inated by the use of 7-deaza deoxyguanosine 5′-triphosphate.
16. Analysis of the nucleic acid sequences and deduced amino acid sequence was by the computer program MacVector, release 3.5 (International Biotechnologies, New Haven, CT).
17. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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19. Computation of homology was performed by the BLAST Network Service (National Center for Biotechnology Information, Bethesda, MD).
20. The assay mixture for the determination of the enzymatic activity of the IAGlu synthetase gene cloned in pBluescript SK$^-$ (Stratagene) in *E. coli* was in a 0.5-ml final volume containing 0.8 mM $^{13}C_6$-labeled IAA and 0.05 μCi of [5-^{3}H]IAA (to facilitate IAGlu purification); 5 mM UDPG; 0.1 mM dithiothreitol; 75 mM Hepes buffer, pH 7.4; 50 mM *myo*-inositol; and 0.1 ml of enzyme extract (27 μg of protein). Incubation was for 4 hours at 37°C. After the sample was processed (*3*, *18*, *21*), the resulting material was scanned from 100 to 1200 daltons in a VG-ZAB2S mass spectrometer (VG-Analytical, Manchester, UK), proving the presence of $^{13}C_6$-labeled IAGlu.
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30. We thank A. Schulze for testing IAGlu catalytic activity, B. A. Larkins and J. E. Habben for the corn cDNA library, J. Leykam for NH_2-terminal amino acid sequencing, A. Ehmann for the fast atom bombardment mass spectroscopy characterization of IAGlu labeled with $^{13}C_6$-[benzene ring]-indole-3-acetic acid, and F. J. de Bruijn for advice and financial support to K.S. (NSF grant DCB 9105392 to F.J.deB.). Supported by grants from the NSF (IBN 92-07743 and IBN 9407617) (to R.S.B.).

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An Interleukin-4–Induced Transcription Factor: IL-4 Stat

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Interleukin-4 (IL-4) is an immunomodulatory cytokine secreted by activated T lymphocytes, basophils, and mast cells. It plays an important role in modulating the balance of T helper (Th) cell subsets, favoring expansion of the Th2 lineage relative to Th1. Imbalance of these T lymphocyte subsets has been implicated in immunological diseases including allergy, inflammation, and autoimmune disease. IL-4 may mediate its biological effects, at least in part, by activating a tyrosine-phosphorylated DNA binding protein. This protein has now been purified and its encoding gene cloned. Examination of the primary amino acid sequence of this protein indicates that it is a member of the signal transducers and activators of transcription (Stat) family of DNA binding proteins, hereby designated IL-4 Stat. Study of the inhibitory activities of phosphotyrosine-containing peptides derived from the intracellular domain of the IL-4 receptor provided evidence for direct coupling of receptor and transcription factor during the IL-4 Stat activation cycle. Such observations indicate that IL-4 Stat has the same functional domain for both receptor coupling and dimerization.

Studies of the mammalian immune system have led to the discovery of small, secreted proteins that provide circuitry to both dedicated and peripheral components of the system (1). These proteins, broadly termed cytokines, act to modify the growth and differentiated function of cells harboring cognate receptors. Cytokines are typically expressed under tight regulation with respect to cell type and physiologic state. The biological effects of a given cytokine are, in turn, limited to target cells bearing the corresponding receptor.

Biochemical and somatic cell genetic studies have begun to resolve the mechanisms by which cytokines selectively modify receptor-bearing target cells. Studies of interferon α (IFN-α) and interferon γ (IFN-γ) have revealed essential components of the cytokine signaling pathway. Cells treated with IFN-γ rapidly activate an otherwise latent DNA binding protein (*2*). Concomitant with activation, the DNA binding protein, variously termed p91 or Stat1, becomes tyrosine-phosphorylated by way of a receptor-associated tyrosine kinase. Mutant cells lacking either Jak1 or Jak2 tyrosine kinase fail to respond to IFN-γ signaling. Shortly after phosphorylation of a single and specific tyrosine residue (Y701), p91 translocates from cytoplasm to nucleus where it activates an array of genes containing its specific binding site. The p91 protein has emerged as the founding member of a family of cytokine-activated transcription factors. Likewise, resolution of the role of Jak kinases in interferon signaling has uncovered a biological function for this class of tyrosine kinases. These observations provide a conceptual coupling between a specific cytokine and the battery of genes it induces in target cells.

Interleukin-4 (IL-4), like IFN-γ, rapidly alters the pattern of gene expression in cells bearing its cognate receptor. When exposed to IL-4, B lymphocytes activate the synthesis of sterile transcripts of the immunoglobulin locus and subsequently undergo class switching to the immunoglobulin E heavy chain isotype (*3*). IL-4 also activates genes encoding cell surface proteins including various immunoglobulin receptors and the MHC (major histocompatibility complex) class II antigen (*4*). IL-4 modulates the activity of DNA binding proteins in B lymphocytes (*5*). Three studies have indicated that IL-4 may regulate gene expression in a manner analogous to IFN-γ. A latent DNA binding protein is rapidly phosphorylated on tyrosine and translocated to the nucleus in receptor-bearing cells treated with IL-4 (*6*). In order to study the molecular mechanisms governing this process we have purified the IL-4–induced DNA binding protein and cloned its encoding gene.

Human monocytic Thp-1 cells were grown in suspension, treated briefly with IL-4, harvested, disrupted, and fractionated to separate nuclear and cytoplasmic proteins (*7*). Nuclear extracts from IL-4–treated cells, but not control cells, contained a DNA binding activity capable of specific interaction with a double-stranded, synthetic oligonucleotide corresponding to the IL-4 response element located upstream of the human FcγRI gene (*6*). This activity was purified by three chromatographic steps (*8*) and found to be specified by a polypeptide that migrated with a molecular size of 100 kD as indicated by SDS–polyacrylamide gel electrophoresis (Fig. 1). The 100-kD polypeptide reacted with an antibody that recognized phosphotyrosine, consistent with studies that implicated tyrosine phosphorylation as an essential step required for its activation (*6*).

The purified, 100-kD polypeptide was digested with lysine-C and resulting peptides were fractionated by capillary high-performance liquid chromatography. Amino acid sequences were obtained from six peptide fragments (*9*). Synthetic oligonucleotides designed from these sequences were used for

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polymerase chain reaction (PCR) amplification of complementary DNA (cDNA) prepared with mRNA from Thp-1 cells (*10*). A PCR fragment that encoded three of the sequenced peptides was isolated. Complementary DNA clones were obtained and sequenced, allowing prediction of the open reading frame corresponding to the 100-kD polypeptide (Fig. 2). Starting with an initiator methionine codon located 182 base pairs (bp) downstream from 5′ terminus of the longest cDNA clone, the sequence predicts an open reading frame 848 residues in length which, in an unmodified state, should generate a polypeptide of 94 kD. All six of the peptide sequences generated by lysine-C digestion of the purified 100-kD polypeptide were found in the conceptually translated open reading frame (Fig. 2).

A search of the NCBI BLAST database revealed substantial similarity between the primary amino acid sequence of the 100-kD, IL-4–induced protein and that of mammary gland factor (MGF), a prolactin-induced DNA binding protein that belongs to the Stat family of transcription factors (*11*). Some sequence similarity was also observed between the IL-4–induced protein and the remaining four members of the Stat family (*12*) (Table 1). In all cases, the most significant segments of sequence similarity corresponded to three regions, one consisting of about 50 amino acids located adjacent to the NH_2-termini of all six proteins, and two more centrally located regions that have been predicted to specify contiguous SH3 and SH2 domains (Src homology). Because of similarity of the IL-4–induced protein to Stat proteins, coupled with its rapid, phosphotyrosine-associated conversion from latent to active state, we have designated this protein IL-4 Stat.

The distance from the 5′ terminus of the longest IL-4 Stat cDNA clone to the polyadenylated tail was 4.0 kb. RNA (Northern) blotting assays (*13*) confirmed the presence of a 4-kb IL-4 Stat mRNA. This mRNA species was observed in various human tissues, with significant amounts in peripheral blood lymphocytes, colon, intestine, ovary, prostate, thymus, spleen, kidney, liver, lung, and placenta (Fig. 3). Northern blotting also revealed a slightly smaller mRNA that was most abundant in kidney, as well as three larger mRNAs about 4.8, 5.5, and 6 kb. The 4.8- and 6-kb species were most abundant in spleen and thymus, whereas the 5.5-kb species was only observed in peripheral blood lymphocytes. The molecular composition and origin of these IL-4 Stat–related mRNAs is yet to be resolved.

The IL-4 receptor complex is composed of two distinct polypeptides, a 139-kD ligand-binding subunit (IL-4R) and a smaller polypeptide (IL-2Rγ) also utilized for IL-2, IL-7, and IL-13 signaling (*14*). Inspection of the primary amino acid sequences of the intracellular domains of the two receptor subunits has not revealed obvious motifs capable of mediating signal transduction. Treatment of cultured cells with IL-4 does, however, bring about rapid tyrosine phosphorylation of the intracellular domain of the IL-4R subunit (*15*). Indeed, phosphorylation of tyrosine 472 (Y472) of the IL-4R has been implicated in signaling through a large cytoplasmic protein termed either insulin receptor substrate–1 (IRS-1) or 4PS (*16*). In that the primary amino acid sequence of IL-4 Stat may specify SH3 and SH2 domains (Fig. 2), these domains might facilitate direct interaction with the intracellular domain of the IL-4 receptor at some point in the IL-4 Stat activation cycle.

This interpretation and the conceptual basis of the experiments designed to test it rely on prior studies of interferon signaling. The Stat protein induced by IFN-γ, p91, is rapidly converted from the monomeric to dimeric state after presentation of ligand (*17*). On the basis of observations that the IFN-γ receptor becomes tyrosine-phosphorylated shortly after binding of its cognate cytokine and that p91 contains contiguous SH3 and SH2 domains, Schreiber and colleagues tested whether a phosphotyrosine peptide derived from the IFN-γ receptor might inhibit activation of p91. Using a disrupted cell assay that recapitulates the conversion of latent p91 to the tyrosine-phosphorylated, DNA binding competent state, they showed that a receptor-derived phosphopeptide potently inhibits p91 activation (*18*). The inhibitory peptide corresponded to a region of the IFN-γ receptor that is tyrosine-phosphorylated after ligand binding and contained a specific tyrosine (Y440) that is essential for IFN-γ signaling. These observations are consistent with the idea that the SH3 and SH2 domains of p91 might facilitate direct binding to the IFN-γ receptor.

DNA binding by activated p91 can be selectively inhibited by synthetic phospho-

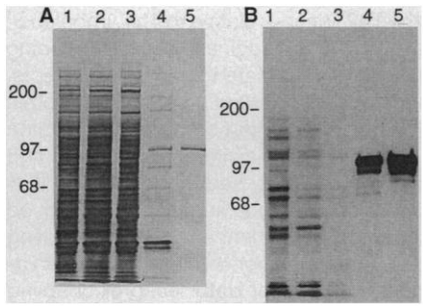

Fig. 1. Purification of IL-4 Stat. Nuclear extract from Thp-1 cells treated with IL-4 was precipitated with ammonium sulfate and then chromatographed in three steps that led to the purification of IL-4 Stat (*8*). (**A**) Coomassie staining pattern of polypeptides separated by SDS-gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane. (**B**) Immunoblot of the same PVDF membrane with a monoclonal antibody to phosphotyrosine. Bound antibody was visualized by chemiluminescence with horseradish peroxidase–coupled antibody to mouse immunoglobulin. (Lane 1) Crude Thp-1 nuclear extract. (Lanes 2 to 5) protein samples that had been precipitated with ammonium sulfate (*2*), S-Sepharose chromatography (*3*), DNA affinity chromatography (*4*), and Q-Sepharose chromatography (*5*). Molecular markers (left) correspond to positions of electrophoretic migration of myosin heavy chain (200 kD), phosphorylase B (97 kD), and bovine serum albumin (68 kD).

Fig. 2. Conceptually translated amino acid sequence of IL-4 Stat. Recombinant clones encoding IL-4 Stat were isolated from a cDNA library prepared with mRNA from human umbilical vein endothelial cells (HUVEC). DNA sequence was resolved on both DNA strands by the chain termination method of DNA sequencing. The predicted IL-4 Stat protein sequence is shown starting at an initiator methionine residue located 182-bp downstream of the longest cDNA. The predicted IL-4 Stat open reading frame was interrupted by a translation termination codon 116 bp upstream of the presumed initiator methionine, consistent with the interpretation that the 182-bp region corresponds to the 5′-untranslated region of the IL-4 Stat mRNA. The IL-4 Stat open reading frame predicts a polypeptide consisting of 848 amino acids•that, in an unmodified state, specifies the monomeric size of 94 kD. Peptides resolved from microsequencing of lysine-C fragments (*9*) are underlined. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L. Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W. Trp, and Y. Tyr.

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MSLWGLVSKM PPEKVQRLYV DFPQHLRHLL GDWLESQPWE FLVGSDAFCC   50
NLASALLSDT VQHLQASVGE QGEGSTILQH ISTLESIYQR DPLKLVATFR  100
QILQGEKKAV MEQFRHLPMP FHWKQEELKF KTGLRRLQHR VGEIHLLREA  150
LQKGAEAGQV SLHSLIETPA NGTGPSEALA MLLQETTGEL EAAKALVLKR  200
IQIWKRQQQL AGNGAPFEES LAPLQERCES LVDIYSQLQQ EVGAAGGELE  250
PKTRASLTGR LDEVLRTLVT SCFLVEKQPP QVLKTQTKFQ AGVRFLLGLR  300
FLGAPAKPPL VRADMVTEKQ ARELSVPQGP GAGAESTGEI INNTVPLENS  350
IPGNCCSALF KNLLLKKIKR CERKGTESVT EEKCAVLFSA SFTLGPGKLP  400
IQLQALSLPL VVIVHGNQDN NAKATILWDN AFSEMDRVPF VVAERVPWEK  450
MCETLNLKFM AEVGTNRGLL PEHFLFLAQK IFNDNSLSME AFQHRSVSWS  500
QFNKEILLGR GFTFWQWFDG VLDLTKRCLR SYWSDRLIIG FISKQYVTSL  550
LLNEPDGTFL LRFSDSEIGG ITIAHVIRGQ DGSPQIENIQ PFSAKDLSIR  600
SLGDRIRDLA QLKNLYPKKP KDEAFRSHYK PEQMGKDGRG YVPATIKMTV  650
ERDQPLPTPE LQMPTMVPSY DLGMAPDSSM SMQLGPDMVP QVYPPHSHSI  700
PPYQGLSPEE SVNVLSAFQE PHLQMPPSLG QMSLPFDQPH PQGLLPCQPQ  750
EHAVSSPDPL LCSDVTMVED SCLSQPVTAF PQGTWIGEDI FPPLLPPTEQ  800
DLTKLLLEGQ GESGGGSLGA QPLLQPSHYG QSGISMSHMD LRANPSWX    848
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tyrosine peptides because phosphopeptides derived both from the transcription factor itself (*17*) and the IFN-γ receptor (*19*) have inhibitory activity. Hence it is possible that the SH3 and SH2 domains of p91 serve two distinct roles—facilitating direct interaction with the IFN-γ receptor early during the p91 activation cycle and later mediating dimerization once p91 has been tyrosine-phosphorylated. Such dual function might explain the inhibitory activities of receptor- and transcription factor–derived phosphopeptides. If the dimerization interface of an activated Stat protein were specified by reciprocal coupling of SH3:SH2 domains and phosphotyrosine substrates, dimerization and, in turn, DNA binding might be inhibited by tyrosine-phosphorylated peptides capable of occupying the SH3:SH2 domain.

In order to investigate possible coupling between the IL-4 receptor and the transcription factor it appears to activate, we examined the inhibitory effects of five phosphotyrosine peptides derived from the intracellular domain of the human IL-4R subunit on DNA binding by activated IL-4 Stat. As a control we also tested the inhibitory activity of the phosphotyrosine peptide of the IFN-γ receptor that blocks p91 activation (*18*). Each peptide contained a centrally located phosphotyrosine flanked on the amino and carboxyl sides by seven amino acids specified by the native sequence of the human IL-4R subunit (*20*). Samples of nuclear extract prepared from IL-4–induced Thp-1 cells were incubated with individual phosphopeptides then tested by the electrophoretic mobility shift assay for the retention of active IL-4 Stat. Two of the five phosphopeptides derived from the intracellular domain of the IL-4R subunit inhibited the DNA binding activity of IL-4 Stat at concentrations ranging from 100 to 300 μM (Fig. 4A). The IFN-γ–derived phosphopeptide did not affect DNA binding activity. Moreover, the activities of both of the inhibitory, IL-4 receptor–derived peptides were dependent on tyrosine phosphorylation. Nonphosphorylated peptides showed no inhibitory activity (Fig. 4A).

Surprisingly, the two inhibitory peptides derived from the IL-4 receptor are related in primary amino acid sequence (NH_2-GP-PGEAGY^{P}KAFSSLL-COOH and NH_2-ASSGEEGY^{P}KPFQDLI-COOH). Relative to the centrally located phosphotyrosine, the two peptides are identical at the +1 and +3 positions. Detailed studies of interaction of SH2 and phosphotyrosine peptide have indicated that the +1 and +3 positions are important for specifying selectivity of this interaction (*21*).

These observations, like those reported for p91 (*17*, *18*), are consistent with the interpretation that Stat proteins interact directly with phosphotyrosine peptides on their corresponding receptor at an early point during their activation cycle. Whereas the inhibitory activities of receptor-derived phosphotyrosine peptides support a direct interaction model, these and previous observations have been limited to either crude cytoplasmic or nuclear extracts. As such, it is possible that the inhibitory peptides might block other components of the signaling system that either physically adapt or enzymatically link receptor and transcription factor.

To test whether the IL-4 receptor–derived phosphopeptides might interact directly with IL-4 Stat, we examined the effects of five synthetic peptides on the DNA binding activity of the purified transcription factor. IL-4 Stat purified from cytokine-induced Thp-1 cells (Fig. 1) was incubated with the two IL-4 receptor–derived phosphopeptides that had shown inhibitory activity when tested in crude nuclear extracts (Fig. 4A). Corresponding nonphosphorylated versions of each peptide were also tested, as was the tyrosine-phosphorylated peptide derived from the IFN-γ receptor that inhibits activation of p91 (*18*). We again observed phosphotyrosine-dependent inhibition by the two IL-4 receptor–derived peptides and no discernible inhibitory effect by the IFN-γ phosphopeptide (Fig. 4B).

The observations presented in Fig. 4 parallel the results and interpretations of studies of p91 and the IFN-γ receptor (*17*, *18*). One advance, however, comes from the use of purified components. As judged by Coomassie staining, the IL-4 Stat used in our study was substantially pure (Fig. 1). Given that the two receptor-derived, inhibitory peptides were capable of complete elimination of IL-4 Stat DNA binding activity, any indirect mode of inhibition must invoke a catalytic mechanism. One such mechanism might entail dephosphorylation of IL-4 Stat, a possibility eliminated by immunoblot assays with antibodies specific to phosphotyrosine. After complete inhibition of IL-4 Stat DNA binding activity by incubation with the inhibitory phosphopeptides, protein was analyzed by immunoblotting, which showed that IL-4 Stat does not lose phosphotyrosine as a result of exposure to the receptor-derived, inhibitory peptides (Fig. 4C).

We next investigated the mechanism by which the inhibitory peptides derived from the IL-4 receptor block the DNA binding activity of purified IL-4 Stat. The inhibitory activity of both receptor-derived peptides re-

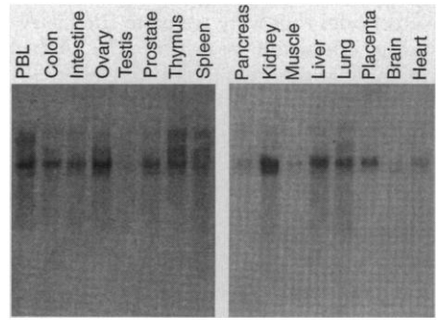

Fig. 3. Tissue distribution of IL-4 Stat mRNA. Nylon membranes containing polyadenylate-enriched mRNA from 16 human tissues were probed by nucleic acid hybridization at high stringency for IL-4 Stat mRNA (*13*). A prominent band of approximately 4 kb was observed in most tissues, with highest amounts occurring in peripheral blood lymphocytes (PBL), colon, intestine, ovary, prostate, thymus, spleen, kidney, liver, lung, and placenta. A slightly smaller band (3.8 kb) was observed in kidney. Larger bands of 4.5 and 6 kb were observed most abundantly in thymus and spleen. The mRNA preparation from peripheral blood lymphocytes also contained a larger mRNA species (5.5 kb) that hybridized to the IL-4 Stat probe.

Table 1. Amino acid sequence similarity comparison of Stat proteins. Pairwise distance matrix comparisons were obtained with CLUSTAL V protein alignment software program distributed by the European Molecular Biology Laboratory. Stat1 (p91), Stat2 (p113), and IL-4 Stat are human sequences, Stat3 (APRF) and Stat4 are mouse sequences (*12*), and Stat5 (MGF) is a sheep sequence (*11*).

Type	IL-4 Stat	Stat 1	Stat 2	Stat 3	Stat 4	Stat 5
IL-4 Stat	100	21.9	18.7	17.9	21.6	34.2
Stat 1 (p91)		100	38.9	49.6	50.4	23.7
Stat 2 (p113)			100	34.5	36.2	21.4
Stat 3 (APRF)				100	45.3	24.0
Stat 4					100	25.7
Stat 5 (MGF)						100

quired phosphorylation on tyrosine. Moreover, the inhibitory peptides are related in primary amino acid sequence on the immediate carboxyl terminal side of the phosphorylated tyrosine, a region known to function in specifying selective interaction between phosphotyrosine peptides and SH2 domains (*21*). Thus, these inhibitory peptides might bind to the SH3:SH2 domain of IL-4 Stat, thereby disrupting reciprocal SH3:SH2-phosphotyrosine interactions that otherwise facilitate dimer adherence.

In order to test whether IL-4 Stat indeed exists in a dimeric state, purified protein was exposed independently to two chemical crosslinkers, glutaraldehyde and disuccinimidyl glutarate (DSG). Both reagents caused time-dependent crosslinking of IL-4 Stat to covalently linked dimers (Fig. 5A). Even when exposed for a length of time sufficient to quantitatively crosslink all IL-4 Stat to covalently linked dimers, no evidence of higher order (trimeric or tetrameric) oligomerization was observed. The limited nature of this crosslinking, coupled with the low protein concentration (100 ng/ml), provides evidence that functional IL-4 Stat exists in a dimeric state. This interpretation is consistent with studies of other Stat proteins (*17*) and likewise fits with the dyad symmetric nature of the seven IL-4 Stat binding sites identified thus far (*6*).

Chemical crosslinking provided a means of testing whether the monomer:dimer equilibrium of IL-4 Stat might be influenced by the IL-4 receptor–derived peptides that inhibited DNA binding. Purified IL-4 Stat was exposed to the same five peptides that were tested in the DNA binding inhibition assay (Fig. 4B). After brief incubation the samples were exposed to DSG under conditions sufficient to quantitatively crosslink IL-4 Stat. The two IL-4 receptor–derived peptides, if phosphorylated on tyrosine, impeded formation of covalently linked IL-4 Stat in a concentration-dependent manner (Fig. 5B). No inhibition was observed with nonphosphorylated variants of the same two peptides or with the phosphopeptide derived from the IFN-γ receptor. The concentration at which receptor-derived phosphopeptides inhibited DNA binding (Fig. 4) corresponded with that required to impede crosslinking of IL-4 Stat dimers (Fig. 5). We therefore conclude that incubation of IL-4 Stat with tyrosine-phosphorylated peptides derived from the intracellular domain of its cognate receptor influences monomer:dimer equilibrium, and that the disassociation of IL-4 Stat dimers represents the mechanism by which receptor-derived phosphopeptides inhibit DNA binding.

Our data allow several concrete predictions. First, we anticipate that IL-4 Stat activation may entail transient coupling with either or both of two specific phosphotyrosine residues, Y^P578 and Y^P606, located in the intracellular domain of the IL-4 receptor. In that the inhibitory activities of synthetic peptides corresponding to these regions of the IL-4 receptor require tyrosine phosphorylation, transient receptor coupling of IL-4 Stat should likewise be dependent upon tyrosine phosphorylation. These interpretations are at odds with functional studies of the IL-4R subunit that have indicated that mutated variants of the receptor lacking all tyrosines native to the intracellular domain can mediate the growth stimulatory effects of IL-4 as tested in the murine pro-B cell line Ba/F3 (*22*). It is possible that mitotic proliferation, the biological process monitored in the latter study, is independent of IL-4 Stat. However, phosphorylation of Y472 of the IL-4R subunit has been firmly implicated in the IL-4–induced activation of IRS-1 and the proliferative response of human macrophage 32D cells (*16*). Further studies are required to resolve these contradictions.

Our second prediction results from the ability of IL-4 receptor–derived phosphopeptides to selectively inhibit DSG-mediated crosslinking of IL-4 Stat. Such inhibition was observed at concentrations similar to those required to inhibit DNA binding activity. These results indicate that the inhibitory peptides dissociate IL-4 Stat dimers, thereby causing an inhibition of DNA binding activity. We therefore propose that IL-4 Stat utilizes the same polypeptide domain, probably the SH3:SH2 domain, to mediate transient receptor

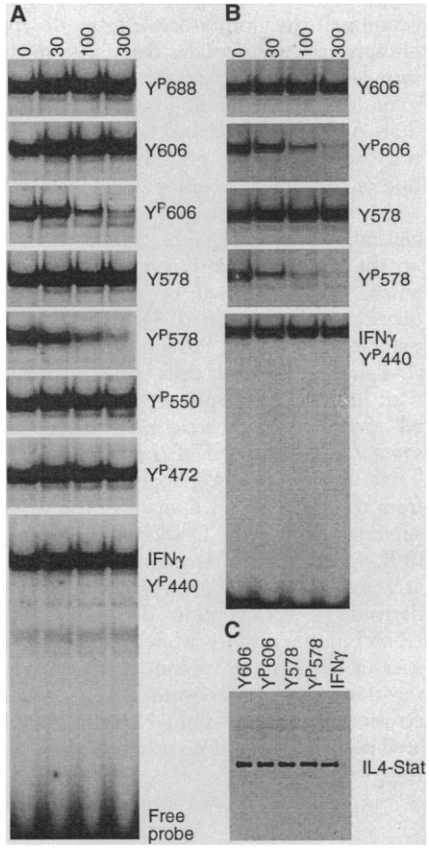

Fig. 4. Inhibition of IL-4 Stat DNA binding activity by phosphopeptides derived from the IL-4 receptor. (**A**) Gel mobility shift assays where crude nuclear extract prepared from IL-4–induced Thp-1 cells was incubated with synthetic peptides derived from either the ligand binding chain (IL-4R) of the IL-4 receptor or the IFN-γ receptor. Synthetic peptides (30, 100, or 300 μM) were incubated with nuclear extract for 30 min at 24°C. Samples were then mixed with a double-stranded, ^{32}P-labeled oligonucleotide corresponding to the IL-4 response element of the gene encoding FcγRI. The nucleotide sequence of the probe DNA was 5′-GTATTTCCCAGAAAAGGAAC-3′. The lower panel is an autoradiographic exposure of the electrophoretic mobility shift resulting from treatment of IL-4 Stat with a tyrosine-phosphorylated peptide derived from the IFN-γ receptor (NH_2-TSFGY^{P}DKPH-COOH). Unbound (free) DNA probe migrated at the bottom of the electropherogram. The probe bound by IL-4 Stat was retarded. Incubation of the nuclear extract with the IFN-γ receptor phosphopeptide did not affect the DNA binding activity of IL-4 Stat. The upper seven panels show only the IL-4 Stat–retarded complexes. Two IL-4 receptor–derived peptides inhibited DNA binding by IL-4 Stat in a concentration- and phosphotyrosine-dependent manner; one corresponding to a 15-residue region centered on Tyr^{578} of the IL-4R subunit (NH_2-GPPGEAGY^{P}KAFSSLL-COOH), and another corresponding to a 15-residue region centered on Tyr^{606} of the IL-4R subunit (NH_2-ASSGEEGY^{P}K-PFQDLI-COOH). Three other peptides, corresponding to tyrosine residues 472 (NH_2-VIAG-NPAY^{P}RSFSNSL-COOH), 550 (NH_2-VSAPTS-GY^{P}QEFVHAV-COOH) and 688 (NH_2-SLGSGIVY^{P}SALTCHL-COOH) of the IL-4 receptor did not affect DNA binding by IL-4 Stat even if phosphorylated on tyrosine. (**B**) The upper panel shows electrophoretic mobility shift assays where receptor-derived peptides were tested for inhibition of DNA binding activity by purified IL-4 Stat. Peptides were mixed with purified IL-4 Stat (Fig. 1) and assayed for inhibition of DNA binding as in (A). Phosphotyrosine-dependent inhibition was observed for the same IL-4R–derived peptides (Y^P578 and Y^P606) as observed in crude nuclear extracts. No inhibitory activity was observed for the phosphopeptide derived from the IFN-γ receptor. (**C**) Immunoblot assays of IL-4 Stat after incubation with synthetic peptides used in (B). Purified IL-4 Stat (Fig. 1) was incubated with synthetic peptides (300 μM) as in (B). Protein samples were then fractionated by SDS-gel electrophoresis, transferred to a nitrocellulose membrane and assayed by immunoblotting with an antibody to phosphotyrosine (Fig. 1). The amount of phosphotyrosine associated with IL-4 Stat was not changed after incubation with any of the five synthetic peptides.

coupling and dimerization. This represents unequivocal evidence of direct coupling of a Stat protein to its cognate receptor.

If our interpretation of the inhibitory effects of IL-4 receptor–derived phosphopeptides is correct, such observations imply that the same functional domain of IL-4 Stat is used both for receptor coupling and dimerization. There are several reasons to predict that the contiguous SH3 and SH2 domains of IL-4 Stat forms this multifunctional interface. First, SH2 (*23*) and SH3:SH2 domain (*24*) are known substrates for binding phosphotyrosine peptides. Second, x-ray crystallographic studies of the SH3:SH2 domain of Lck tyrosine kinase have provided evidence of anti-parallel dimerization, wherein reciprocal protein:protein contacts are formed between the SH2 domain of one subunit and the SH3 domain of its dimeric partner (*24*).

On the basis of these observations we propose the conceptual model shown in Fig. 6, where IL-4 Stat first associates via its SH3:SH2 domain with the freshly tyrosine-phosphorylated IL-4 receptor. Proximity to a receptor-associated tyrosine kinase results in phosphorylation of IL-4 Stat, presumably at a position on the immediate carboxyl terminal side of the SH2 domain [as is known to be the case of p91 (*2*)]. Thereafter, a critical transition occurs involving concomitant release from the receptor and IL-4 Stat dimerization.

The latter reaction may be favored as a result of the concerted, reciprocal interaction between the phosphorylated tail peptide of one IL-4 Stat subunit and the SH2:SH3 domain of its dimeric partner (Fig. 6). In that IL-4 Stat dimers are presumably held together by two SH2:phosphotyrosine couplings, each such interaction might be individually weaker than the corresponding interaction with the IL-4 receptor.

The reciprocal coupling model envisioned for IL-4 Stat dimerization is conceptually similar to the mechanism of dimerization of the Cro protein of bacteriophage λ, wherein a specific phenylalanine of one subunit is embedded into a receptive pocket on its dimeric partner (*25*, *26*). It also closely parallels the mechanism of Lck dimerization, which has been interpreted to represent a negative regulatory step controlling Lck tyrosine kinase activity (*24*). If correct, this interpretation of the IL-4 Stat activation pathway points to subtly controlled dimerization as a key regulatory step in cytokine-mediated signaling.

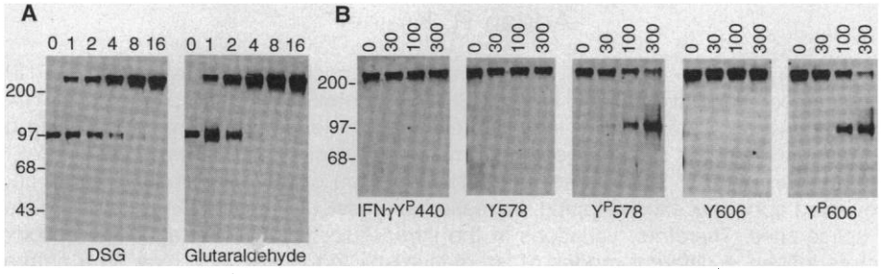

Fig. 5. Inhibition of dimerization of IL-4 Stat by phosphopeptides derived from the IL-4 receptor. (**A**) Patterns of glutaraldehyde and DSG-mediated crosslinking of IL-4 Stat. Purified IL-4 Stat (Fig. 1) was mixed with 0.02% glutaraldehyde or 0.5 mM DSG and incubated at 24°C for 1, 2, 4, 8, and 16 min. Crosslinking was terminated by the addition of 0.1 M tris(hydroxymethyl)aminomethane. Samples were fractionated by SDS-gel electrophoresis and transferred to nitrocellulose membranes for immunoblotting. IL-4 Stat was visualized with antibodies specific to phosphotyrosine (Fig. 1). Both crosslinking reagents caused a time-dependent conversion of IL-4 Stat to covalently linked dimers. Molecular markers correspond to positions of migration of myosin heavy chain (200 kD), phosphorylase B (97 kD), bovine serum albumin (68 kD), and ovalbumin (43 kD). (**B**) Effects of synthetic, receptor-derived peptides on DSG-mediated crosslinking of IL-4 Stat. Purified IL-4 Stat (Fig. 1) was mixed with 30, 100 or 300 μM concentrations of five synthetic peptides as described (Fig. 4). Samples were then exposed to 0.5 mM DSG for 8 min. Crosslinking was stopped by the addition of 0.1 M tris(hydroxymethyl)aminomethane, and samples were fractionated by SDS-gel electrophoresis. IL-4 Stat was visualized by immunoblotting with an antibody specific to phosphotyrosine (Fig. 1). The same two IL-4 receptor–derived peptides that inhibited IL-4 Stat DNA binding activity (Fig. 4) also inhibited DSG-mediated crosslinking of IL-4 Stat. Nonphophorylated forms of the two IL-4 receptor–derived peptides and the IFN-γ receptor–derived phosphopeptide did not dissociate IL-4 Stat dimers.

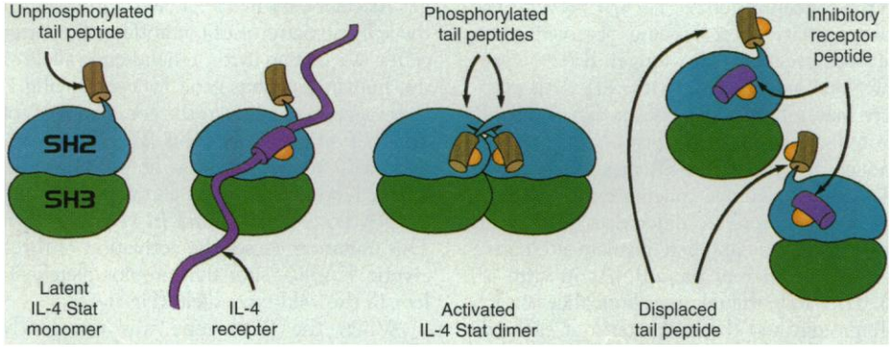

Fig. 6. Conceptual model of IL-4 Stat activation and inhibition by receptor-derived phosphopeptides. Latent IL-4 Stat binds to one of two phosphotyrosine peptides on the intracellular domain of IL-4 receptor (purple). Tyrosine phosphorylation of IL-4 Stat results in dimerization and concomitant release from receptor. Inhibitory, receptor-derived phosphopeptides disrupt IL-4 Stat dimers by occupying part of dimer interface. Phosphotyrosines on IL-4 receptor and IL-4 Stat are shown as gold spheres.

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7. Thp-1 human monocytic cells were grown in CO_2-independent RPMI 1640 culture medium (Gibco) supplemented with 10% fetal bovine serum (FBS), 10 mM Hepes (pH 7.3), 2 mM L-glutamine, streptomysin at 100 μg/ml, penicillin at 100 μg/ml, and 5 μM β-mercaptoethanol. Cells (50 liters) were harvested (7.5×10^5 cells per milliliter) and resuspended in 250 ml of warmed (37°C) culture medium lacking FBS. Cells were incubated for 15 min with recombinant IL-4 (5 ng/ml) (R & D Systems) and harvested by centrifugation. Nuclear extracts were prepared [J. D. Dignam, R. M. Lebovitz, R. G. Roeder, *Nucleic Acids Res.* **11**, 1475 (1983)] and solubilized in an aqueous buffer containing 20 mM Hepes (pH 7.9), 25% (v/v) glycerol, 0.1 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonate, 0.5 mM dithiothreitol (DTT), aprotinin at 1 μg/ml, pepstatin at 1 μg/ml, leupeptin at 1 μg/ml, 1 mM benzamidine, 1 mM sodium vanadate, 1 mM NaF, 5 mM β-glycerolphosphate (buffer C) supplemented with NaCl to a final concentration of 0.42 M.
8. Nuclear extract of IL-4–treated Thp-1 cells (2 g) was precipitated with 30% ammonium sulfate; the proteins were removed by centrifugation and the supernatant was then treated with 60% ammonium sulfate. Proteins precipitating between 30 and 60% ammonium sulfate (1 g) were recovered by centrifugation, resuspended in 200 ml of buffer C and dialyzed against the same buffer but lacking the protease inhibitors. After dialysis, insoluble proteins were removed by centrifugation and the remaining material was chromatographed over a 100-ml S-Sepharose

(Pharmacia) column. Protein flow-through (0.5 g) was mixed with a DNA-affinity resin prepared by coupling synthetic, biotinylated DNA corresponding to the IL-4 response element of the gene encoding FcγR1 (5′-GTATTTCCCAGAAAAGGAAC-3′) to streptavidin agarose (Sigma). After binding (2 hours at 4°C), the affinity matrix was placed on a disposable column and washed sequentially with 10 ml of buffer C, 4 ml of buffer C supplemented with a mutated variant of the IL-4 response element (5′-GTATCACCCAGTCAAGGAAC-3′) at 0.2 mg/ml, and 10 ml of buffer C. Protein (40 μg) was eluted by exposure to 5 ml of buffer C supplemented with 0.35 M NaCl, dialyzed against buffer C, and placed on a 0.5-ml Q-Sepharose (Pharmacia) column. The column was washed with 5 ml of buffer C and protein (10 μg) was eluted with 1 ml of buffer C supplemented with 0.35 M NaCl.

9. Purified IL-4 Stat (Fig. 1) was subjected to SDS-gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore) [P. Matsudaira, *J. Biol. Chem.* **262**, 10035 (1987)]. The membrane was stained with Coomassie blue R-250 in 40% methanol and 0.1% acidic acid for 30 s and then destained for 5 min with 10% acetic acid in 50% methanol. The 100-kD IL-4 Stat protein was excised from the membrane and treated with 1 μl of methanol. Membrane-bound protein was alkylated with isopropylacetamide [H. C. Krutzsch and J. K. Inman, *Anal. Biochem.* **209**, 109 (1993); S. Wong, C. Grimley, A. Padua, J. H. Bourell, W. J. Henzel, *Techniques in Protein Chemistry IV* (Academic Press, New York, 1993), p. 371] and then digested in 50 μl of 0.1 M ammonium bicarbonate, 10% acetonitrile with 0.2 μg of lysine-C (Wako) at 37°C for 17 hours. The solution was then concentrated to 20 μl and directly injected onto a capillary high-performance liquid chromatogram. Peptides were separated on a C18 capillary column (0.32 by 100 mm) (LC Packing) developed with 0.1% aqueous trifluoroacetic acid as buffer A and acetonitrile containing 0.07% trifluoroacetic acid as buffer B [W. J. Henzel, J. H. Bourell, J. T. Stults, *Anal. Biochem.* **187**, 228 (1990)]. Isolated peptides were sequenced on a 470A Applied Biosystems sequencer. Sequence interpretation was performed on a DEC 5900 computer [W. J. Henzel, H. Rodriguez, C. Watanabe, *J. Chromatogr.* **404**, 41 (1987)].
10. Degenerate oligonucleotides corresponding to two sequenced peptides of IL-4 Stat were used for PCR amplification of cDNA prepared from Thp-1 cell mRNA. One primer 5′-AARATGTGYGARACNYTNAA-3′ (R was equimolar mix of G and A; Y was an equimolar mix of T and C; N was an equimolar mix of G, A, T, and C) corresponded to the peptide sequence NH_2-KMCETLN-COOH. The other primer 5′-CATYTGYTCNGGYTTRTARTG-3′ corresponded to the peptide sequence NH_2-HYKPEQM-COOH. Conditions for PCR amplification were 94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min repeated for 35 cycles. The PCR reaction buffer consisted of 10 mM tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 10 pM of each primer and 20 ng of cDNA. The amplified product, 0.5 kb in length, was cloned and sequenced by the chain termination method.
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13. RNA blot hybridization with a uniformly labeled DNA probe prepared from IL-4 Stat cDNA and Multiple Tissue Northern Blot membranes (Clonetech) were used. Probe labeling, hybridization, and membrane washing were performed as described [J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, ed. 2, (1989)].
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Regulation of Alternative Splicing in Vivo by Overexpression of Antagonistic Splicing Factors

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The opposing effects of SF2/ASF and heterogeneous nuclear ribonucleoprotein (hnRNP) A1 influence alternative splicing in vitro. SF2/ASF or hnRNP A1 complementary DNAs were transiently overexpressed in HeLa cells, and the effect on alternative splicing of several cotransfected reporter genes was measured. Increased expression of SF2/ASF activated proximal 5′ splice sites, promoted inclusion of a neuron-specific exon, and prevented abnormal exon skipping. Increased expression of hnRNP A1 activated distal 5′ splice sites. Therefore, variations in the intracellular levels of antagonistic splicing factors influence different modes of alternative splicing in vivo and may be a natural mechanism for tissue-specific or developmental regulation of gene expression.

Alternative splicing is a major mechanism for controlling the expression of cellular and viral genes. SF2/ASF and other members of the SR protein family have an activity required for general splicing in vitro and also regulate alternative splicing by promoting the use of proximal 5′ splice sites (*1–4*). This latter activity is counteracted in vitro by hnRNP A1, which promotes the use of distal 5′ splice sites (*5*, *6*). Thus, the antagonizing activities of SR proteins and hnRNP A1 are key determinants of alternative 5′ splice site selection in vitro. In addition, a small increase in the concentration of SF2/ASF prevents the inappropriate exon-skipping observed when certain precursor messenger RNAs (pre-mRNAs) are spliced in vitro (*7*). This property may reflect a mechanism by which SR proteins ensure the fidelity of splicing. Although any individual SR protein can complement an inactive splicing extract lacking all the SR proteins, differences have been detected in their ability to regulate alternative splicing of different pre-mRNAs in vitro (*8*, *9*). Therefore, the relative abundance of each SR protein and the molar ratio of each SR protein to hnRNP A1, or to other antagonists, may determine the patterns of alternative splicing of many genes expressed in a particular cell type. Tissue-specific variations in the total and relative amounts of SR proteins or their mRNAs have been described (*8*, *10*, *11*), and in addition the molar ratio of SF2/ASF to hnRNP A1 varies over a wide range in different rat tissues (*11*).

Whether changes in the relative amounts of SF2/ASF and hnRNP A1 can affect gene expression in vivo was not known. To address this question, we transiently overexpressed SF2/ASF or hnRNP A1 complementary DNAs (cDNAs) in HeLa cells and analyzed the splicing patterns of cotransfected reporter genes. We first analyzed a thalassemic allele of the human β-globin gene, whose splicing is responsive to changes in the concentration of SF2/ASF in vitro (*1*). This β^{thal} allele contains a G to A transition at position 1 of intron 1, which results in abnormally spliced mRNA both in vitro and in vivo (*12*, *13*). This mutation causes the activation of three cryptic 5′ splice sites that are completely silent in the wild-type allele (Fig. 1A).

When the β^{thal} gene was transiently transfected into HeLa cells (*14*), all three cryptic sites were used in roughly equal proportions (Fig. 1A). Upon cotransfection of a human SF2/ASF cDNA, a substantial change in the relative use of each cryptic

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