluate whether STAT5A and STAT5B could mediate transcription from the β-casein promoter, OTT-1 cells were transiently transfected with murine STAT5A or STAT5B cDNA and a reporter plasmid in which the luciferase gene is placed under the control of the minimal β-casein promoter. As Figure 7 shows, cells transfected with just the reporter gene display IL-3-dependent production of luciferase activity (lanes 1 and 2). This activation is presumably mediated by endogenous STAT5 protein. Transient overexpression of either STAT5A or STAT5B proteins by co-transfection of either STAT5 cDNA augments luciferase expression from the reporter plasmid (Figure 7, lanes 4 and 6).

IL-3 utilizes both STAT5A and STAT5B in IL-3-dependent cells

Since STAT5A and STAT5B cannot be distinguished functionally in the two assays described above, the question arises as to which molecule is normally activated by IL-3 in cells that normally respond to IL-3. To address this question, the β-casein probe binding activity was purified from IL-3-stimulated MC/9 cells using a β-casein oligonucleotide affinity matrix and compared with recombinant STAT5A and STAT5B isolated in the same manner from transfected COS cells. Figure 8A shows

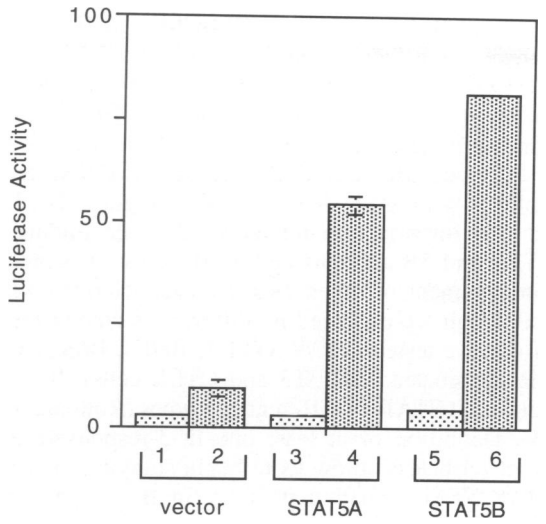

Fig. 7. IL-3 stimulates transactivation of a β-casein luciferase reporter. OTT-1 cells were transiently transfected with the β-casein luciferase reporter plasmid and control vector (lanes 1 and 2), murine STAT5A (lanes 3, 4) or STAT5B (lanes 5 and 6) and luciferase activity measured from cells stimulated (lanes 2, 4, 6) or not (lanes 1, 3, 5) with IL-3. Luciferase activity is expressed in arbitrary units.

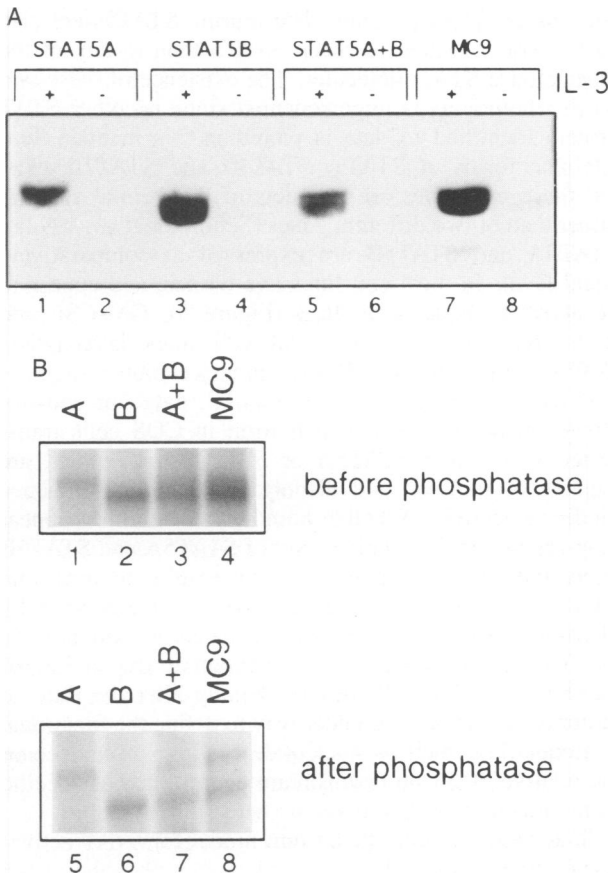

Fig. 8. Comparison of proteins purified using a β-casein oligonucleotide affinity column. In (**A**), proteins were purified from MC9 cells (lanes 7 and 8) or COS cells transfected with IL-3Rα, AIC2A and STAT5A (lanes 1 and 2), STAT5B (lanes 3 and 4) or STAT5A plus STAT5B (lanes 5 and 6). The proteins were resolved by SDS−PAGE and analyzed by immunoblotting with anti-phosphotyrosine antibodies. In (**B**), proteins from the IL-3-stimulated samples in (A) were radioiodinated and treated (lanes 5–8) or not (lanes1–4) with alkaline phosphatase before separation by SDS−PAGE and autoradiography.

an anti-phosphotyrosine Western blot of the β-casein oligonucleotide affinity-purified proteins. The apparent molecular weights of phosphorylated STAT5A and STAT5B differ by ~1 kDa. Interestingly, the β-casein binding activity purified from either MC9 cells or COS cells transfected with both STAT5A and STAT5B exhibits an intermediate mobility.

Since phosphorylation alters the electrophoretic mobility of proteins in SDS−PAGE gels, the purified STAT5A and STAT5B proteins were radioiodinated and treated with alkaline phosphatase before analysis by SDS−PAGE. Figure 8B shows the autoradiograph of the proteins before (lanes 1–4) and after (lanes 5–8) alkaline phosphatase treatment. The untreated samples display the same heterogeneity seen in Figure 8A. However, after alkaline phosphatase treatment, STAT5A and STAT5B are clearly resolved (compare lane 5 with lane 6). Importantly, the broad band representing the β-casein binding activity purified from MC/9 cells (Figure 8A, lane 7, and Figure 8B, lane 4) resolves into two bands with mobilities corresponding to those of STAT5A and STAT5B. Thus, both STAT5A and STAT5B appear to be activated in response to IL-3 in MC9 cells. Similar observations were seen in other IL-3-dependent cells, OTT-1 and Ba/F3 (data not shown).

The membrane-proximal domain of the GM-CSFR β chain is required for activation of STAT5

Previously, we defined two regions in the cytoplasmic domain of the human GM-CSFR β chain which transduce distinct signals, both of which are necessary for support of long-term proliferation (Sakamaki *et al.*, 1992; Sato *et al.*, 1993). Using these carboxy deletion mutants, the region required for activation of JAK2 kinase was mapped between amino acids 455 and 517 of the β chain (Quelle *et al.*, 1994). It was thus interesting to determine whether this region of the receptor is also required for activation of STAT5. As shown in Figure 9A, the wild-type receptor, as well as every deletion mutant except for β_{455}, was able to induce phosphorylation of STAT5, as assessed by anti-phosphotyrosine immunoblotting of anti-MGF immunoprecipitated proteins. This correlated with their ability to induce tyrosine phosphorylation of JAK2. Since the murine IL-3 response in β_{455} is comparable with that of wild type, this diminished phosphorylation is not due to other defects downstream of the receptor, such as decreased expression of STAT5 in the β_{455} cells. However, tyrosine phosphorylation may not be sufficient either for activation of DNA binding or transactivation ability, so these two parameters were also examined. As Figure 9B and C shows, truncation of the β chain at residue 455 abolishes its ability to stimulate DNA binding or transactivation of the β-casein luciferase reporter gene. Interestingly, the transactivational ability of β_{763} is ~30% greater than that of the wild-type receptor. Increased levels of JAK2 kinase activity (Quelle *et al.*, 1994), as well as the activity of several other signaling molecules (Sakamaki *et al.*, 1992; Sato *et al.*, 1993), have also been previously observed when the β_{763} deletion mutant is compared with the full-length receptor.

Discussion

In this paper, we report the first identification of an IL-3-responsive STAT. We show, using several different criteria,

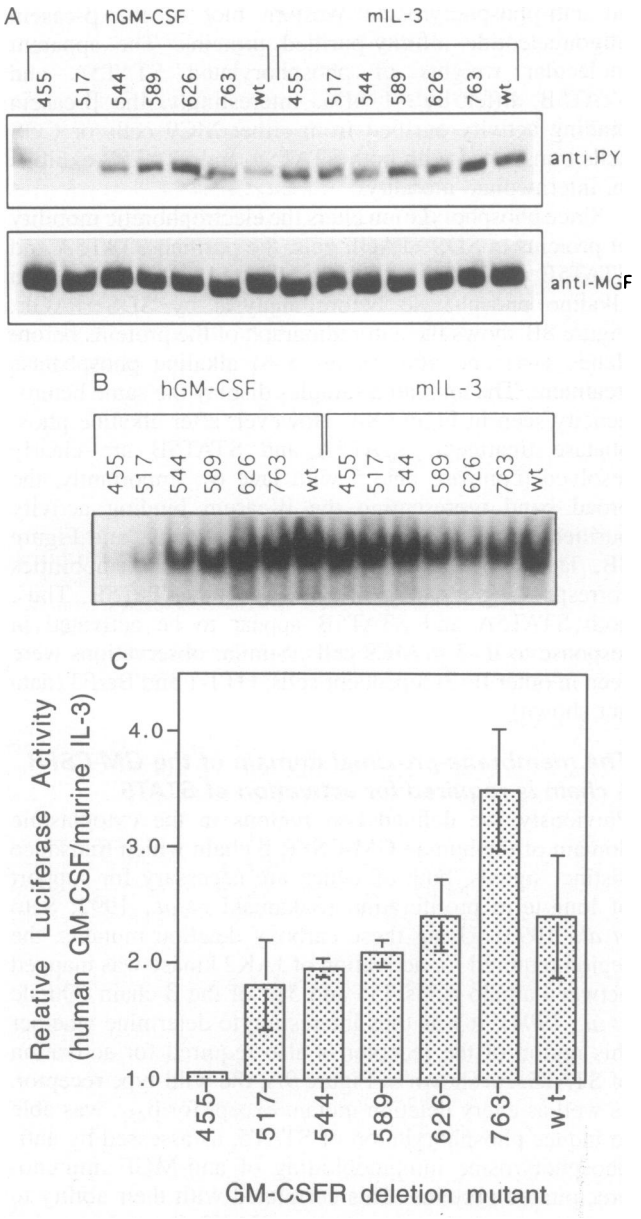

Fig. 9. (**A**) Tyrosine phosphorylation of STAT5 by human GM-CSF receptor mutants. Ba/F3 transfectants expressing wild-type or mutant receptors were stimulated with murine IL-3 or human GM-CSF and immunoprecipitated with or anti-MGF. The immunoprecipitates were analyzed by Western blotting with anti-phosphotyrosine (top panel) or anti-MGF (bottom panel). (**B**) Stimulation of β-casein binding. Nuclear extracts from Ba/F3 cells expressing wild-type or mutant receptors stimulated with murine IL-3 or human GM-CSF were analyzed for β-casein probe binding activity. (**C**) Transactivation by human GM-CSF receptor mutants. Ba/F3 cells expressing the various GM-CSF receptors were transiently transfected with murine STAT5 and the β-casein luciferase reporter plasmid. The cultures were then divided into two and cultured in either murine IL-3 or human GM-CSF for 10 h. Cell extracts were then prepared and luciferase activity determined. Results are plotted as the ratio of the activity detected from human GM-CSF versus murine IL-3-stimulated cells to normalize for transfection efficiency.

that IL-3 (and GM-CSF and IL-5) activates molecules related to STAT5, a molecule originally purified as a prolactin-responsive factor from sheep. Using the sheep cDNA, we isolated two highly related murine homologs. Both are expressed widely and are activated in response not only to IL-3, but also to GM-CSF and IL-5. While IL-3-responsive factors that bind to either GRR (Larner *et al.*, 1993) or IRF-1 (Rothman *et al.*, 1994) probes have been reported, at least a portion of these GRR or IRF-1 binding activities is also related to STAT5 since we were able to supershift both GRR and IRF-1 DNA−protein complexes with antibodies to STAT5 (Figure 1B). However, it is important to remember that the finding that STAT5A and 5B are activated by IL-3 does not preclude the involvement of other, as yet uncharacterized STATs, and although we observed no differences among the cell lines that we tested (MC/9, OTT-1, Ba/F3, B6SUtA$_1$ and IL-3R transfected NIH 3T3 and CTLL cells), IL-3 may use different STATs in different cell types (Rothman *et al.*, 1994). Definition of at least one IL-3-responsive STAT does, nevertheless, allow us to begin studying the role of the JAK-STAT signaling pathway in IL-3 signal transduction.

Unexpectedly, two different but highly homologous STAT5 molecules were isolated. Sequence analysis of the murine STAT5 cDNAs indicates that the sheep and mouse molecules are highly conserved. The homology, ~90% at the amino acid level, is significantly higher than the similarity between any of the previously identified mouse and human STAT proteins. The murine STAT5s are also more closely related to sheep STAT5 than to any of the other mouse STAT molecules. The existence of these two STAT5 homologs is unprecedented since no other STAT protein identified to date is paired in this manner. Two alternate forms of STAT1, STAT1α and STAT1β, exist but these molecules are products of differential splicing rather than of two different genes (Schindler *et al.*, 1992a). STAT5A and STAT5B are expressed at comparatively equal levels in most cell lines and tissues, and appear to be activated equally by IL-3 (Figure 5), GM-CSF and IL-5 (data not shown) in all cell lines investigated (MC/9, OTT-1 and Ba/F3). Preliminary evidence suggests that the two proteins heterodimerize (data not shown). However, homodimers, which form in COS cells transfected with either STAT5A or 5B cDNA by itself, are capable of function and display similar GAS sequence binding selectivity. Whether homo or heterodimers appear may depend on the relative level of STAT5A and STAT5B expressed. In this regard, it is interesting to note that STAT5A, but not STAT5B, appears to be enhanced by phorbol myristic acid (PMA) and calcium ionophore in the Th1 T-cell clone, D10. Differential expression of STAT5A and STAT5B may potentially alter the ratio of dimer types. Efforts are under way to define the functional consequences, such as nucleotide binding preference or the relative effect on downstream events such as specific gene induction, of homo versus heterodimerization.

In accordance with the current model for STAT activation (Darnell *et al.*, 1994), STAT5 becomes phosphorylated on tyrosine residues and translocates to the nucleus in response to IL-3. Significantly, tyrosine phosphorylation occurs even when cells are pre-cooled to and stimulated at 4°C, suggesting that STAT5 and the kinase responsible for this phosphorylation may be closely associated with the IL-3 receptor prior to ligand binding. Indeed, JAK2, a candidate kinase which may mediate STAT5 phosphorylation, does appear able to associate directly with the human GM-CSF β chain in a ligand-independent

manner (Quelle *et al.*, 1994). Whether STAT5 also directly interacts with the receptor, in a manner analogous to that of STAT1 association with the IFNγ (Greenlund *et al.*, 1994) and EGF (Fu and Zhang, 1993) receptors or of STAT3 with the IL-6R (Lutticken *et al.*, 1994), remains to be clarified since attempts to co-immunoprecipitate STAT5 with anti-β chain antibodies have not been successful. Interestingly, cells stimulated at 4°C also display β-casein probe binding activity (data not shown), suggesting that all the events which are required to confer DNA-binding activity on STAT5 occur very rapidly. However, nuclear translocation of STAT5 was observed only when cells were incubated at 37°C (data not shown).

Analysis of the region of the GM-CSFR β chain required for activation of STAT5 revealed that the membrane-proximal domain previously shown to be important for DNA synthesis (Sakamaki *et al.*, 1992), c-*myc* induction (Sato *et al.*, 1993) and JAK2 activation (Quelle *et al.*, 1994) is also necessary for STAT5 activity. This is consistant with other data suggesting that STAT5 may function downstream of JAK2 and may indeed be a direct substrate of JAK2 (Gouilleux *et al.*, 1994). However, the relationship between STAT5 activation and the other signals directed from the membrane-proximal domain, namely c-*myc* induction and DNA synthesis, remains to be determined. Specific inhibition of the STAT5 pathway is required in order to address this question. The genes regulated by STAT5, and thus the contribution of STAT5 to IL-3 signaling, are not yet clear.

Using these GM-CSFR deletion mutants, STAT5 tyrosine phosphorylation, activation of β-casein probe binding and transactivation ability could not be dissociated. Thus, the signal emanating from the membrane-proximal domain appears to be sufficient for signaling STAT5 transcriptional activity. Nevertheless, other regions of the receptor may contribute signals that alter the net effect of STAT5 activation on gene expression. This can occur through signals that lead to modification, i.e. phosphorylation, of the STAT5 complex that modifies its target gene specificity, or through signals which activate other transcription factors which also interact with the promoters of STAT5-regulated genes. Interestingly, neither truncated receptors β_{517} nor β_{544} contain tyrosine phosphorylation sites, yet both are able to activate STAT5. This implies that although STAT proteins normally bind tyrosine-phosphorylated receptors (Greenlund *et al.*, 1994; Hou *et al.*, 1994), this interaction is not essential for STAT phosphorylation and activation. Alternatively, since we were unable to co-immunoprecipitate STAT5 with antibodies which recognize the β chain, the apparent lack of requirement for β-chain phosphorylation suggests that the GM-CSF receptor complex may contain components in addition to the α and β chains which have been defined.

In all parameters examined in this study, STAT5 responds to IL-3, GM-CSF and IL-5 in an identical manner. This might be expected since the three cytokines share a common receptor signaling subunit. However, IL-3, GM-CSF and IL-5 do have unique ligand-binding subunits and exert different signals in some cell types (Heyworth *et al.*, 1990). If STAT5 provides a basis for this difference, then some more subtle modification of STAT5, i.e. specific phosphorylation sites or association with other proteins, must alter its specificity of action. The mechanism of specificity is an important but unresolved issue. Despite obviously different cellular responses, implying distinct gene inductions, all of the signaling events described thus far to the IL-3, GM-CSF and IL-5 receptor systems (Miyajima *et al.*, 1993) are common to all three cytokines and also to others. The STAT pathway, because of the directness of its route from the receptor to the nucleus, may potentially generate specific signals depending on the nature of the STAT family member that interacts with the receptor. It is, therefore, important to determine not only what genes are regulated by STAT5 in response to IL-3, GM-CSF and IL-5, but also whether these cytokines differentially regulate STAT5A and STAT5B.

Materials and methods

Reagents

The antibodies to STAT1 and phosphotyrosine were purchased from UBI (Lake Placid, New York). Polyclonal chicken antibodies raised against the N-terminal 120 amino acid region of sheep MGF have been described previously (Wakao *et al.*, 1994). Recombinant murine IL-5 was from R & D Systems (Minneapolis, MN); IL-3 and GM-CSF were produced and purified as described previously (Miyajima *et al.*, 1986, 1987). All other reagents were from Sigma unless specified.

Cells and cell culture

OTT-1 (Hawley *et al.*, 1991) and MC/9 (Nabel *et al.*, 1981) cells were maintained in RPMI medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin and streptomycin, and 10 ng/ml murine IL-3. Prior to each experiment, these cells were deprived of IL-3 and FCS for 10–12 h in RPMI containing bovine serum albumin (BSA)/linoleic acid (Sigma) and 10 μg/ml transferrin (Boehringer Mannheim). Cell viability was 95–100%, as assessed by trypan blue dye exclusion. COS cells were cultured in DMEM containing 10% FCS.

Nuclear extract preparation and electrophoretic mobility shift assay

Nuclear extracts were prepared essentially as described previously (Masuda *et al.*, 1993), except that the following buffers were used. After washing with phosphate-buffered saline (PBS), cells were resuspended in 50 mM HEPES (pH 7.5), 100 mM sodium flouride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 2 mM sodium molybdate, 2 mM EDTA (PSB buffer) supplemented with 0.2% NP40, 10 mM magnesium chloride, 10 μg/ml aprotinin, 2 μg/ml leupeptin and 2 mM Pefabloc (Boehringer Mannheim). After 1 min on ice, the nuclei were pelleted and washed in the same buffer, but without NP40. The nuclei were again pelleted and then extracted with vigorous agitation at 4°C in PSB containing 0.1% NP40, 0.3 M sodium chloride, 10% glycerol and protease inhibitors as above.

Mobility shift assays were performed in a total volume of 10 μl in the following buffer: 10 mM Tris−HCl (pH 8.0), 100 mM potassium chloride, 5 mM magnesium chloride, 1 mM dithiothreitol, 0.5 mg/ml BSA, 10% glycerol. Each reaction, also containing 1 μg of poly(dI-dC) and 10 fmol of ^{32}P end-labeled probe, was initiated by the addition of ~2 μg of nuclear extract and allowed to incubate at room temperature for 30 min prior to electrophoretic analysis on a 5% polyacrylamide gel in 0.25×TBE buffer.

The oligonucleotide probes used were: wild-type β-casein (5′-AGATTTCTAGGAATTCAATCC-3′), mutant β-casein (5′-AGATTTATTTTAATTCAACC-3′), APRE (5′-GATCCTTCTGGGAATTCCTA-3′), IRF-1 (5′-GATCCATTTCCCGAAATGA-3′), FcεRIIb (5′-GAATTTCTAAGAAGGGA-3′), FcγRI (5′-GTATTTCCCAGAAAAGGAAC-3′), Iε (5′-GTCAACTTCCCAAGAACAGAA-3′), Ly6/E (5′-CATGTTATGCATATTCCTGTAAGTG-3′), GBP(5′-AAGTACTTTCAGTTTCATATTACTCTAAATC-3′), SIE (5′-GTCGACAGTTCCCGTCAATC-3′) and ISRE (5′-GATCGGGAAAGGGAAACCGAAACTGAAGCC-3′).

Immunoprecipitation and Western blotting

After factor stimulation, cells (2×10^7cells/sample) were quickly pelleted and resuspended in 1 ml of PSB containing 0.5% NP40 and protease inhibitors, and gently rocked at 4°C for 45 min. Insoluble material was

then removed by microcentrifuging for 15 min at 15 kr.p.m. and the clarified cell lysate was incubated with either anti-MGF or control IgY at a final dilution of 1/100. After 2 h at 4°C, 10 µl (packed volume, Sigma) of protein A pre-coated with goat anti-chicken IgY (Sigma) was added and the mixture was incubated for an additional 2 h. The beads were then washed three times with 0.1% NP40 in PSB before being boiled for 2 min in SDS sample buffer.

Samples were fractionated on 7.5% polyacrylamide–SDS gels and electrotransferred to Immobilon (Millipore, San Francisco, CA) membranes. The membranes were blocked for 1 h at room temperature in 3.5% BSA in 20 mM Tris–HCl (pH 7.5), 150 mM sodium chloride and 0.5% Tween 20 (TBST) before being incubated with either 1 µg/ml 4G10 anti-phosphotyrosine or 1 µg/ml affinity-purifed anti-MGF for 2 h. After washing three times for 10 min with TBST, the membrane was incubated with 0.1 µg/ml anti-mouse IgG- or anti-chicken IgY-conjugated horseradish peroxidase for 45 min. The membranes were washed as before and bound antibody complexes were visualized by Enhanced Chemilumeniscence (Amersham, Arlington Heights, IL).

Isolation of murine STAT5 cDNA

The entire sheep MGF cDNA was used to screen a MC/9, mouse mast cell line, library in pME18S. Hybridization was carried out in Amersham Rapid Hyb buffer for 2 h at 50°C. Membranes were then given one wash at 50°C with 2×SSPE and 0.1% SDS for 20 min, followed by two 30 min washes at 55°C with the same buffer.

S1 nuclease protection analysis

The S1 nuclease protection assay was performed as described previously (Berk and Sharp, 1978). STAT5A plasmid was digested with *Bst*EII, treated with shrimp alkaline phosphatase and the 4.5 kb fragment isolated by agarose gel electrophoresis. This fragment was end labeled with [γ-^{32}P]ATP to a specific activity of 10^7 c.p.m./µg DNA. RNA, 1 µg of poly(A)$^+$ or 20 µg of total, was hybridized to 4×10^4 c.p.m. of probe in 15 µl of 80% formamide, 0.4 M sodium chloride, 40 mM PIPES (pH 6.4) and 1 mM EDTA at 55°C. After 12 h, 15 µl of cold S1 solution containing 400 U/ml S1 nuclease, 250 mM sodium chloride, 30 mM sodium acetate (pH 4.5) and 1 mM zinc chloride were added, and the mixture incubated at 37°C for 45 min. The protected DNA was then analyzed on a 5% polyacrylamide/8 M urea gel.

Transient transfection in COS, OTT-1 and BaF/3 cells

COS cells were transfected using the DEAE–dextran method and allowed to grow for 24 h in complete media before being deprived of serum for 24 h. Cells were then treated for 10 min with the appropriate cytokine and total cell lysates were prepared as described above. OTT-1 cells were electroporated at 960 µF and 270 V with 15 µg each of a reporter plasmid consisting of a luciferase gene under the control of a minimal β-casein promoter and either murine STAT5, in pME18S, at room temperature in RPMI supplemented with 10 µg/ml DEAE–dextran. After a 12 h recovery period in IL-3-containing media, cells were deprived of IL-3 for 12 h before a 6 h incubation with 10 ng/ml IL-3. Cytoplasmic extracts were prepared by three cycles of freeze–thaw in 0.25 M Tris (pH 7.5), normalized by protein concentration and assayed for luciferase activity (Promega). Ba/F3 cells were electroporated under the same conditions as OTT-1 cells; however, the 12 h recovery period was in IL-4-containing media instead of IL-3 and the subsequent IL-3 deprivation step was omitted.

Oligonucleotide affinity purification of proteins

Affinity matrices consisting of wild-type and mutant β-casein sequences were prepared using standard procedures. Briefly, the complementary oligonucleotides for wild-type and mutant β-casein GAS sequences were annealed, phosphorylated with T4 kinase and ligated with T4 ligase. The resulting multimers were coupled to CNBr Sepharose (Pharmacia) at room temperature in PBS supplemented with 1 mM EDTA. After 16 h, the beads were washed with 0.4 M sodium chloride, 1 mM EDTA in PBS and equilibrated with 0.1% NP40 in PSB before use.

Samples for oligoprecipitation were prepared as described above for immunoprecipitation. Lysate from 2×10^7 cells was pre-cleared with 100 µl, packed volume, of mutant β-casein DNA–Sepharose for 30 min and then incubated with 10 µl of wild-type β-casein DNA–Sepharose for 2 h at 4°C. After washing 3×10 min with 0.1% NP40 in PSB, proteins were eluted with 0.4 M sodium chloride in the same buffer. Eluates were then made 2% SDS, 5% ME and 10% glycerol for SDS–PAGE analysis or diluted 2-fold with 50 mM Tris–HCl (pH 8), 0.1% NP40 for radioiodination.

Radioiodination and alkaline phosphatase treatment of proteins

Protein samples, in 50 µl, were incubated at room temperature with 100 µCi of Na^{125}I and 100 µg/ml chloramine T for 30 min. The reaction was terminated with 200 µg/ml Na metabisulfite and 10 µg/ml NaI. Unincorporated ^{125}I was removed by centrifugation through a 1 ml Sephadex G-25 (Pharmacia) column equilibrated with 0.2% SDS, 150 mM sodium chloride in 50 mM Tris–HCl (pH 8).

Labeled proteins (usually 2 µl) were heated for 2 min at 100°C before 10-fold dilution into shrimp alkaline phosphatase buffer (NEB) supplemented with 0.1% NP40 and protease inhibitors as above. One unit of shrimp alkaline phosphatase was added and the samples incubated at 37°C for 2 h before boiling in 3% SDS, 5% ME and 10% glycerol and separation by SDS–PAGE.

Acknowledgements

The authors thank D.Gorman for many helpful suggestions. We also thank Drs T.Hara for the MC/9 library, Y.Naito and T.Kinoshita for RNA samples, and K.Moore, M.McMahon, T.Kitamura and G.Manning for critical reading of the manuscript. The DNAX Research Institute is fully supported by Schering-Plough Corp.

References

Akira,S., Nishio,Y., Inoue,M., Wang,X.J., Wei,S., Matsusaka,T., Yoshida,K., Sudo,T., Naruto,M. and Kishimoto,T. (1994) *Cell*, **77**, 63–71.

Arai,K., Lee,F., Miyajima,A., Miyatake,S., Arai,N. and Yokota,T. (1990) *Annu. Rev. Biochem.*, **59**, 783–836.

Artgetsinger,L.S., Cambell,G.S., Yang,X., Witthuhn,B.A., Silvennoinen,O., Ihle,J.N. and Carter-Su,C. (1993) *Cell*, **74**, 237–244.

Berk,A.J. and Sharp,P.A. (1978) *Proc. Natl Acad. Sci. USA*, **75**, 1274–1278.

Darnell,J.J., Kerr,I.M. and Stark,G.R. (1994) *Science*, **264**, 1415–1421.

Duronio,V., Clark,L.I., Federsppiel,B., Wieler,J.S. and Schrader,J.W. (1992) *J. Biol. Chem.*, **267**, 21856–21863.

Fu,X.Y. and Zhang,J.J. (1993) *Cell*, **74**, 1135–1145.

Fu,X.Y., Schindler,C., Improta,T., Aebersold,R. and Darnell,J.J. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 7840–7843.

Gorman,D.M., Itoh,N., Kitamura,T., Schreurs,J., Yonehara,S., Yahara,I., Arai,K. and Miyajima,A. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 5459–5463.

Gouilleux,F., Wakao,H., Mundt,M. and Groner,B. (1994) *EMBO J.*, **13**, 4361–4369.

Greenlund,A.C., Farrar,M.A., Viviano,B.L. and Schreiber,R.D. (1994) *EMBO J.*, **13**, 1591–1600.

Hara,T. and Miyajima,A. (1992) *EMBO J.*, **10**, 1875–1884.

Hawley,T., McLeish,W. and Hawley, R. (1991) *Cytokine*, **3**, 60–71.

Heyworth,C., Dexter,T., Kan,O. and Whetton,A. (1990) *Growth Factors*, **2**, 197–211.

Hou,J., Schindler,U., Henzel,W., Ho,T., Brasseur,M. and McKnight,S. (1994) *Stat. Sci.*, **265**, 1701–1706.

Ihle,J., Witthuhn,B., Quelle,F., Yamamoto,K., Thierfelder,W., Krieder,B. and Silvennoinen,O. (1994) *Trends Biochem. Sci.*, **19**, 222–227.

Isfort,R., Stevens,D., May,W.S. and Ihel,J.N. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 7982–7986.

Isfort,R.J. and Ihle,J.N. (1990) *Growth Factors*, **2**, 213–220.

Itoh,N., Yonehara,S., Schreurs,J., Gorman,D.M., Maruyama,K., Ishii,A., Yahara,I., Arai,K. and Miyajima,A. (1990) *Science*, **247**, 324–327.

Koyasu,S., Tojo,A., Miyajima,A., Akiyama,T., Kasuga,M., Urabe,A., Schreurs,J., Arai,K., Takaku,F. and Yahara,I. (1987) *EMBO J.*, **6**, 3979–3984.

Larner,A.C., David,M., Feldman,G.M., Igarashi,K., Hackett,R.H., Webb,D.S., Sweitzer,S.M., Petricoin,E. and Finbloom,D.S. (1993) *Science*, **261**, 1730–1733.

Linnekin,D., Evans,G., Michiel,D. and Farrar,W.L. (1992) *J. Biol. Chem.*, **267**, 23993–23998.

Lutticken,C. *et al.* (1994) *Science*, **263**, 89–92.

Masuda,E.S., Tokumitsu,H., Tsuboi,A., Shlomai,J., Hung,P., Arai,K. and Arai,N. (1993) *Mol. Cell. Biol.*, **13**, 7399–7407.

Minami,Y., Kono,T., Yamada,K., Kobayashi,N., Kawahara,A., Perlmutter,R.M. and Taniguchi,T. (1993) *EMBO J.*, **12**, 759–768.

Miyajima,A., Otsu,K., Schreurs,J., Bond,M.W., Abrams,J.S. and Arai,K. (1986) *EMBO J.*, **5**, 1193–1197.

Miyajima,A., Schreurs,J., Otsu,K., Kondo,A., Arai,K. and Maeda,S. (1987) *Gene*, **58**, 273–281.
Miyajima,A., Hara,T. and Kitamura,T. (1992) *Trends Biochem. Sci.*, **17**, 378–382.
Miyajima,A., Mui,A.L.-F., Ogorochi,T. and Sakamaki,K. (1993) *Blood*, **82**, 1960–1974.
Nabel,G.S., Galli,J., Dvorak,A.M., Dvorak,H.F. and Cantor,H. (1981) *Nature*, **291**, 332–334.
Nicholson,S.E., Oates,A.C., Harpur,A.G., Ziemiecki,A., Wilks,A.F. and Layton,J.E. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 2985–2988.
Quelle,F.W., Sato,N., Witthuhn,B.A., Inhorn,R.C., Eder,M., Miyajima,A., Griffin,J.D. and Ihle,J.N. (1994) *Mol. Cell. Biol.*, **14**, 4335–4341.
Rothman,P. *et al.* (1994) *Immunity*, **1**, 457–468.
Sakamaki,K., Miyajima,I., Kitamura,T. and Miyajima,A. (1992) *EMBO J.*, **11**, 3541–3549.
Sato,N., Sakamaki,K., Terada,N., Arai,K. and Miyajima,A. (1993) *EMBO J.*, **12**, 4181–4189.
Schindler,C., Fu,X.Y., Improta,T., Aebersold,R. and Darnell,J.J. (1992a) *Proc. Natl Acad. Sci. USA*, **89**, 7836–7839.
Schindler,C., Shuai,K., Prezioso,V.R. and Darnell,J.J. (1992b) *Science*, **257**, 809–13.
Schrader,J.W. (1986) *Annu. Rev. Immunol.*, **4**, 205–230.
Shuai,K., Schindler,C., Prezioso,V.R. and Darnell,J.J. (1992) *Science*, **258**, 1808–1812.
Shuai,K., Stark,G.R., Kerr,I.M. and Darnell,J.J. (1993) *Science*, **261**, 1744–1746.
Shuai,K., Horvath,C.M., Huang,L.H., Qureshi,S.A., Cowburn,D. and Darnell,J.J. (1994) *Cell*, **76**, 821–828.
Silvennoinen,O., Witthuhn,B.A., Quelle,F.W., Cleveland,J.L., Yi,T. and Ihle,J.N. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 8429–8433.
Sorensen,P., Mui,A.L.-F. and Krystal,G. (1989) *J. Biol. Chem.*, **264**, 19253–19258.
Sorensen,P.H., Mui,A.L., Murthy,S.C. and Krystal,G. (1989) *Blood*, **73**, 406–418.
Stahl,N. *et al.* (1994) *Science*, **263**, 92–95.
Torigoe,T., O'Conner,R., Santoli,D. and Reed,J.C. (1992) *Blood*, **80**, 617–624.
Wakao,H., Schmitt,N.M. and Groner,B. (1992) *J. Biol. Chem.*, **267**, 16365–16370.
Wakao,H., Gouilleux,F. and Groner,B. (1994) *EMBO J.*, **13**, 2182–2191.
Witthuhn,B.A., Quelle,F.W., Silvennoinen,O., Yi,T., Tang,B., Miura,O. and Ihle,J.N. (1993) *Cell*, **74**, 227–236.
Zhong,Z., Wen,Z. and Darnell,J.J. (1994a) *Proc. Natl Acad. Sci. USA*, **91**, 4806–4810.
Zhong,Z., Wen,Z. and Darnell,J.J. (1994b) *Science*, **264**, 95–98.

Received on November 11, 1994; revised on December 20, 1994