STF–IL-4: a novel IL-4-induced signal transducing factor

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The mechanism by which interleukin-4 (IL-4) regulates the expression of particular genes is unknown. We have determined that IL-4 induces a DNA binding factor (termed STF–IL-4) which has a strong affinity for an IFN- γ activation site (GAS). Interestingly, STF–IL-4 also binds to the IL-4 responsive promoter for the Ig heavy chain germline ϵ transcript. The IL-4 dependent activation of STF–IL-4 is rapid, does not require protein synthesis and results in the sequential appearance of binding activity first in the cytoplasm and then later in the nucleus. Activation of STF–IL-4 is sensitive to tyrosine kinase inhibitors and the active factor is tyrosine phosphorylated. This pattern of activation is similar to the activation of interferon-induced transcription factors. STF–IL-4 appears to be a new member of a growing family of cytokine-induced transcriptional regulators.

Key words: cytokine/interferon/interleukin-4/signal transduction

Introduction

Interleukin-4 (IL-4), which is produced by T cells, mast cells and basophils, has potent biological activities on many different cells, including B and T lymphocytes, mast cells and macrophages (reviewed in Spits, 1992). When cells are cultured with IL-4, it often stimulates a variety of different functions within a given cell type. For instance, IL-4 can stimulate B cells to proliferate and differentiate (reviewed in Paul and Ohara, 1987), alter the level of cell surface receptors such as MHC class II (Noelle *et al.*, 1984; Roehm *et al.*, 1984) and CD23 (Defrance *et al.*, 1987; Hudak *et al.*, 1987), and stimulate the B cells to undergo Ig heavy chain class-switching to IgG1 and IgE (reviewed in Coffman *et al.*, 1993). Many of these biological activities rely on IL-4's ability to stimulate the transcription of particular genes. For instance, the ability of IL-4 to stimulate Ig heavy chain class-switching to IgG1 and IgE is dependent on its ability to stimulate germline $\gamma 1$ and ϵ transcription prior to switching (reviewed in Coffman *et al.*, 1993).

The gene encoding the IL-4 cellular receptor has been cloned (reviewed in Izuhara *et al.*, 1993) and is a member of the hematopoietin receptor superfamily (Cosman, 1993). The gene encodes a 140 000 kDa molecule which contains

a conserved pattern of cysteine residues as well as a WSXWS box, which are found in other members of this superfamily of cell surface receptors (Miyajima *et al.*, 1992). In addition, the IL-4 receptor does not contain recognizable kinase domains. Although the ability of IL-4 to induce the transcription of certain genes has been well documented, the mechanism by which the binding of IL-4 to its receptor stimulates transcription of these genes remains unknown. Murine IL-4 does not seem to activate a cell through the mobilization of Ca^{2+} or by activation of the phosphoinositol pathway (Justement *et al.*, 1986; Mizuguchi *et al.*, 1986).

Recently, the pathway by which the interferons (IFNs) stimulate transcription of early response genes has been elucidated (reviewed in Pellegrini and Schindler, 1993). The binding of IFN (either α/β or γ) to its receptor stimulates the activation by tyrosine phosphorylation, of latent cytoplasmic transcription factors, termed signal transducing factors (STF)–IFN- α/β or –IFN- γ , respectively. After this phosphorylation event, these factors translocate to the nucleus where, after binding to specific DNA elements [ISRE and the IFN- γ activation site (GAS) respectively] in the promoter of responsive genes, they function as transcriptional activators. The components of these STF complexes have been purified and cloned, and appear to represent a novel class of transcriptional activators. One of these proteins, p91, is a component of both STF–IFN- α and STF–IFN- γ (reviewed in Pellegrini and Schindler, 1993).

This system of signal transduction is important in the biological response to IFNs which require the rapid activation of specific target genes. It appears that other cytokines may employ a related signaling paradigm. For instance, both CNTF (Bonni *et al.*, 1993) and EGF (Sadowski *et al.*, 1993) rapidly activate STFs after binding to their respective receptors. Interestingly, the p91 protein appears to be a component of both STF–CNTF and STF–EGF (Bonni *et al.*, 1993; Silvennoinen *et al.*, 1993a,b). Recent evidence indicates that other cytokines also induce STFs and that several of these STFs bind to a site in the promoter of the *IRF-1* (ISGF2) gene (termed IRF-1 GAS) which has been shown to bind STF–IFN- γ (Pine *et al.*, 1994; P.Rothman and C.Schindler, unpublished observations).

IL-4 and IFN- γ are produced by different subsets of CD4⁺ helper T cells (reviewed in Mosmann and Coffman, 1989). These two cytokines regulate several steps in B cell development. One model by which two different cytokines could regulate the transcription of the same genes would involve similar pathways of signal transduction. In an effort to characterize a putative STF that could mediate the effects of IL-4 on B cell development, we determined that IL-4 rapidly activates a novel STF which has an affinity for the GAS element. The activation and activity of this factor (termed STF–IL-4) share many biological features with the IFN pathway. In addition, STF–IL-4 binds to a sequence at the IL-4 inducible promoter of the Ig germline ϵ transcript that differs significantly from the GAS element.

Results

IL-4 induces a novel GAS binding factor

It is known that the ability of IFN- γ to stimulate transcription can be mediated through STF-IFN- γ (also referred to as GAF; reviewed in Pellegrini and Schindler, 1993). This factor, which appears to consist of p91 homodimers (Shuai *et al.*, 1992; Pellegrini and Schindler, 1993), is known to bind to GAS elements in responsive promoters. Recently it has been determined that several other cytokines can activate factors that bind the GAS site of the *IRF-1* gene or closely related sequences (Bonni *et al.*, 1993; J. Yuan *et al.*, manuscript submitted; P. Rothman and C. Schindler, unpublished observations). We wanted to determine if IL-4 stimulates a factor(s) that bind(s) to this element. The M12.4.1 B cell line was cultured with IL-4 or IFN- γ or both IL-4 and IFN- γ for 2 h and cellular extracts were isolated. These extracts were assayed using electrophoretic mobility shift assays (EMSA) with an oligonucleotide corresponding to the IRF-1 GAS consensus element (Figure 1). Extracts isolated from untreated M12.4.1 cells do not shift the IRF-1 GAS probe (Figure 2, lane 1). However, when cells are cultured with IL-4 for 2 h, they contain a novel binding activity (termed STF-IL-4; Figure 2, lane 2). This complex was competed with both the IRF-1 GAS element and a site (termed RR1/2) from the Ig germline ϵ promoter (shown in Figure 1 and described in more detail below), but was not competed with a random (the octamer binding site) competitor (Figure 2, lanes 5–7). When the cells are stimulated with IFN- γ they activate a binding activity identical to STF-IFN- γ (Figure 2, lane 3). The STF-IFN- γ complex migrates more quickly than the STF-IL-4 complex. When these cells are co-cultured with IL-4 and IFN- γ , both activities are induced at levels comparable with those seen when the cytokines are added individually (Figure 2, lane 4).

We wanted to determine if other murine B lymphoid cells were able to produce this complex. Extracts were prepared from untreated 18.81A20 cells (AMuLV-transformed pre-B line), M12.4.1 cells (B cell line) and normal splenocytes that had been cultured with IL-4 for 2 h and examined by EMSA with an IRF-1 GAS probe. Extracts from the IL-4 cultures of the pre-B cell line (Figure 3, lane 2), the B cell line (Figure 3, lane 6) and freshly isolated splenocytes (Figure 3, lane 10) all contained an IL-4 inducible binding activity with a similar mobility. Human peripheral blood leukocytes stimulated with IL-4 also contain a GAS binding activity with a similar mobility (data not shown). In spleen cells, a doublet of bands is observed in some experiments; while in 18.81A20 cells, several complexes with faster mobilities are observed. It is likely that these complexes represent degradative products of STF-IL-4 (data not shown). Interestingly, the 18.81A20 cell line seems to contain a small amount of STF-IL-4 activity prior to the addition of IL-4 to the culture (Figure 3, lane 1).

The binding activities in these three cells share a similar pattern of competition with a panel of different oligonucleotides. All three complexes are competed with the IFP-GAS element (Strehlow *et al.*, 1993; Figure 3, lanes 4, 8 and 12) and an element from the Ig germline ϵ promoter (Figure 3, lanes 5, 9 and 13), but are not competed with the Ly6-GAS element (Khan *et al.*, 1993; Figure 3, lanes 3, 7 and 11). These studies indicate that the factors identified in all three cell types are likely to be the same factor.

IRF-1 PROMOTER

-179

GGGGCGGAGACGCCCGCTGACTTCCCTTCGCCGCTAGCTCTACAACAGCC
TGATTTCCCCGAATGACGGCAGCAGCCGCCAATGGGCGCCGCGGGCTGT
 CCGGGGGCGGGGCCGGCCAGGGCTGGGGAATCCCGCTAAGTGTGGATTGCT
 CGGTGGCGCCGCGCCCTGGCAGAGCTCGCCACTCCTTAGTCGAGGCAAGACGT
 GCGCCAGCCCGCCGAACCGA⁺⁵⁶

I ϵ PROMOTER

-122

GTCAACTTCCAAGAACAAGATCAAAAGGGAAGTCCAAGGCTGCTAAGG
 CCGGGGGTCCACCCCACTTTAGCTGAGGGCACTGAGGCAGAGCGGCCCC
 TAGGTACTACCATC TGGGCATGAATTAATGGTTACTAGA GATTCAACGCG
 CTGGGAGCCTGCACAGGGGCGAGAAG⁺⁵⁷

Fig. 1. IRF-1 and I ϵ promoter sequences. The sequences of the promoters of the *IRF-1* gene (from bp -179 to +56, top) (Sims *et al.*, 1993) and I ϵ germline (from bp -122 to +57, bottom) (Rothman *et al.*, 1991) transcript are shown. The IRF-1 GAS element (top) and I ϵ RR1/2 element (bottom) are underlined. The major initiation sites are shown by arrows.

PROBE

IRF-1 GAS

RR1/2

Fig. 2. Mobility shift analysis of IRF1-GAS and I ϵ binding activities in IL-4 and IFN- γ -stimulated cells. Whole cell extracts were prepared from M12.4.1 cells before (lanes 1 and 8) and after 2 h of culture with IL-4 (lanes 2, 5–7 and 9–12), IFN- γ (lane 3) or both IL-4 and IFN- γ (lane 4) and examined by mobility shift assay with a labeled IRF-1 GAS probe (lanes 1–7) or I ϵ RR1/2 probe (lanes 8–12). The probes were derived from the sequences shown in Figure 1. Competition assays were performed with the unlabeled IRF-1 GAS (lanes 6 and 10), I ϵ RR1/2 (lanes 5 and 11) or octamer binding sequence (lanes 7 and 12) oligonucleotide competitors at a 50-fold molar excess.

Since both STF-IL-4 and STF-IFN- γ can bind to GAS elements, we wanted to determine the relationship between these complexes. We first examined the binding specificity of these factors by determining the ability of a panel of related GAS elements to compete for binding of these factors to the IRF-1 GAS element (Figure 4). We find that the binding of STF-IFN- γ to the IRF-1 GAS element is competed with three (IFP, Ly6 and β CAS) related GAS elements (Figure 4, lanes 4–6). In contrast, STF-IL-4 is

Fig. 3. Activation of STF-IL-4 in different B lymphoid cells. Whole cell extracts were prepared from 18.81A20 (pre-B cells) before (lane 1) and after 2 h of culture with IL-4 (lanes 2–5) and from M12.4.1 cells (lane 6–9) and splenocytes (lanes 10–13) after 2 h of culture with IL-4. The extracts were examined for STF-IL-4 activity (indicated by arrow) by mobility shift assay with a labeled IRF-1 GAS probe. Competition assays were performed with the unlabeled Ly6-GAS (lanes 3, 7 and 11), IFP-GAS (lanes 4, 8 and 12) or I ϵ RR1/2 (lanes 5, 9 and 13) oligonucleotide competitors at a 50-fold molar excess.

Fig. 4. STF-IL-4 and STF-IFN- γ have different mobilities and competition profiles. Whole cell extracts were prepared from M12.4.1 cells after 2 h of culture with IFN- γ (lanes 1–6) or IL-4 (lanes 7–12), and assayed by mobility shift with a labeled IRF-1 GAS probe. Competition assays were performed either with the unlabeled I ϵ RR1/2 (lanes 2 and 8), I ϵ RR 3/4 (lanes 3 and 9), Ly6-GAS (lanes 4 and 10), β casein-GAS (lanes 5 and 11) or IFP-GAS (lanes 6 and 12) oligonucleotide competitors at a 50-fold molar excess.

competed by the β CAS (Wakao *et al.*, 1992; Figure 4, lane 11) and IFP (lane 12) sites, but not by the Ly6 site (lane 10). In addition, an oligonucleotide derived from the Ig germline ϵ promoter (termed RR1/2), which has previously been shown to bind an IL-4 inducible binding complex (Rothman *et al.*, 1991), competes the binding of STF-IL-4 (lane 8) but not STF-IFN- γ (lane 2). A truncated form of the RR1/2 site (termed RR3/4) does not compete the binding of either STF-IL-4 (lane 9) or STF-IFN- γ (lane 3). These studies indicate that although

Fig. 5. p91 is not tyrosine phosphorylated in response to IL-4. Whole cell extracts which were prepared from M12.4.1 (lanes 2 and 3) or 18.81A20 (lanes 4 and 5) cells after 2 h of culture with (lanes 3 and 5) or without IL-4 (lanes 2 and 4) were immunoprecipitated using an antiserum raised to p91 (p91ab1) and fractionated by 7% SDS-PAGE and immunoblotted. Control extracts from 2fTGH cells cultured with IFN- α (1000 U/ml) for 15 min (lane 1) were immunoprecipitated with an antiserum against p113 (anti-113) (Schindler *et al.*, 1992a,b). In the top panel, the samples were probed with an antiphosphotyrosine antibody (4g10) (UBI). In the bottom panel the blot was stripped and reprobed with a second antiserum raised to p91 (anti-p91) (Schindler *et al.*, 1992a,b).

both STF-IL-4 and STF-IFN- γ bind to a common site (IRF-1 GAS), albeit with different mobilities, they also have distinctive DNA binding affinities.

The p91 protein has been shown to be a component of several cytokine-induced STF that bind to GAS elements (Bonni *et al.*, 1993; Lutticken *et al.*, 1994; Sadowski and Gilman, 1993; Silvennoinen *et al.*, 1993a,b; C.Schindler, unpublished data). In addition, it has been demonstrated that activation of p91 requires tyrosine phosphorylation (Shuai *et al.*, 1992). To determine if p91 is a component of STF-IL-4, we assayed whether the p91 protein is tyrosine phosphorylated in response to IL-4. Extracts were isolated from both the M12.4.1 and 18.81A20 cell lines that were cultured with or without IL-4. These extracts were immunoprecipitated with an antiserum raised against p91. These precipitants were resolved by SDS-PAGE and immunoblotted with an antiphosphotyrosine antibody. IFN- α activated (i.e. tyrosine phosphorylated) STF-IFN- α components were immunoprecipitated from 2fTGH cells and served as a positive control. All three activated component proteins (p113, p91 and p84) (Schindler *et al.*, 1992a,b) are detected with the antiphosphotyrosine antibody (Figure 5, lane 1). However, in neither the 18.81A20 or M12.4.1 cell lines do we find p91 tyrosine phosphorylated in response to IL-4 (Figure 5, top panel, lanes 2–5). The presence of the p91 protein within these precipitants was confirmed by reprobing the blot with another antisera that recognizes p91 and p84 (Figure 5, bottom panel). We have also determined that several anti-p91 sera which block STF-IFN- γ binding in EMSA experiments fail to interact with STF-IL-4 (data not shown). These data indicate that despite similar DNA binding properties, STF-IFN- γ and STF-IL-4 are distinct factors, and that p91 is only a component of STF-IFN- γ .

Fig. 6. Characterization of STF-IL-4 activation. M12.4.1 cells were cultured either alone (lanes 1, 8 and 13), with IL-4 (lanes 2–4, 9–12 and 14–17) or IFN- γ (lanes 5–7). Whole cell extracts were prepared from cells that were cultured with the cytokines alone (lanes 2 and 5) or with preincubation with cycloheximide (lanes 3 and 6) or staurosporine (lanes 4 and 7). Nuclear (lanes 13–17) and cytoplasmic extracts (lanes 8–12) were isolated from M12.4.1 cells cultured with IL-4 for varying lengths of time as indicated. Extracts were assayed by mobility shift with a labeled IRF-1 GAS probe.

IL-4 rapidly induces a latent cytoplasmic factor to translocate into the nucleus

After binding to their receptors, the IFNs rapidly induce transcription of target genes. Essential to this rapid activation is the presence of a latent cytoplasmic pool of IFN STF. Binding of the IFNs to their receptors activates these preformed cytoplasmic factors, rendering them competent for nuclear translocation and DNA binding (reviewed in Pellegrini and Schindler, 1993). To ascertain whether STF-IL-4 is induced by a similar mechanism, M12.4.1 cells were cultured with IL-4 for various lengths of time and nuclear and cytoplasmic extracts prepared for analysis by EMSA with the IRF-1 GAS probe (Figure 6). STF-IL-4 activity appears in cytoplasmic extracts after 1 min of culture with IL-4 (Figure 6, lane 9). The level of STF-IL-4 activity continues to increase for the first 15 min of culture with IL-4 (Figure 6, lanes 9–12). In other experiments, STF-IL-4 activity increases for the first 2 h of culture and then begins to decrease slowly (data not shown). Interestingly, STF-IL-4 activity appears slightly later in the nucleus than the activity in the cytoplasm. A small level of STF-IL-4 activity is first seen in the nucleus 2 min after cells are cultured with IL-4 (Figure 6, lane 15), and gradually continues to increase after this time (Figure 6, lanes 16 and 17). This pattern is similar to that seen with IFN- γ and is consistent with the translocation of a cytoplasmically activated factor into the nucleus.

The activation of the IFN STFs is resistant to cycloheximide, which is consistent with the rapid activation of preformed latent cytoplasmic factors (Levy *et al.*, 1988; Schindler *et al.*, 1992a,b). If IL-4 activates a preformed factor, then induction of this factor should not require protein synthesis and should therefore be resistant to cycloheximide. M12.4.1 cells were precultured with cycloheximide and then either IFN- γ or IL-4 was added for 15 min. Extracts from these cells were assayed for STF-IFN- γ and STF-IL-4 activity. As previously shown, the activation of STF-IFN- γ is resistant to cycloheximide (Figure 6, lane 6). We find

Fig. 7. STF-IL-4 contains phosphotyrosine residues. Whole cell extracts were prepared from M12.4.1 cells after 2 h of culture with IL-4 and assayed by mobility shift with a labeled IRF-1 GAS probe. The 4g10 (lanes 3 and 4) or the Py20 (lane 5) antiphosphotyrosine antibodies or an isotype control OKT4 (lanes 6–8) antibody were added in the amounts indicated to the extracts prior to incubation with the probe.

that the activation of STF-IL-4 by IL-4 is similarly resistant to cycloheximide (Figure 6, lane 3). These studies revealed that protein synthesis is not required for the induction of STF-IL-4. Together with the experiment above, these findings are consistent with a model in which IL-4 activates a latent cytoplasmic DNA binding protein rendering it competent for nuclear translocation.

STF-IL-4 activation requires tyrosine phosphorylation

Previous studies have shown that activation of the IFN STFs requires tyrosine phosphorylation (Schindler *et al.*, 1992a,b; Shuai *et al.*, 1992). The rapid stimulation of tyrosine phosphorylation by many cytokines suggests that this may be a general paradigm for cytokine-induced signal transduction (reviewed in Pellegrini and Schindler, 1993). Since the rapid activation of STF-IL-4, described above, is consistent with activation by a post-translational modification, we tested the possibility that the activation of STF-IL-4 might also be mediated by a tyrosine kinase. M12.4.1 cells were grown in the presence of the tyrosine kinase inhibitor staurosporine, and STF-IL-4 activity was assayed by EMSA. In a pattern similar to STF-IFN- γ (Figure 6, lane 7), STF-IL-4 activation is inhibited by the presence of staurosporine (Figure 6, lane 4). Furthermore, since p91 (STF-IFN- γ) is a direct substrate of a tyrosine kinase, we wanted to determine whether activated STF-IL-4 contained phosphotyrosyl residues. Extracts isolated from M12.4.1 cells cultured with IL-4 for 2 h were used in an EMSA experiment with the IRF-1 GAS probe. Two antibodies reactive against phosphorylated tyrosines were added to the EMSA reactions prior to the addition of the probe. When these reactions were run on the gel we found that these antibodies inhibited the formation of the STF-IL-4 complex (Figure 7, lanes 3–5), while equivalent amounts of an isotype-matched control antibody had no effect (Figure 7, lanes 6–8). The observation that STF-IL-4

activation is blocked by staurosporine and that active STF-IL-4 contains phosphotyrosines is consistent with a model in which the activation of STF-IL-4 requires tyrosine phosphorylation.

STF-IL-4 binds to the germline ϵ promoter

An important function of IL-4 is the regulation of IgE production. In this regard, IL-4 stimulates the transcription of the germline murine ϵ locus prior to Ig class-switch recombination to this region (Rothman *et al.*, 1988; Stavnezer *et al.*, 1988). We have partially characterized a lipopolysaccharide (LPS)/IL-4 responsive promoter (shown in Figure 1) at the initiation site of the murine germline ϵ transcript (Rothman *et al.*, 1991). Using EMSA, we found that when spleen cells were cultured with IL-4 they contained a new binding activity which has affinity for a sequence near the initiation site of the germline ϵ transcripts (Rothman *et al.*, 1991). We wanted to determine if this site could bind STF-IL-4. We used an oligonucleotide (termed RR1/2) from this site as a cold competitor in EMSA. The RR1/2 site competed the binding of STF-IL-4 to the IRF-1 GAS site using extracts from three different types of lymphoid cell (Figure 3, lanes 5, 9 and 13). To confirm that these complexes were related, we used the RR1/2 site from the Ig germline ϵ promoter as a probe in EMSA experiments. The RR1/2 probe binds two complexes in IL-4-stimulated M12.4.1 cell extracts (Figure 2, lane 9). The slower migrating complex, which is also seen in unstimulated M12.4.1 cells (Figure 2, lane 8), is not competed with an IRF-1 site (Figure 2, lane 10). However, the faster migrating complex is induced by IL-4 in these cells. This complex also migrates with the same mobility as STF-IL-4 (compare Figure 2, lanes 2 and 9) and is competed by the IRF-1 site (Figure 2, lane 10). These data indicate that STF-IL-4 binds at both the IRF-1 GAS site and the RR1/2 site from the germline ϵ promoter.

As described above, STF-IL-4 binds to a sequence at the initiation site of Ig germline ϵ transcripts. Detectable levels of these transcripts are first seen after 24 h of culture with LPS and IL-4 (Rothman *et al.*, 1990). If STF-IL-4 is involved in the induction of this promoter, there should be a correlation between the presence of STF-IL-4 and germline ϵ transcription. 18.81A20 cells were cultured under various conditions and extracts prepared after both 2 and 24 h. The extracts were assayed for STF-IL-4 activity using the IRF-1 probe (Figure 8). We found that STF-IL-4 is clearly present after 24 h of culture with IL-4 (Figure 8, lane 10). These studies indicate that, despite its rapid induction, STF-IL-4 activity can still be found in cells cultured with IL-4 for 24 h, and its presence is consistent with a possible role in the induction of later transcriptional events, such as the induction of Ig germline ϵ transcripts.

The IL-4 induction of several genes, including the Ig germline ϵ (Rothman *et al.*, 1988; Stavnezer *et al.*, 1988) and γ 1 transcripts (Berton *et al.*, 1989; Esser and Radbruch, 1989), *c-fos* (Klemsz *et al.*, 1989) and MHC class II (reviewed in Glimcher and Kara, 1992), is either dependent on, or synergistic with, the effects of the mitogen LPS. We wanted to determine if LPS had any effect on the induction of STF-IL-4. 18.81A20 cells were cultured with IL-4 with or without LPS. In addition, we performed parallel cultures with IFN- γ with or without LPS. Extracts from these cells were assayed for STF-IL-4 and STF-IFN- γ activity with

Fig. 8. STF-IL-4 activation is unaffected by LPS and persists for over 24 h. Whole cell extracts were prepared from M12.4.1 cells after 2 (lanes 1–8) or 24 h (lanes 9–16) of culture with cytokines alone, as indicated (lanes 2–4 and 10–12), or with cytokines and LPS (lanes 5–8 and 13–16), and assayed by mobility shift with a labeled IRF-1 GAS probe.

the IRF-1 GAS probe. We found that the addition of LPS to these cultures had no effect on the induction of STF-IL-4 (Figure 8, compare lanes 2 and 10 with lanes 6 and 14) or STF-IFN- γ (compare Figure 8, lanes 3 and 11 with lanes 7 and 15). This suggests that the role of LPS in regulating the transcription of these genes does not involve direct effects on the activation of STF-IL-4 and may involve a second activation factor.

Discussion

We have characterized the ability of IL-4 to activate a cytoplasmic DNA binding factor that we have termed STF-IL-4. The induction of STF-IL-4 occurs extremely rapidly (within 1 min), suggesting that it may be involved in the cell's early response to IL-4. We have also seen STF-IL-4 activity in cells cultured with IL-4 for 2 days. This may indicate that, in addition to its involvement in the early response to IL-4, STF-IL-4 may also be involved in later IL-4-induced transcriptional activation.

The activation of STF-IL-4 shares several features with the activation of the IFN-induced factors STF-IFN- γ and STF-IFN- α/β (also referred to as ISGF3). These factors are all rapidly induced in a cycloheximide-resistant manner, indicating that these factors are present in the cytoplasm prior to activation. Furthermore, STF-IL-4 appears in the cytoplasm prior to its appearance in the nucleus. This finding is similar to that shown for the IFN STFs, indicating that the binding of IL-4 to its receptor induces both the activation of STF-IL-4 and its subsequent translocation into the nucleus where it binds target DNA sequences. This system for signal transduction may allow extremely rapid activation of target genes.

Our data also suggest that the activation of STF-IL-4 may involve tyrosine phosphorylation. Not only is STF-IL-4 activation sensitive to a kinase inhibitor, but activated STF-IL-4 contains phosphotyrosyl residue(s). These

observations are consistent with previous studies demonstrating that the binding of IL-4 to its receptor induces the tyrosine phosphorylation of a number of proteins, one of which appears to be the receptor itself. Another of these proteins, termed 4PS, is structurally similar to a factor (IRS-1) which is phosphorylated in response to insulin (Wang *et al.*, 1992). It seems likely that some of the other tyrosine phosphorylated proteins are components of the STF-IL-4 complex. Even though IL-2 and IL-4 share many biological functions (such as the ability to stimulate T cell proliferation), the pattern of tyrosine phosphorylation of cytoplasmic factors induced by these cytokines is different. These and other observations are consistent with the notion that IL-4 and IL-2 may use distinct mechanisms of signal transduction.

We present substantial evidence that, although STF-IL-4 and STF-IFN- γ can recognize and bind to the same sequence, they are composed of distinct proteins. These factors can be differentially competed by different GAS-related elements and they run with distinct mobilities in EMSA. In addition, the p91 protein, which as a homodimer forms STF-IFN- γ (p91), is not phosphorylated in response to IL-4. In preliminary studies we also find that a cell line that lacks p91 can activate STF-IL-4 (P. Rothman and C. Schindler, unpublished data). Therefore, although these factors share many biological features, they are distinct. Interestingly, IL-4 and IFN- γ are produced by different subsets of CD4⁺ T lymphocytes, termed TH1 (IFN- γ -producing) and TH2 (IL-4-producing). It appears that the stimulation of one or the other of these subsets has a profound effect on the immune response elicited. TH1 cells mediate several functions associated with cell-mediated immunity, while TH2 cells induce functions associated with humoral immunity. Therefore, at least a portion of the biological differences between a TH1 and TH2 response may be secondary to the antagonistic effects IL-4 or IFN- γ have on the transcription of several genes. For instance, IL-4 stimulates MHC class II expression in B cells (Noelle *et al.*, 1984; Roehm *et al.*, 1984), germline Ig ϵ and γ 1 transcripts (reviewed in Coffman *et al.*, 1993), and CD23 (Defrance *et al.*, 1987; Hudak *et al.*, 1987). IFN- γ inhibits all of these effects. Similarly, IL-4 can repress the IFN- γ induction of Ig germline γ 2a and γ 3 germline transcripts (reviewed in Coffman *et al.*, 1993). The fact that STF-IL-4 binds to IFN- γ response sites may allow these cytokines to modulate the transcription of the same genes. The ability of STF-IL-4 to bind to other distinct sites may allow IL-4 to exert an effect on other genes as well.

It appears that the IFN signaling pathway may serve as a general paradigm for cytokine-induced transcriptional regulation. Both CSF-1 and the IL-6 family of ligands have been shown to induce STF factors in a pattern similar to the IFNs (Bonni, 1993; Eilers *et al.*, 1993; Wegenka *et al.*, 1993; Lutticken, 1994). We have recently also found that IL-3 and erythropoietin may also fit this paradigm (J. Ihle, P. Rothman and C. Schindler, unpublished observations). This suggests that cytokines have related signaling systems that enable them to rapidly and specifically activate a series of genes that are involved in a biological response. Although many of the STFs that have been characterized (STF-CNTF, STF-EGF, STF-IL-6, STF-IFN- γ and STF-IFN- α/β) contain activated p91 protein, p91 is not a component of STF-IL-4. This would suggest that p91 is not an essential component for all cytokine signal

transduction pathways, but rather is important only for a subset of cytokines.

Although IL-4 has multiple biological functions, the most profound defect in IL-4-deficient mice is their inability to produce IgE (Kuhn *et al.*, 1991). The requirement for IL-4 in the production of IgE is secondary to its ability to stimulate germline ϵ transcription, which appears to target Ig class-switch recombination to the ϵ locus. We have shown that STF-IL-4 binds to an element in the germline ϵ promoter. Our previous work has demonstrated two IL-4 inducible nucleoprotein complexes (termed complexes 1 and 2) using the region around the germline ϵ promoter in EMSA (Rothman *et al.*, 1991). Although STF-IL-4 binds to this same site, the migration pattern of STF-IL-4 and complexes 1 and 2 are clearly different (complexes 1 and 2 migrate considerably slower than STF-IL-4) in EMSA experiments. Therefore, it is unclear if STF-IL-4 is a component of these complexes or is a distinct factor.

There are several lines of evidence suggesting that STF-IL-4 is a transcriptional regulator. First, it binds at the initiation site of the IL-4 responsive germline ϵ promoter. Second, it binds with high avidity to the IRF-1 GAS element which is also recognized by the transcriptional activators STF-CNTF, STF-IFN- γ and STF-IL-6 (Pine *et al.*, 1994; J. Yuan *et al.*, manuscript submitted; P. Rothman and C. Schindler, unpublished data). However, our initial experiments have shown that, although IFN- γ could induce transcription in a minimal promoter reporter construct using the IRF-1 GAS element, IL-4 had no effect on transcription with this reporter construct (data not shown). Cloning of the STF-IL-4 factor will allow for more definitive studies of its role in IL-4-mediated gene regulation.

STF-IL-4 joins a growing number of cytokine-induced factors that share a unique pattern of rapid activation. This network of STFs may allow a cell to rapidly integrate several different signals in a coordinated manner. In addition to coordinating different cytokine signals, this system must allow each cytokine to specifically activate important target genes. Our studies of STF-IL-4 provide evidence that different STFs will have both common and specific binding sites and suggest a model by which both specificity and coordination are achieved.

Materials and methods

Cell culture

The isolation and culture of murine splenic B cells has been described previously (Rothman *et al.*, 1988). The M12.4.1 (Glimcher *et al.*, 1982) and 18.81A20 cell (Alt *et al.*, 1982) lines have been described previously. Splenocytes, M12.4.1, and 18.81A20 cells were cultured in RPMI medium [supplemented with 10% fetal bovine serum (FBS) and 50 μ M β -mercaptoethanol] with LPS (10 μ g/ml), recombinant IL-4 (400 U/ml) or IFN- γ (Genzyme, Cambridge, MA) as described in the text. The 2fTGH cell line (McKendry *et al.*, 1991) was grown in DMEM supplemented with 10% FBS and was cultured with 1000 IU/ml IFN- α supplied by Roche (P. Sorter). In cycloheximide experiments, M12.4.1 cells were cultured with 10 μ g/ml cycloheximide for 1 h prior to the addition of cytokine. In staurosporine experiments, M12.4.1 cells were cultured in medium containing 500 μ M staurosporine for 20 min prior to the addition of cytokine.

Mobility shift assays and cell extracts

Double-stranded oligonucleotide probes were as follows: the IRF-1 GAS (as described by Bonni *et al.*, 1994; Pine *et al.*, 1994) probe spans from -138 to +107 of the IRF-1 promoter TACAACAGCCTGATTTCCCC-GAATGACGGC and includes the IRF-1 GAS (shown in bold); the RR1/2 element spans from -7 to +18 of the germline ϵ promoter (shown in bold), gatcTGGGCATGAATTAATGGTTACTAGA (Rothman *et al.*, 1991);

the RR3/4 element spans from +8 to +19 of the germline ϵ promoter gatcTGGTTACTAGAG; the IFP-GAS element, gatcCAGATTCTCAGAAA (Strehlow et al., 1993); the β CAS-GAS, gatcGACTTCTTGAATT (Wakao et al., 1992); the Ly6-GAS, gatcATATTCCTGTAAAGT (Khan et al., 1993); and the Oct probe which contains an octamer binding site (shown in bold), gatcTGTCGAATGCAAATCACTAGAA.

The preparation and employment of DNA oligonucleotide probes in mobility shift assays were performed as previously described (Bonni et al., 1993; Pine et al., 1994), and fractionated on 0.22 $\times$ TBE, 4.5% acrylamide (29:1) gels. Whole cell extracts (WCE), nuclear extracts and cytoplasmic extracts were prepared as previously described (Levy et al., 1988; Schindler et al., 1992a,b; Pine et al., 1994). Antiphosphotyrosine antibodies, which were purchased from UBI (4G10) or Zymed (PY-20), were added to the extracts for 30–60 min at 4°C prior to addition of the DNA probe.

Whole cell extracts were used for immunoprecipitations (IP). Extracts were precleared by the addition of either 2–3 μ l preimmune or nonimmune sera with protein A Sepharose (PAS) (Oncogene Sciences, Manhasset, NY) beads per 300–1000 μ l extract. After agitation at 4°C for 60 min, the supernatants were collected and divided into 300 μ l aliquots. To each of these samples, 3 μ l antisera (1:100 dilution) were added and incubated at 4°C for 90 min. Subsequently, 20 μ l protein A Sepharose were added and the samples agitated for an additional 60 min at 4°C. The IP were then collected by centrifugation and washed three times in 200 μ l buffer. The first two washes were with cold lysis buffer and the final wash was with cold PBS. The pellets were resuspended in 30 ml of 1.3 $\times$ SDS gel loading buffer and heated at 80°C for 5–10 min. Half of this sample was loaded on 7% PAGE. Western blotting was performed as previously described (Schindler et al., 1992a,b). The anti-91 and anti-p113 antisera have been described previously (Schindler et al., 1992a,b). The p91ab2 polyclonal rabbit antiserum was generated as described previously (Schindler et al., 1992a,b) against a GST fusion protein in which a region of p91 (representing amino acids 598–705) was fused to the glutathione-S transferase protein as described previously (Schindler et al., 1992a,b).

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Note added in proof

Preliminary data indicate that IL-13, which shares many biologic properties with IL-4, induces a mobility shift complex indistinguishable from that of STF–IL-4.