



SPI-C and STAT6 can cooperate to stimulate IgE germline transcription

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Abstract

SPI-C is a novel ETS protein that is expressed in B lymphocytes. No target gene for SPI-C has so far been defined. We have performed a yeast two-hybrid screen using SPI-C as bait in order to further analyze the functional role of this orphan transcription factor. We found that SPI-C interacted specifically with the C-terminus of STAT6 in yeast. By co-immunoprecipitation in transfected COS7 cells the physical interaction between SPI-C and STAT6 was confirmed. Furthermore, this protein–protein interaction is functional since we could demonstrate that SPI-C and STAT6 stimulated IL4 induced IgE transcription synergistically but only when both proteins bound to DNA. Thus, a protein interaction between SPI-C and STAT6 is the basis for a novel mechanism for regulation of IL4 induced gene expression.

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SPI-C is an ETS transcription factor that is differentially expressed during B-cell development and in some macrophage populations [1]. Within the ETS transcription factor family SPI-C is most homologous to the SPI-group proteins SPI-B and PU.1 (SPI-1) [2]. Although the DNA binding ETS domain, and especially the DNA contacting helices, are highly homologous to other SPI-group proteins, the regions flanking the ETS-domain are unique to SPI-C [2]. We have previously shown that mouse SPI-C has an N-terminal, acidic trans-activation domain, and that this domain is conserved in its net charge at identical positions between mouse, human, and fish SPI-C orthologues [3]. PU.1 and SPI-B also carry trans-activation domains that can act as heterologous trans-activating regions [4,5]. However, interaction with transcriptional co-activators is a prerequisite for transcriptional activation. For example, PU.1 and SPI-B can interact with the PU.1 interaction partner (PIP/IRF4) [6], CREB binding protein CBP [7] and PU.1 with GATA-1 [8], pRb [9], and Pub [10],

respectively, interactions that regulate transcriptional activity of SPI-B or PU.1.

In order to further clarify the functional role of SPI-C and its potential target genes, we searched for proteins interacting with the human orthologue (hSPI-C) using the yeast two-hybrid system. We report here that the hSPI-C protein specifically interacts with the signal transducer and activator of transcription 6 (STAT6) in yeast, as well as in COS7 cells. Furthermore, this interaction has a functional consequence in that IL4 induced transcription of the IgE germline promoter (I ϵ) is synergistically trans-activated by hSPI-C and STAT6.

Materials and methods

Yeast two-hybrid screening. The yeast two-hybrid system kit (matchmaker two-hybrid system 3, cat# K1612-1, Clontech, Palo Alto, USA) was used to screen a human spleen cell library (human spleen matchmaker cDNA library, cat# HL4054AH, Clontech) according to the 2-hybrid transformation protocol $\times 30$ [11]. Positive interacting clones were rescued by isolation from the yeast clones using the yeast plasmid isolation kit (DNA-pure, #DPMY 0050, CPG, NJ, USA) and subsequent transformation into electrocompetent *Escherichia coli* strain JM109 (Promega, Madison, USA) bacteria. Successfully transformed plasmids were

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prepared for test transformations from bacteria using the Jetprep miniprep system (Genomed, Saveen, Malmö). Sequencing was performed with the ABI sequence kit (Applied Biosystems, Cheshire, UK). The sequences obtained were translated in-frame with the Gal4 BD sequence and homologous proteins were identified using the EBI protein search engine.

Plasmid construction. pGBKT7hSPI-C was constructed by PCR amplification of the partial hSPI-C cDNA identified at that time, lacking the first methionine and threonine codons, with the primers 5'-TTTGAATTCACCATGTGTGTGAACAAGACAAGCTGG-3', as 1087 5'-TTTTGTGCGACTTAGCAATCATGGTGATTAGCTC-3' from a spleen cell library (cat# HL4054AH, Clontech). The hSPI-C PCR product was digested with *EcoRI*, *SalI* and ligated into an *EcoRI/SalI* digested pGBKT7 vector (cat# K1612-1, Clontech). The pGBKT7 plasmids containing N-terminal hSPI-C (hSPI-CA112-249), etshSPI-C (hSPI-CA1106,208-249), and C-terminalhSPI-C (hSPI-CA1191) were constructed by PCR amplification of the corresponding regions from the pGBKT7hSPI-C clone using the primers N-sense (s) 1084, N-anti-sense (as) 1099 5'-TTTGTGCGACTCGGAGCTTCTTCTGCCTT-3', ETS-s1106 5'-TTTGAATTCGAAGAGCTCCGACTGTTTGA-3', ETS-as1085 5'-TTTTGTGCGACCCAGGAAATAGGATGGAGAGAGTC-3', C-s 1107 5'-TTTGAATTCCTTCAGTGAGGCCATTCTCCA-3', C-as 1087 and digesting the PCR-amplified DNA with the enzymes *EcoRI* and *SalI* prior to ligation into an *EcoRI* and *SalI* pGBKT7 cut vector. In order to produce mammalian expression vectors containing the SPI-C, 13:45STAT6, and STAT6 sequences, we PCR amplified the hSPI-C cDNA minus the ATG translational start with the primers s1236 5'-GAATTCACGTGTGTGAACAAGACAAGC-3', SP6 as 5'-ATTTAGGTGACACTATA-3', from a pCDNA3FLAG plasmid containing the full length hSPI-C sequence cut the PCR product with *EcoRI* and *XbaI*, and ligated the digested DNA fragment into an *EcoRI/XbaI* cut pCDNA3FLAG [12] vector. The 13:45STAT6 was PCR amplified from the parent clone plasmid pACT13:45 with the primers s1242 5'-TTTGAAATCGCCACCATGCTCTTCTGCGCCAGAGATC-3', as1241 5'-TTTCTCGAGCCAACTGGGGTTGGCCCTT-3', digested with *EcoRI* and *XhoI*, and inserted into an *EcoRI/XhoI* digested pCDNA3mychis vector [3]. The luciferase reporter vector pGL3Ie was constructed by separately phosphorylating 5'-GTTGCTCAATCGACTTCCCAAGAAGAGAGAAAAGGGAACTTCACGGGGCGG-3', 5'-CCGCCCTGGAAGTTCCCTTTCTCTCTGTCTCTTGGAAAGTCGATTGAGCAAC-3' oligonucleotides with Polynucleotide Kinase (PNK, Roche, Bromma, Sweden). The annealed oligonucleotides were subsequently ligated into a dephosphorylated *SmaI* cut pGL3SP6TATA vector [1]. pGL3Ie Sm and pGL3Ie Em were constructed in the same way using the oligonucleotides 5'-GTTGCTCAATCGACAATCC AAGAAGAGAGAGAAAAGGGAACTTCACGGGGCGG-3', 5'-CCGCCCTGGAAGTTCCCTTTCTCTCTGTCTTGGTTAGTCGATTGAGCAAC-3' (pGL3Ie Sm) or 5'-GTTGCTCAATCGACTTCCCAAGAAGAGAGAAAAGTTAACTTCCAGGGCGG-3', 5'-CCGCCCTGGAAGTTAACTTTTCTCTCTGTTCTTGGGAAGTCGATTGAGCAAC-3' (pGL3Ie Em). The sequence of all constructs was confirmed by sequence analysis on an ABI sequence analyzer using sequence reactions prepared with the ABI sequencing kit v.3.1.

Cell culture conditions. COS7 were grown at 37 °C, 5% CO₂ in RPMI containing 7–8% FCS, 10 mM Hepes, 1 mM sodium pyruvate, and 50 µg/ml gentamicin (Gibco BRL).

Co-immunoprecipitation. COS7 cells were seeded in 6-well plates and lipofected (Lipofectamine 2000, Invitrogen AB, Sweden) at 80–90% confluency with 2 µg plasmids according to the manufacturer's instructions. Lipofection was always performed in the absence of antibiotics to avoid toxic effects. Twenty-four hours after transfection, the cells were scraped from the bottom of the wells and total cell lysates were prepared in 500 µl NP-40 lysis buffer (1% NP-40, 150 mM NaCl, and 50 mM Tris-Cl, pH 8.0) and sonication. The lysates were pre-cleared with 50 µl (bead volume) Sepharose beads (Protein G Sepharose 4 fast flow, Amersham Biosciences, Uppsala, Sweden) for 1 h and the supernatant was incubated with 1 µg isotype control IgG₁ (554121, BD), anti-myc (9E10, Santa Cruz) or anti-FLAG (M2 Sigma) overnight. To immunoprecipitate the proteins, 50 µl of Sepharose beads was added for 1 h at 4 °C. After the incubation, the Sepharose was washed three times in lysis buffer and once in 50 mM Tris-Cl, pH 8.0. The proteins bound to the Sepharose were eluted by incubation in 95 °C for 7 min in SDS load. The beads were removed and

the immunoprecipitated proteins loaded onto 10% (FLAGhSPI-C, 13:45STAT6) or 5% (full length STAT6) SDS-PAGE gels. The separated proteins were electroblotted onto nitrocellulose membranes (Hybond Extra, Amersham Pharmacia) and the membranes were blocked in 5% dried fat free milk in PBS 0.05% Tween 20 (PBS-T). Detection of FLAG-tagged SPI-C was performed with the M2 anti-FLAG antibody (Ab) (Sigma) at 1:10000 dilution in PBS-T and incubation with the goat-anti-mouse-IgG-HRP (sc-2005, Santa Cruz) in a second step. The myc-tagged proteins were detected with the anti-mycobiotin (9E10B, Santa Cruz) or anti-myc (9E10, Santa Cruz) Abs diluted 1:3000 followed by incubation with the SA-HRP diluted 1:5000 (7100-05, Southern Biotechnology) or anti-mouse IgG HRP (sc-2005) Ab. Membranes were washed three times in PBS-T after incubation with antibody or SA-HRP. The ECL (Amersham Biosciences) kit was used to detect the proteins recognized by the antibodies according to the instructions of the manufacturer and the signal was detected on blue sensitive RP films.

Luciferase assay. COS7 cells were seeded in 24-well plates and lipofected with 0.4 µg total plasmid/well (0.05 µg pGL3Ie, 0.175 µg SPI-C or PU.1 expression vectors and 0.175 µg STAT6 expression vector, empty pCDNA3 was used where none, only one or a lesser amount of expression vector was used to keep total amount of plasmid constant) at 80–90% confluency using the Lipofectamine 2000 reagent according to instructions of the manufacturer. After o/n incubation in transfection mix, the cells were starved in serum free optimum medium (Gibco BRL) for 6 h and subsequently incubated in serum containing medium in the absence or presence of 10 ng/ml human IL4 (Peprotech, London, England). The transfected cells were harvested 43 h after transfection by incubation in 100 µl reporter lysis buffer (Promega) at 4 °C, followed by a –70 °C freeze thaw. The lysates were spun and 20 µl of each lysate measured for luciferase activity in 100 µl luciferase assay substrate (Promega) in a micro-lumat luminometer (microlumat LB96P, EG&G Berthold).

Nuclear extracts and gel-shift assays. COS7 cells were transfected and grown in 6-well plates as above but the reaction scaled up four times for each well. Cells were collected after incubation with IL4 and 1/10 of the cells were used for luciferase measurement while the rest were used to extract nuclear proteins as described previously [13]. Probes for gel-shifts were made by end-labeling one pmol of sense oligonucleotide with γ -³²P ATP (Amersham) using PNK (Roche) for 30 min at 37 °C. The labeled oligonucleotide was annealed to cold anti-sense oligonucleotide and the double-stranded probes were purified using gel-filtration spin-columns (Bio-Rad Bio-Spin 30 chromatography columns). Gel-shifts were performed as described in [13]. In short, 3 µl of nuclear extracts was incubated with 2 µg poly(dI-dC) in bind buffer for 5 min at RT before addition of unlabeled competitor oligonucleotides and a further 15 min incubation. Probes were added last and the samples were incubated for another 30 min at RT before they were separated on a 6% polyacrylamide gel. The sequence of the Ie oligonucleotides is shown above while the oct oligonucleotide is described in [13].

Results and discussion

We have earlier shown that SPI-C can activate transcription from a reporter gene [1,3]. However, the target genes and potential co-activators involved in transcriptional regulation by SPI-C remained unknown. In an effort to increase our understanding of these phenomena, we exploited the yeast two-hybrid system. Hence, a human spleen cell cDNA library cloned into the yeast expression vector pACT2 was screened using the human orthologue of the SPI-C transcription factor fused to the GAL4 DNA binding domain as a bait. A total of 1×10^6 transformants were screened by growth on selective media and expression of the β -gal reporter gene. Surviving, β -gal expressing clones were subsequently analyzed by DNA

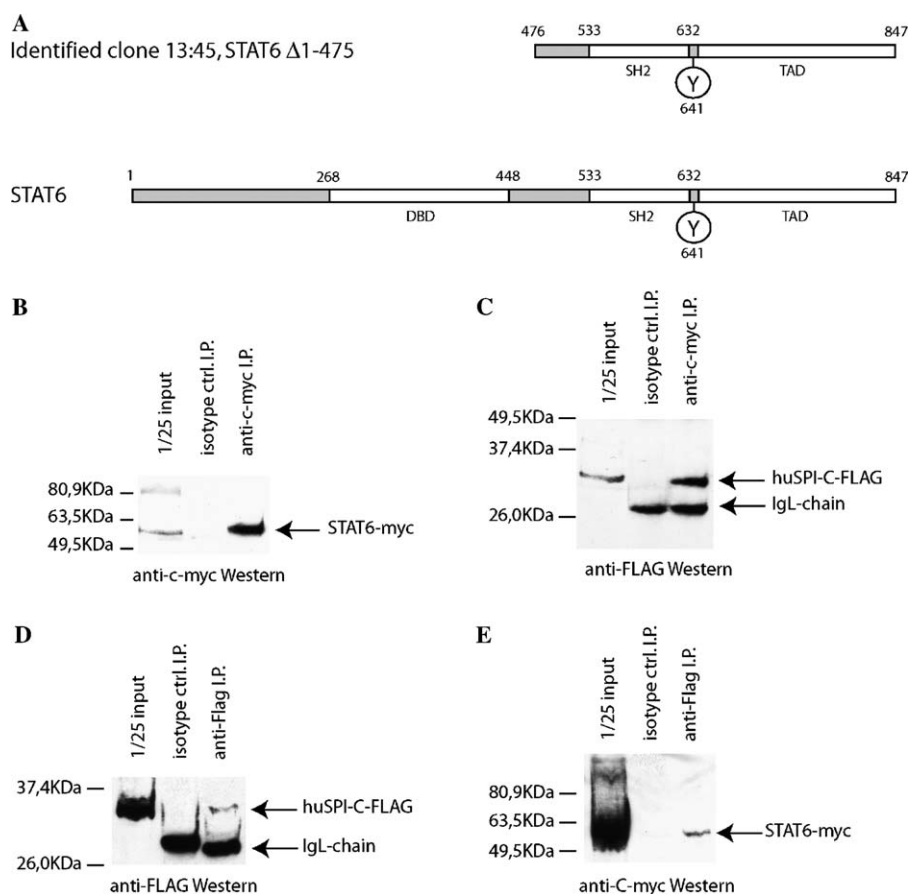


Fig. 1. Schematic presentation of the truncated 13:45STAT6 clone (A) compared to the full length STAT6 protein with the DNA binding (DBD), SH2 and transcription activation (TAD) domains in white and the phosphorylated tyrosine residue at position 641 highlighted. (B) Immunoprecipitation of 13:45STAT6myc with an anti-myc antibody. Western blot analysis by incubation with an anti-mycbiotin primary antibody followed by an SA-HRP secondary step. (C) Detection of co-immunoprecipitated proteins with the anti-FLAG antibody followed by an anti-mouse IgG secondary step that also recognizes the IgG light chain. (D) Immunoprecipitation of FLAGSPI-C with an anti-FLAG antibody. Western blot analysis with anti-FLAG as above. (E) Detection of co-immunoprecipitated proteins with the anti-myc antibody, as above.

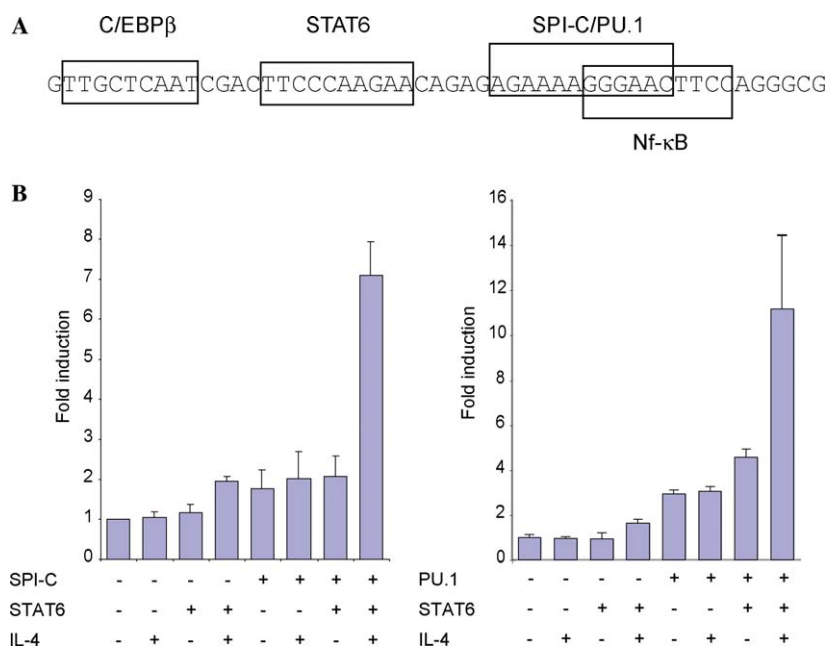


Fig. 2. Transfection of COS7 cells with reporter plasmid pGL3I ϵ (containing the promoter sequence depicted in (A)) and FLAGSPI-CpCDNA3 and/or STAT6mychispCDNA3 (B). The error bars show standard deviation of three independent experiments performed in triplicate.

sequencing and database search. One of the clones (the 13:45 clone) was found to be a partial, in-frame cDNA of the transcription factor signal transducer and activator of transcription 6 (STAT6) encoding the 476–847 amino acid portion of the STAT6 protein (Fig. 1A). The STAT6 clone contained the SH2 domain that mediates homodimerization of the STAT6 protein upon phosphorylation on tyrosine residue 641 (Y641). As a negative control, the 13:45 clone was cotransformed with the pBD and pBD p53 plasmids and no yeast growth was observed (data not shown).

Protein–protein interactions may depend on post-translational modifications that in turn can be different between yeast and mammalian cells. Ideally, such interactions should therefore be validated using endogenously expressed proteins. We attempted such experiments but failed, most likely due to the fact that SPI-C is expressed at very low levels (data not shown and below). We there-

fore had to turn to a transfection system in order to test whether STAT6 and SPI-C can interact also in mammalian cells. To this end, SPI-C was cloned into the vector pCDNA3FLAG [12] to produce a fusion protein with an N-terminal FLAG tag, while the 13:45 STAT6 cDNA was cloned into the pCDNA3mycis to produce a fusion protein with a C-terminal mycis-tag. These vectors were co-transfected into COS7 monkey epithelial cells, whole cell lysates were prepared and analyzed by immunoprecipitation. The STAT613:45myc fusion protein was specifically immunoprecipitated with the anti-myc antibody as shown in Fig. 1B. When the membrane was reprobed with an anti-FLAG antibody, it could readily detect the co-immunoprecipitated FLAGSPI-C (Fig. 1C). Furthermore, we also immunoprecipitated FLAGSPI-C with the anti-FLAG antibody (Fig. 1D) and subsequently detected co-immunoprecipitated myc-tagged 13:45STAT6 with an anti-myc antibody (Fig. 1E), confirming that under these

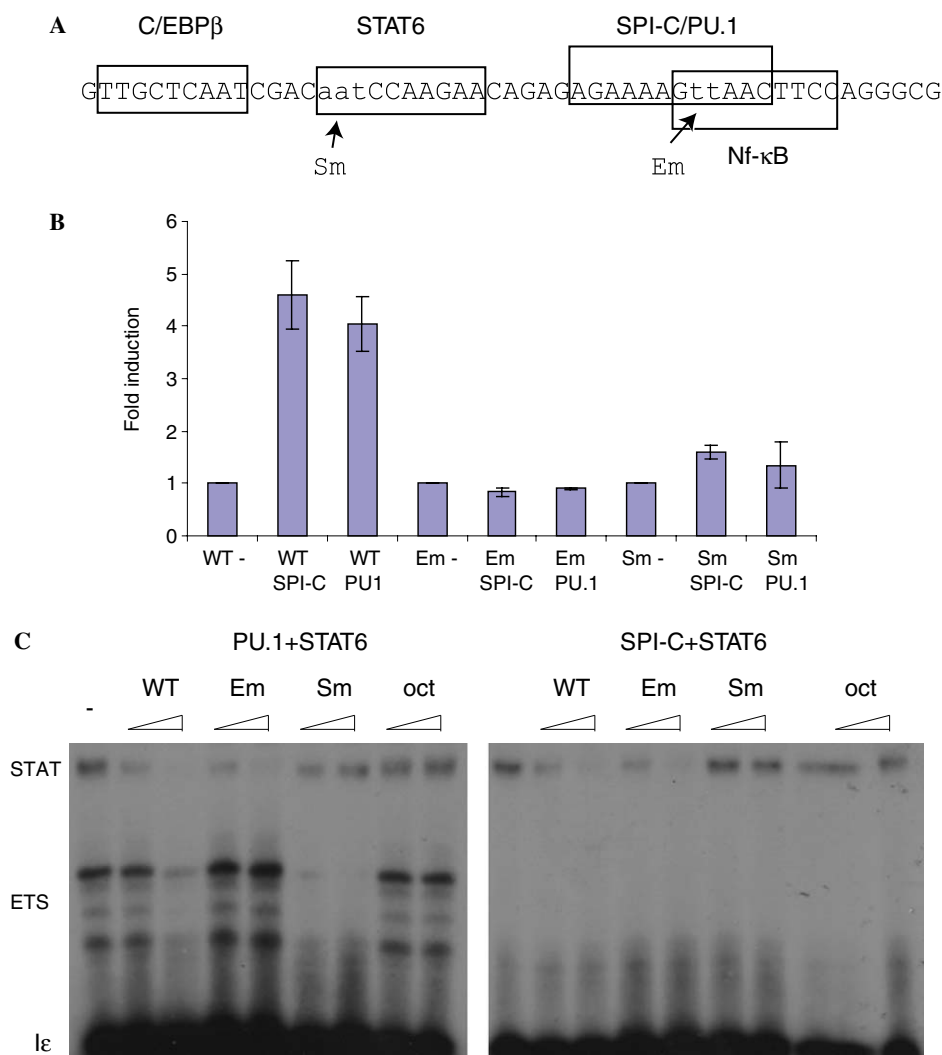


Fig. 3. Transfection of COS7 cells with reporter plasmid pGL3Iε (WT), pGL3Iε with a mutated STAT-site (Sm) or pGL3Iε with a mutated Ets-site (Em). Mutations present in Sm or Em are indicated by small caps in (A), (B) shows the resulting data from cells transfected with reporter plasmid alone (–) or reporter plasmid + SPI-C and STAT6 (SPI-C) or PU.1 and STAT6 (PU.1). (C) EMSA of nuclear extracts prepared from COS7 cells transfected with SPI-C + STAT6 or PU.1 + STAT6. The WT Iε oligonucleotide was used as probe and shifts were competed with the indicated unlabeled oligonucleotides at 100- and 1000-fold excess. The oct oligonucleotide has a sequence unrelated to Iε and was here included as a negative control.

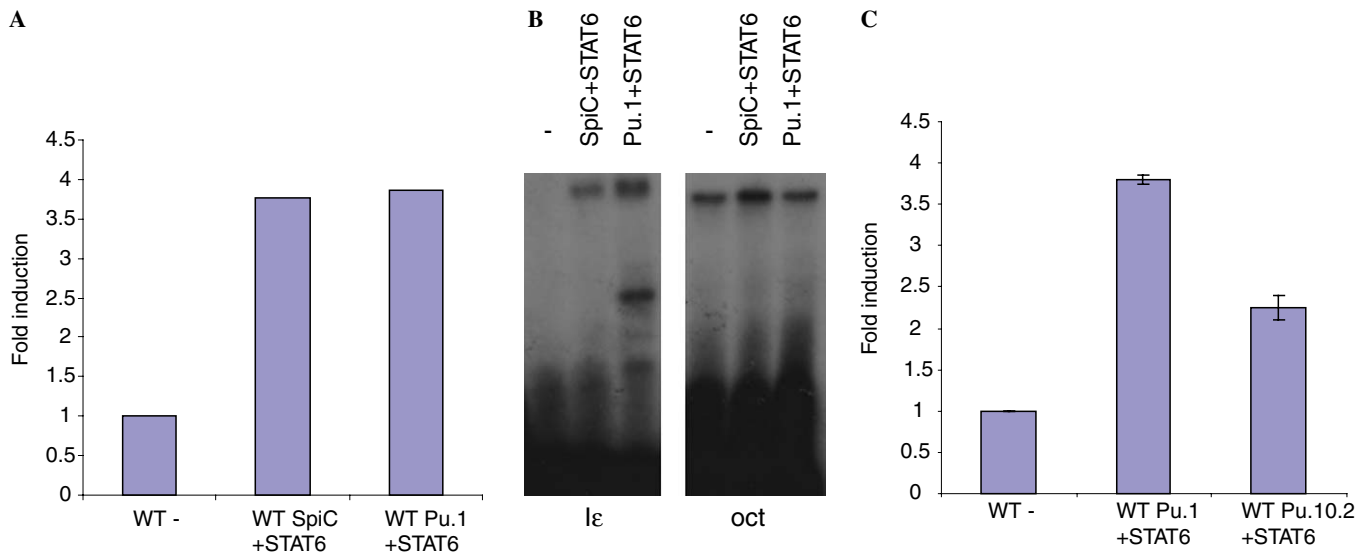


Fig. 4. COS7 cells were transfected with pGL3I ϵ and SPI-C + STAT6 or PU.1 + STAT6 and cells were collected for luciferase measurement and preparation of nuclear extracts. (A,B) Luciferase and gel-shift data from one representative experiment. Shifts obtained with the oct probe (binding ubiquitous OCT-proteins) were used to compare the protein content of the different extracts. (C) Luciferase levels obtained using different amounts of PU.1 expression vector in combination with pGL3I ϵ reporter plasmid and STAT6 expression vector.

conditions STAT6 interacts with SPI-C also in mammalian cells.

Having established that the SPI-C and STAT6 proteins potentially could interact, we proceeded to investigate whether such interaction could have an effect on transcriptional stimulation. We therefore constructed a reporter vector carrying a promoter site from the IgE germ line promoter where the PU.1 and STAT6 proteins had previously been shown to stimulate transcription in a cooperative fashion [14]. This reporter construct (Fig. 2A) was transfected into COS7 cells together with SPI-CFLAG or/and full length STAT6 expression vectors. Since STAT6 is an important molecule in IL4 signaling, we also performed the experiment both in the presence or absence of IL4 [15]. The result showed that the I ϵ reporter plasmid was activated by STAT6 expression in an IL4 dependent manner (2 $\times$ induction). SPI-C expression induced expression of the reporter gene to a level similar to that of the STAT6 + IL4, but this induction was independent of IL4 signaling. SPI-C and STAT6 co-expression lead to the induction of transcriptional activity to a similar level of that observed after transfection of SPI-C alone. However, IL4 stimulation promoted a transcriptional activation of seven times over background (Fig. 2B). As a control we also performed the same experiment using an expression vector for PU.1 in combination with STAT6 and obtained a similar result to that seen with SPI-C (Fig. 2B).

To elucidate whether the cooperative activation of transcription between SPI-C or PU.1 and STAT6 was dependent on DNA binding of the ETS and STAT6 proteins, respectively, we proceeded to construct some mutant reporter vectors (Fig. 3A). Subsequently the mutant vectors were co-transfected with STAT6 expression vector together with either a SPI-C or a PU.1 expression vector

and induced with IL4, as above. As shown in Fig. 3B, when either the ETS or STAT6 binding site was mutated in the I ϵ reporter plasmid, all the IL4 induced transcriptional enhancement was reduced to baseline activity. To verify that the introduced mutations were specific we also performed gel-shifts using the I ϵ promoter as probe and extracts from COS7 cells transfected with either PU.1 and STAT6 expression vectors or SPI-C and STAT6 expression vectors (Fig. 3C). When the gel-shift was competed with cold probes containing the indicated mutations, specific competition of ETS binding was observed with the STAT6 mutant competitor while specific competition of the STAT6 complex was observed with the ETS mutant competitor using PU.1 and STAT6 extracts (Fig. 3C, left panel). No ETS gel-shift complexes were detected with the SPI-C and STAT6 extract (Fig. 3C, right panel), while the competition pattern for STAT6 complexes was identical to that observed with the PU.1 and STAT6 extract. Thus, we conclude that the cooperativity between SPI-C or PU.1 and STAT6 is strictly dependent on DNA binding of both proteins and that SPI-C is expressed at significantly lower levels than is PU.1 in our transfection system.

As noted above, transfected SPI-C is expressed at very low levels and hence it was somewhat surprising that cotransfection of SPI-C induced as high transcriptional activation of the reporter gene as did cotransfection of PU.1. To further investigate the quantitative relationship between SPI-C and PU.1 in transcriptional activation of the I ϵ reporter plasmid, we repeated the cotransfection experiment with both SPI-C and PU.1, both in combination with STAT6 expression vector and induced by IL4, and analyzed luciferase activity and gel-shift in parallel from the same extracts (Fig. 4A and B). In this type of experiment it was again obvious that the same luciferase

activity was obtained in the SPI-C transfectants, while the ETS protein level was significantly lower than that seen in PU.1 transfectants. To rule out the possibility that PU.1 protein levels after transfection were saturated and that difference in levels of PU.1 would not correspond to differences in reporter gene activity, we performed transfections where the amount of PU.1 expression vector was reduced fivefold (Fig. 4C). Although PU.1 binding to the I ϵ probe was clearly visible in gel-shifts from these experiments (data not shown), the results show a reduced activation of the I ϵ reporter plasmid with reduced amount of PU.1. We conclude from this finding that SPI-C is, on a molar basis, more efficacious in costimulating IL4 induced transcription from an I ϵ reporter plasmid together with STAT6.

In summary, the physical interaction between STAT6 and SPI-C can be confirmed by their cooperative activity in stimulating IL4-dependent, IgE germline transcription. This interaction represents a novel mechanism for STAT6 and IL4 dependent activation of target genes in the process of B lymphocyte differentiation. SPI-C can be envisioned to compete with PU.1 for binding to the IgE germline promoter, but also by its direct binding to STAT6. Furthermore, we propose that the physical interaction between SPI-C and STAT6 is more stable than the interaction between PU.1 and STAT6 [14], in order to compensate for the low level of SPI-C expression and enable SPI-C to be an efficient costimulator of combined ETS/STAT6 templates. However, the exact interplay between STAT6 and the various ETS proteins in the control of IL4 induced transcriptional regulation merits further investigation.

Acknowledgments

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