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IL-2 and long-term T cell activation induce physical and functional interaction between STAT5 and ETS transcription factors in human T cells

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Activation of Stat5 by many cytokines implies that it cannot alone insure the specificity of the regulation of its target genes. We have evidenced a physical and functional interaction between members of two unrelated transcription factor families, Ets-1, Ets-2 and Stat5, which could contribute to the proliferative response to interleukin 2. Competition with GAS- and EBS-specific oligonucleotides and immunoassays with a set of anti-Stat and anti-Ets families revealed that the IL-2-induced Stat5-Ets complex recognizes several GAS motifs identified as target sites for activated Stat5 dimers. Coimmunoprecipitation experiments evidenced that a Stat5/Ets-1/2 complex is formed *in vivo* in absence of DNA. GST-pull down experiments demonstrated that the C-terminal domain of Ets-1 is sufficient for this interaction *in vitro*. Cotransfection experiments in Kit225 T cells resulted in cooperative transcriptional activity between both transcription factors in response to a combination of IL-2, PMA and ionomycin. A Stat5-Ets protein complex was the major inducible DNA-binding complex bound to the human IL-2rE GASd/EBSd motif in long-term proliferating normal human T cells activated by CD2 and CD28. These results suggest that the inducible Stat5-Ets protein interaction plays a role in the regulation of gene expression in response to IL-2 in human T lymphocytes. *Oncogene* (2000) **19,** 2086–2097.

Keywords: T lymphocytes; IL-2/IL-2R; STAT5; ETS; transcription

Introduction

Signal transducer and activators of transcription 5 (Stat5a and Stat5b) and Ets-1, and Ets-2, belong to two distinct groups of transcription factors, the Stat family and the Ets family. Both families play important roles in cell differentiation and in immune responses induced by cytokines, and a wealth of evidences has demonstrated their key roles in oncogenesis (Catlett-Falcone *et al*., 1999; Ghysdael and Boureux, 1997). Stat proteins are transcription factors activated by tyrosine phosphorylation in the cytoplasm or in the vicinity of the cytoplasmic membrane prior to their translocation to the nucleus where they bind to DNA sequences, predominantly related to the gamma interferon (IFNγ) activated site (GAS) (Darnell, Jr, 1997; Leonard and O'Shea, 1998). Seven mammalian Stat genes have been cloned: Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b and Stat6. In contrast to Stat4 and Stat6 activation by a limited number of cytokines, Stat5a and Stat5b are activated by many cytokines, including prolactin (PRL), growth hormone, IFNγ and interleukin-2 (IL-2). Specific gene activation by these cytokines cannot depend solely on Stat5 but likely involve association with other cell type and/or induction specific factors.

It is now well established that the lineage-specific and inducible expression of eukaryotic genes is controlled by assembly of multipartite complexes of transcription factors on regulatory regions composed of sequence-specific protein binding sites. In that respect, some Stat proteins bind to other transcription factors or coactivators to activate transcription (Bhattacharya *et al*., 1996; Look *et al*., 1995; Muhlethaler-Mottet *et al*., 1998; Qureshi *et al*., 1995; Schaefer *et al*., 1995; Shen and Stavnezer, 1998; Zhang *et al*., 1996). More specifically, MGF/Stat5a has only been reported to form a complex with the glucocorticoid receptor (GR) on the β-casein promoter devoid of GR binding site (GRE) (Cella *et al*., 1998; Stöcklin *et al*., 1996). However, Stat5 has not been shown to bind to any known transcription factors in the immune system.

The Ets gene family encodes a family of transcription factors involved in a variety of biological processes, including growth control, transformation, T cell activation and developmental programs (Anderson *et al*., 1999; Bassuk and Leiden, 1997; Ghysdael and Boureux, 1997). Over 30 members belonging to this family share a highly conserved DNA binding domain and bind to distinct sites containing a core purine rich motif (EBS). Several Ets factors interact with other regulators of gene expression and cell cycle progression. Ets-1 binds various transcription factors, including Sp1 (Gegonne *et al*., 1993), PEBP2α/AML-1 (Giese *et al*., 1995), GHF-1/Pit-1 (Bradford *et al*., 1995), MafB (Sieweke *et al*., 1996), NF-κB, AP-1 (Thomas *et al*., 1997), and USF-1 (Sieweke *et al*., 1998). Despite its high degree of structural similarity with Ets-1, only one protein-protein interaction between Ets-2 and p300/CBP (Jayaraman *et al*., 1999) and two transcriptional cooperations with GATA3 (Blumenthal *et al*., 1999) or ATF/Jun family members (Cirillo *et al*., 1999) have been reported. Two Stat-Ets cooperations have been described. One established that PU.1/Spi-1 and Stat1 are required for IFN induced expression of the FcγRI receptor (Perez *et al*., 1994),

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whereas the second suggested a functional cooperation between Stat5 and the lymphoid Ets factor Elf-1 (John *et al*., 1996; Serdobova *et al*., 1997). The transcriptional synergies between both families required the presence of their respective cognate binding sites.

A positive regulatory region (PRRIII/IL-2rE) has been identified as a target site for IL-2-activated Stat5a and Stat5b proteins both in human and mouse CD25/IL-2Rα genes (John *et al*., 1996; Lecine *et al*., 1996; Soldaini *et al*., 1995). Consistent with a role of both Stat5 proteins in the transcriptional activation of the CD25/IL-2Rα gene, expression of the inducible chain of IL-2 was not maintained on lymphocytes of Stat5a/b-deficient mice in presence of IL-2 (Moriggl *et al*., 1999). PRRIII is a composite region composed of both consensus and non-consensus GAS motifs (GASd and GASp), two distinct Ets binding sites (EBSd and EBSp) and one GATA motif. IL-2 induced formation of two GAS-specific protein complexes revealed by a GASd/EBSd probe. Both inducible complexes contained Stat5 but their different migration was not elucidated. We now report that, in tumoral Kit225 T cells and in human peripheral blood T lymphocytes, the GASd/EBSd regulatory element was mainly bound constitutively by GAPBα/β and that Ets-1 and Ets-2 proteins were associated with Stat5 in the slower migrating IL-2-inducible protein-DNA complex. IL-2 induced a physical association of Stat5b with either Ets-1 or Ets-2 in absence of DNA and transient cotransfection experiments revealed a transcriptional cooperation between these two unrelated transcription factor families.

Results

IL-2 induces a GAS-specific protein-DNA binding complex containing Stat5b, Ets-1 and Ets-2

IL-2 induces formation of two distinct GAS-specific protein-DNA complexes on the GASd/EBSd regulatory element within PRRIII enhancer of IL-2Rα gene (Lecine *et al*., 1996). Competition with an unlabeled GASd/EBSd oligonucleotide abolished the constitutive complex (C1) and the two IL-2-induced complexes (C2 and C3) revealed by EMSAs (Figure 1a, compare lanes 1, 2 and 3). In contrast, a mutant GASd/EBSd oligonucleotide with a disrupted GAS had no effect on C2 and C3 but significantly reduced C1 (Table 1 and Figure 1a, lane 4). The oligonucleotide containing a disrupted EBS motif fully abolished both inducible complexes and reinforced the constitutive C1 complex (Figure 1a, lane 5) whereas an oligonucleotide containing both disrupted motifs had no effect (Figure 1a, lane 6). EBSa, an high affinity Ets binding site ((Bosselut *et al*., 1993) and Table 1), abrogated C1 without affecting the inducible C2 and C3 complexes whereas EBSz, its mutated counterpart, had no effect (Figure 1a, compare lane 2 with lanes 7 and 8). IL-2 induced C2 and C3 complexes are specific for the GAS consensus in the human GASd/EBSd element while the constitutive C1 complex depends on the EBS motif.

Immunoassays revealed that C2 and C3 complexes contained Stat5b proteins but their different migration was not explained and could be due either to the degree of Stat5 protein multimerization or to the

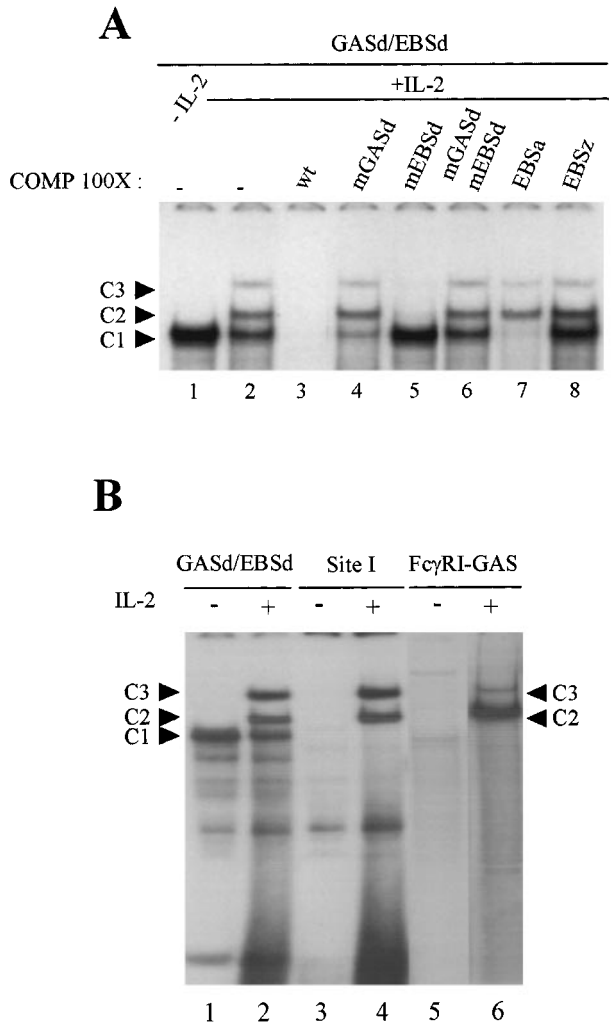

Figure 1 Two GAS-specific protein-DNA complexes are induced by IL-2 in Kit225 leukemic T cells. (**a**) EMSA competition experiments were performed using the GASd/EBSd probe and Kit225 nuclear extracts IL-2 deprived (lane 1) or IL-2 stimulated (lanes 2–6) without (lanes 1 and 2) or in the presence of a 100 × excess of unlabeled competitors: wild-type GASd/EBSd (lane 3, wt); GASd/EBSd mutated in GASd site (lane 4, mGASd); GASd/EBSd mutated in EBSd site (lane 5, mEBSd); GASd/EBSd mutated on both site (lane 6, mGASd/mEBSd); an high affinity Ets-binding site (lane 7, EBSa,) and its mutated form (lane 8, EBSz) (Rabault and Ghysdael, 1994). (**b**) EMSAs were performed with human GASd/EBSd (lanes 1 and 2), murine Site I (lanes 3 and 4), or human FcγRI-GAS (lanes 5 and 6) probes and nuclear extracts from Kit225 T cells IL-2 starved (lanes 1, 3 and 5) or IL-2 stimulated during 1 h (lanes 2, 4 and 6)

presence of additional polypeptides. The EBS element is absent in site I, the murine counterpart of GASd/EBSd, although the IL-2 responsive enhancer is highly conserved in both species (Bucher *et al*., 1997). To characterize the composition of the IL-2-inducible complexes, EMSAs were performed with three probes corresponding to human GASd/EBSd, FcγRI GAS and mouse site I (Table 1). In contrast to previous reports (John *et al*., 1999; Meyer *et al*., 1997), the three probes revealed both complexes in human T cell nuclear extracts, evidencing that they were not a singularity of the human GASd/EBSd element (Figure 1b). Supershift and inhibition of binding assays were performed with specific antisera. Anti-Sta5b serum supershifted C2 and C3 complexes (Figure 2a, lane 3), confirming that both complexes contained Stat5b.

Table 1 Synthetic double-stranded oligonucleotide probes used in EMSAs

Name	*Sequence*	*Origin*
GASd/EBSd	TTTCTTCTAGGAAGTACC	PRRIII/hIL-2Rα (−3772, −3755)
	AAAGAAGATCCTTCATGG	
mGASd	TTTCT**GG**TAGGAAGTACC	PRRII I/hIL-2Rα (−3772, −3755)
	AAAGACCATCCTTCATGG	
mEBSd	TTTCTTCT**CC**GAAGTACC	PRRIII/hIL-2Rα (−3772, −3755)
	AAAGAAGAGGCTTCATGG	
mGASd/mEBSd	TTTCT**GG**T**CC**GAAGTACC	PRRII I/hIL-2Rα (−3772, −3755)
	AAAGACCAGGCTTCATGG	
Site I	TTTCTTCTGAGAAGTACC	PRRIII/mIL-2Rα (−1369, −1352)
	AAAGAAGACTCTTCATGG	
FcγRI-GAS	GTATTTCCCAGAAAAGGAAC	hFcγRI (−51, −32)
	CATAAAGGGTCTTTTCCTTG	
EBSa	GATAAACAGGAAGTGGTTGTA	–
	CTATTTGTCCTTCACCAACAT	–
EBSz	GATAAACA**CC**AAGTGGTTGTA	–
	CTATTTGTGGTTCACCAACAT	–

Sequences are in 5′ to 3′ orientation. GAS motifs are underlined and EBS motifs are doubly underlined. The mutated nucleotides are in boldface

Inhibition of binding by antisera specific for Ets-1 and Ets-2 revealed that the slower migrating inducible C3 complex contained both Ets proteins (Figure 2a, lanes 4, 5 and 6) whereas faster migrating C2 was unaffected. Antisera against other members of the Ets family (Elf-1, GABPα/β, Fli-1 and Tel) failed to affect C3 complex (data not shown). Similar results were obtained using a FcγRI probe (Figure 2b), a well-defined GAS motif devoid of any functional EBS (Beadling *et al*., 1994).

The IL-2-induced proteins were further identified by DNA affinity purification using a GASd/EBSd biotinylated probe and Kit225 cell extracts (Figure 2c). Western blotting analysis confirmed that IL-2 induced not only Stat5b but also Ets-1 or Ets-2 binding (Figure 2c, compare lanes 1 and 2). The doublet observed with anti-Stat5b was likely due to the level of serine phosphorylation of this protein (Beadling *et al*., 1996). The same assay using biotinylated EBSa revealed the constitutive binding of Ets-1 or Ets-2 proteins but did not reveal Stat5b. Furthermore, IL-2 induced a weak but reproducible increase of Ets protein binding (Figure 2c, lanes 3 and 4).

Taken together, these results strongly supported that Ets-1, Ets-2 and Stat5b form a functional GAS-specific DNA-binding complex in response to IL-2 in Kit225 T cells, which is not a peculiarity of the GASd/EBSd motif of the human CD25/IL-2Rα gene.

In vivo *and* in vitro *interactions of Stat5b, Ets-1 and Ets-2 in response to IL-2*

Identification of an IL-2-induced Stat5-Ets DNA binding complex with several GAS probes prompted us to investigate whether these proteins might interact in absence of DNA. Unstimulated or IL-2-stimulated Kit225 cell extracts were immunoprecipitated with anti-Ets-1 and/or -Ets-2 sera and analysed by immunoblotting with an anti-Stat5b serum. Stat5b was coimmunoprecipitated with Ets-1 and Ets-2 in response to IL-2 (Figure 3a, lanes 2, 4 and 6) but not by an anti-c-Rel (Figure 3a, lanes 7 and 8). The doublet suggested that Ets-1 and Ets-2 interact with both forms of Stat5 tyrosine phosphoprotein (Beadling *et al*., 1996). To confirm this interaction in absence of DNA, Ets-1 recombinant proteins were used in GST-pull down assays (Figure 3b). Full-length Ets-1 and an N-terminal truncated form, containing the Ets domain, efficiently interacted with IL-2-activated Stat5b (Figure 3c). These results demonstrated the formation of an IL-2 inducible protein complex associating Stat5b, Ets-1 and/or Ets-2 proteins in absence of their cognate DNA binding sites.

Cooperative transcriptional activation by Stat5b, Ets-1 and Ets-2

Identification of an IL-2-induced Stat5-Ets complex suggested a transcriptional synergy between these transcription factors. To analyse this putative synergy and to avoid the complex interactions observed within the native IL-2rE (John *et al*., 1996, 1999; Meyer *et al*., 1997), wild-type and mutant GASd/EBSd trimers were inserted upstream of HSV TK minimal promoter (Figure 4a). The design of these artificial targets was validated by the binding properties of WT and mutants GASd/EBSd motifs (Figure 1a). After transfection in Kit225 cells, IL-2 induced the wild-type GASd/EBSd construct (Figure 4b) whereas disruption of the GASd motif fully abrogated IL-2-induced CAT activity. EBSd disruption dramatically reduced the overall transcriptional activity without affecting the induction level and the double mutation completely abolished both constitutive and inducible activities.

Cotransfection assays evidenced that IL-2 alone was unable to trigger a cooperation between exogenous Stat5b, Ets-1 and Ets-2 (data not shown). In Jurkat T

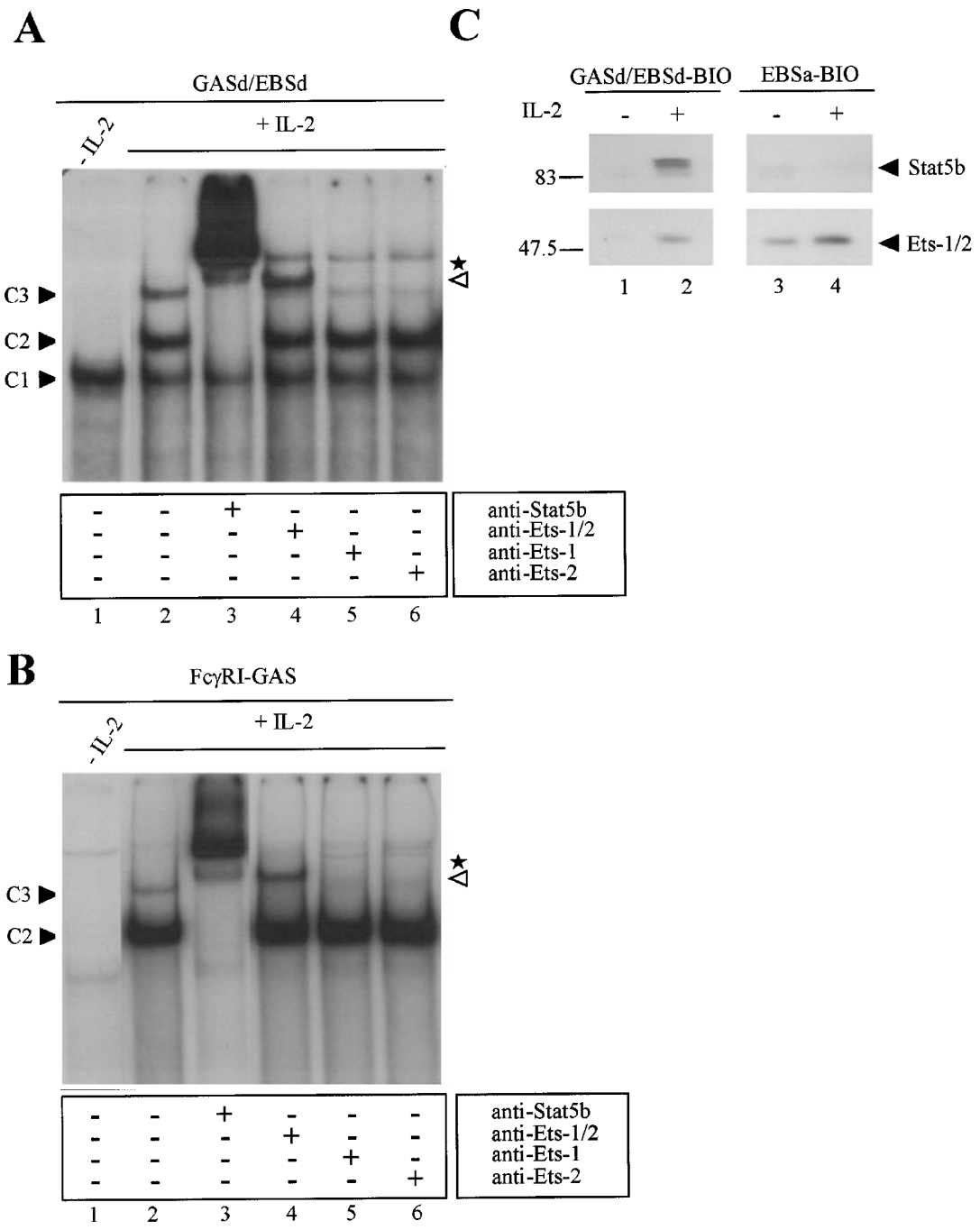

Figure 2 The IL-2-inducible C3 complex contains Stat5b, Ets-1 and Ets2 proteins. (**a**) and (**b**) Immunoassays were performed with GASd/EBSd (**a**) or FcγRI-GAS (**b**) probes and nuclear extracts from Kit225 T cells unstimulated (lane 1) or stimulated during 1 h (lanes 2–6) with IL-2 and incubated (lanes 3–6) or not (lanes 1 and 2), prior the addition of the probe, with antisera to Stat5b (lane 3), Ets-1/2 (lane 4), Ets-1 (lane 5), Ets-2 (lane 6). Proteins-DNA complexes were resolved in non-denaturing acrylamide gels and revealed by autoradiography. Filled arrowheads, position of the specific protein-DNA complexes; open arrowheads, position of the complexes supershifted by the specific antisera. Stars indicate the positions of complexes due to nonspecific DNA-binding activities contributed by sera. (**c**) Affinity purification of DNA-binding proteins were performed with 5′-biotinylated GASd/EBSd (lanes 1 and 2) or EBSa (lanes 3 and 4) probe and whole extracts from Kit225 T cells unstimulated (lanes 1 and 3) or IL-2 stimulated (lanes 2 and 4). Bound proteins were identified by Western blotting with Stat5b- and Ets-1/2-specific antisera. Estimated sizes in kilodaltons are shown on the left of the panel

cells, efficient transactivation of a GM-CSF or IL-5 promoter reporter construct by Ets-1 and Ets-2 required a combined stimulation with PMA and ionomycin, that mimics engagement of the T cell receptor (Blumenthal *et al*., 1999; Thomas *et al*., 1997). We postulated that endogenous transduction pathways in response to IL-2 alone did not efficiently activate overexpressed Ets-1 and Ets-2. Accordingly, the transcriptional activity of a pentameric EBS reporter gene construct transfected in Kit225 cells was slightly activated by IL-2 whereas addition of PMA plus ionomycin resulted in a strong induction dependent of a *bona fide* EBS (Figure 5a). Therefore, further assays were performed in Kit225 T cells activated by a combination of IL-2, PMA and ionomycin.

Transfection of wild-type GASd/EBSd trimeric construct with Stat5b, Ets-1 and Ets-2 resulted in a 27-fold induction in response to a triple IL-2, PMA

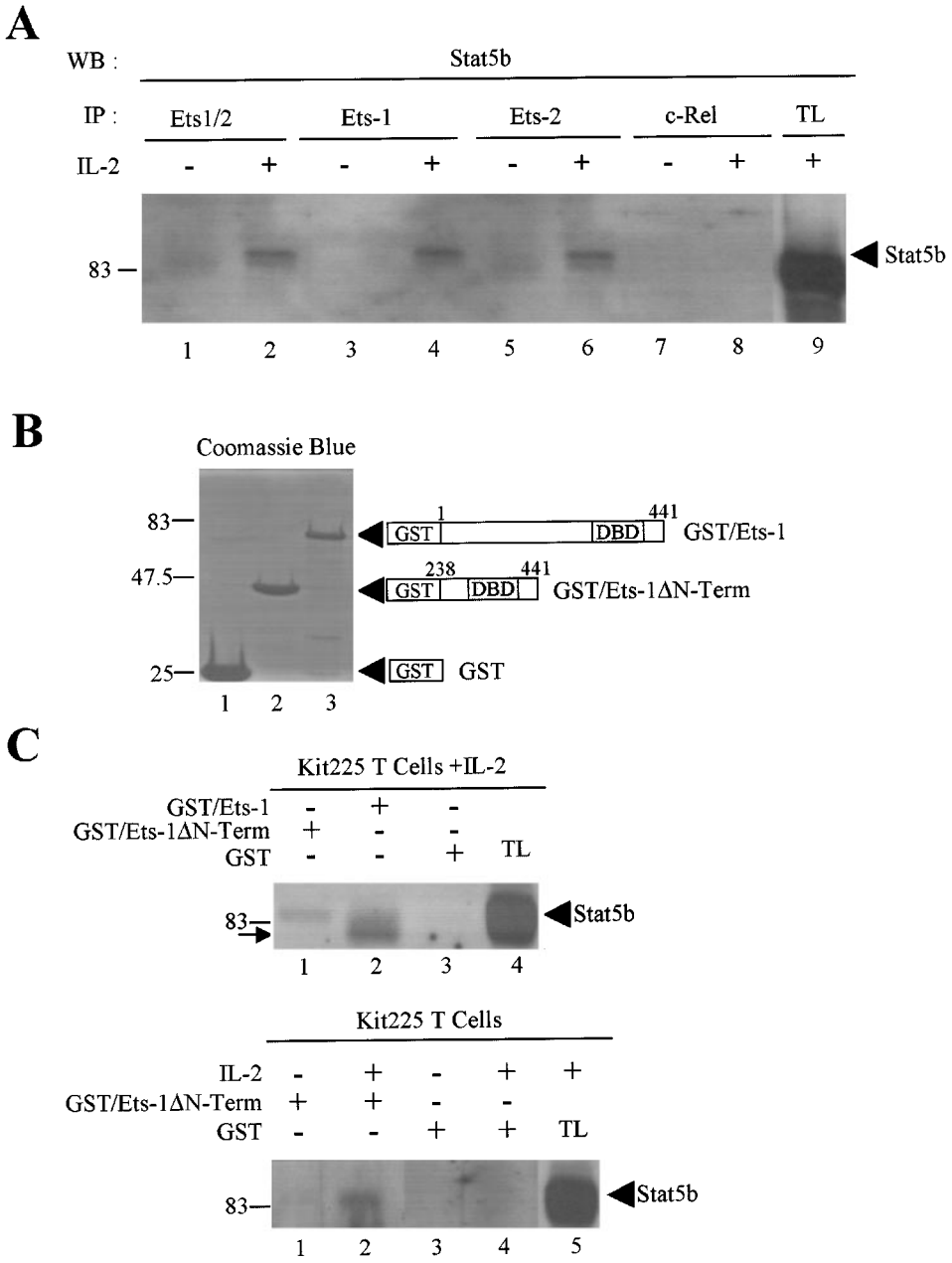

Figure 3 Stat5b, Ets-1 and Ets-2 interact in absence of their cognate binding site in IL-2-stimulated Kit225 human T cells. (**a**) Whole cell extracts from unstimulated (lanes 1, 3, 5 and 7) or IL-2 stimulated (lanes 2, 4, 6, 8 and 9) were immunoprecipitated with antisera directed against either Ets-1 (lanes 3 and 4), Ets-2 (lanes 5 and 6), c-Rel (lanes 7 and 8) or Ets-1/2 (lanes 1 and 2). The resulting immune complexes were denatured, separated by SDS–PAGE and analysed by immunoblotting with an anti-Stat5b serum. Lane 9: IL-2-stimulated Kit225 cell total lysate (TL). Estimated sizes in kilodaltons are shown on the left of the panel. (**b**) Schematic representation of the GST constructs and purity control of the GST-Ets-1 fusion proteins by coomassie blue staining. (**c**) Interaction in solution of IL-2-activated Stat5b with GST-Ets-1 recombinant proteins. IL-2 stimulated whole cell extracts were incubated with 5 μg of various GST-Ets-1 recombinant proteins (upper part, lanes 1–3). Unstimulated (lower part, lanes 1 and 3) and IL-2-stimulated whole cell extracts (lower part, lanes 2 and 4) were incubated with 5 μg of truncated N-terminal GST-Ets-1 recombinant protein. Lane 4 (upper) and lane 5 (lower) contain IL-2-stimulated Kit225 cell total lysate (TL). Immunoblots were revealed with an anti-Stat5b serum. The arrow on the left side of upper part corresponds to the GST protein revealed by the secondary antibody

and ionomycin stimulation, compared to 8–9-fold induction with either Ets-1, Ets-2 or Stat5b (Figure 5b, $P<0.05$). EMSAs confirmed that the protein/DNA complexes induced by the triple stimulation were indistinguishable from those induced by IL-2 alone (Figure 5c). To highlight effects of either factor addition, data in Figure 5d represent the ratio of the CAT activity of the double or the triple cotransfection relative to the averaged CAT activity observed with single cotransfections. Ets-1 and Ets-2 overexpression in the same recipient cells resulted in a minor increase not significantly different from transactivation with either Ets protein (Figure 5b and d) and there was no significant difference with either Ets-1 or Ets-2 cotransfected with Stat5b (data not shown). In contrast, cotransfection of Stat5b, Ets-1 and Ets-2 resulted in a 3.5-fold increase relative to the transcriptional activity observed with single cotransfection (i.e. 30–40-fold increase in comparison to the empty reporter gene vector). Requirement of a *bona fide*

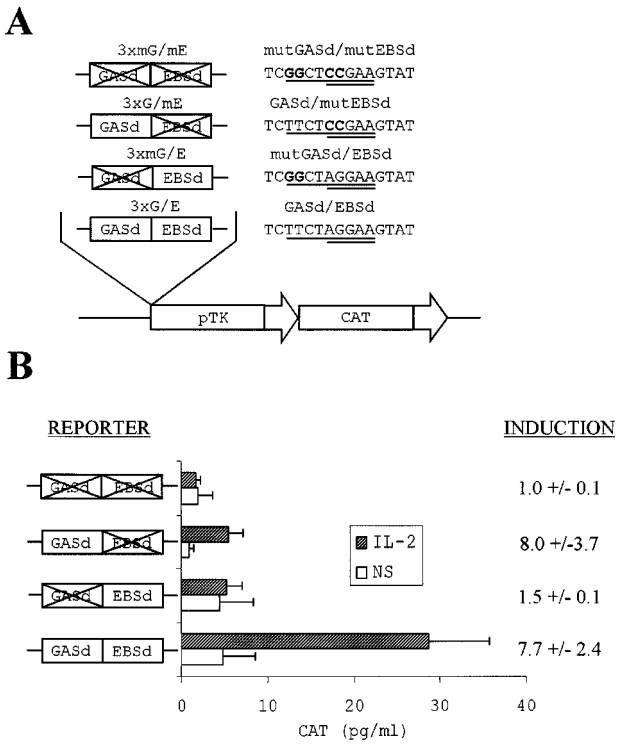

Figure 4 Functional characterization of the wild type and mutant GASd/EBSd trimeric CAT reporter gene constructs. (**a**) Schematic representation of the wild type and mutant GASd/EBSd trimeric constructs cloned upstream of HSV TK minimal promoter/CAT reporter gene vector (see Materials and methods). The nucleotidic sequences of the wild type and mutant motifs are shown on the right part of (**a**). GAS motif is underlined and EBS motif is doubly underlined. Substituted nucleotides are in boldface. (**b**) Unstimulated or IL-2-stimulated Kit225 T cells were transfected with wild-type and mutated GASd/EBSd trimeric constructs (20 μg). Induction level for each transfection assay is indicated at the right of the histogram. All data are standardized to CAT activity obtained with the empty pTK4-CAT vector. Values presented are means of at least four independent determinations and error bars represent standard deviations of the mean (s.e.m.)

GAS consensus for the cooperation between Ets and Stat proteins was further confirmed by mutations of either GASd or EBSd motif. Disruption of GASd site reduced induction to single transfection level (Figure 5d). This residual transcriptional activity is most probably due to direct binding of Ets proteins to the preserved EBSd motif in agreement with its binding activity revealed by EMSAs. In comparison, the synergistic effect was conserved with the trimeric construct containing a disrupted EBSd site. As expected, the double mutation fully abolished cooperation between Stat and Ets.

Taken together, these results demonstrated a cooperative transactivation of GAS regulatory elements by Stat5b, Ets-1 and Ets-2 proteins.

IL-2-dependent mitogenic stimulation of human primary T cells induces a major protein-DNA complex containing Stat5a, Stat5b, Ets-1 and/or Ets-2

To investigate whether the IL2-induced Stat-Ets complex identified in the Kit225 tumoral T cell line represented a component of the physiological IL-2 response, EMSAs were performed with nuclear extracts from human primary T cells activated via CD2+CD28. This potent costimulation triggers an IL-2-dependent proliferation of human primary T cells, mimicking the antigen-dependent T lymphocyte activation (Olive *et al*., 1994). Time-course analysis with the GASd/EBSd probe revealed two major modifications (Figure 6a): (1) a strong constitutive fast migrating protein-DNA complex, C1p, dramatically reduced 30 min after stimulation (Figure 6a, compare lanes 1 and 2–8); (2) a major inducible slower migrating complex, C2p after 4–6 days (Figure 6a, lanes 5 and 6) that was strongly reduced after 8 days (Figure 6a, lanes 7 and 8). Other minor modifications were not reproducibly observed and not further characterized.

Immunoassays with antisera against several Ets proteins revealed that the C1p constitutive complex was composed mostly, if not exclusively, of GABPα and GABPβ (Figure 7a). A similar assay with Kit225 nuclear extracts revealed that, beside a previously characterized minor Elf-1 component (Lecine *et al*., 1996), the C1 complex was also mainly composed of GABPα/β (data not shown). Since IL-2 secretion by CD2+CD28-stimulated T lymphocytes peaked at day 4–6 (Costello *et al*., 1993), we focused on the DNA-binding specificity and composition of the inducible C2p in T cells 6 days after stimulation. Competition experiments with wild-type, GAS and/or EBS disrupted oligonucleotides evidenced that C2p was specific for the GASd motif (Figure 6b), as reported above for IL-2-induced C2/C3 complexes in the leukemic T cell model Kit225. Anti-Stat5, Ets-1 or Ets-2 sera alone partially supershifted C2p complex whereas their combination abolished its formation (Figure 7b, compare lanes 3–5 with lane 6). An irrelevant anti-Sp1 did not affect C2p (Figure 7b, lane 7). Further controls were performed with sera directed against other Stat family members (Stat3, Stat4 and Stat6) confirming the composition of complex C2p (Figure 7c). Taken together, these results demonstrated that the major complex C2p induced by CD2+CD28 in human primary T lymphocytes is a GAS-specific macromolecular complex containing Stat5, Ets-1 and/or Ets-2 proteins.

Despite their near identical antigenic and DNA-binding specificities, the constitutive and inducible complexes revealed by EMSAs performed with nuclear extracts of tumoral Kit225 and primary T cells did not exhibit the same mobility (Figure 7d). This discrepancy suggests that tumoral and primary T cell complexes either differ in the structural conformation and/or the stoichiometry of their various constituents, or contain one or more different partners. For example, we have already reported that Kit225 T cells lack Stat5a protein in contrast to primary T cells which express comparable amounts of Stat5a and Sta5b (Lecine *et al*., 1996). However, further experiments aimed to identify the components of each complex are required to elucidate the unexpected differences in the migration pattern.

Discussion

We have evidenced a physical and functional association between the Ets-1, Ets-2, and Stat5 transcription factors. These associations occur not only in human leukemic Kit225 T cells in response to IL-2 but also in human primary T lymphocytes activated *in vitro* by

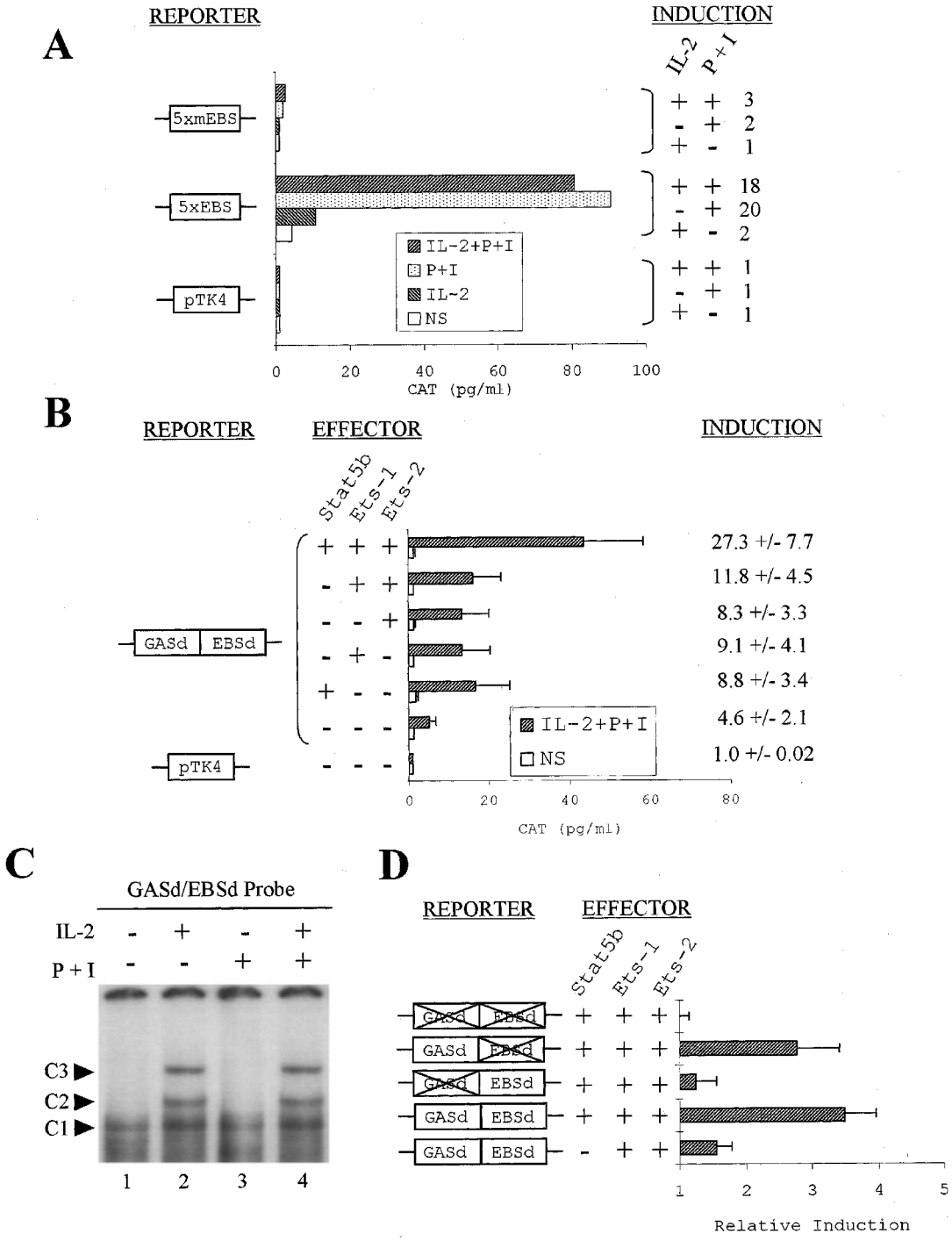

Figure 5 Functional cooperation between Stat5b, Ets-1 and Ets-2 in human Kit225 T Cells. (**a**) PMA + ionomycin treatment is required to efficiently activate a wild-type pentameric EBS reporter gene construct. Unstimulated or IL-2−, PMA + ionomycin- and IL-2+ PMA + ionomycin-stimulated Kit225 T cells were transfected with wild-type and mutated pentameric EBS constructs (20 μg). Induction level for each stimulation condition relative to unstimulated level is indicated at the right of the histogram. The data shown are from one representative experiment. (**b**) Cotransfection of Stat5b, Ets-1 and Ets2 expression vectors with wild-type pTK4-CAT/(GASd/EBSd) × 3. Unstimulated or IL-2 + PMA + ionomycin stimulated Kit225 T cells were cotransfected with the wild-type GASd/EBSd trimeric construct (20 μg) and expression vectors for either Stat5b (2 μg), Ets-1 (2 μg) or Ets-2 (2 μg). Induction level for each transfection assay is indicated at the right of the histogram. (**c**) EMSA were performed with the GASd/EBSd probe and nuclear extracts from Kit225 without (lane 1) or in the presence of IL-2 alone (lane 2), PMA plus ionomycin (lane 3) or a combination of these three inducers (lane 4). (**d**) Effect of mutation within GASd/EBSd element on Stat5b, Ets-1 and Ets-2 functional cooperation. Cotransfections were performed in Kit225 T cells in absence or presence of IL-2 + PMA + ionomycin with wild type or mutant trimers and either Ets-1, Ets-2, or Ets-1, Ets-2 and Stat5b. To underline the cooperation between the three transcription factors and the effects of GASd/EBSd mutations, data are expressed as ratio of CAT activity of triple cotransfections relative to the CAT activity observed with single cotransfections. All data are standardized to CAT activity obtained with the empty pTK4-CAT vector. Values presented are means of at least four independent determinations and error bars represent s.e.m.

CD2 + CD28 costimulation, a potent IL-2-dependent inducer of long-term proliferation, which mimics the physiological antigenic activation of human peripheral blood T lymphocytes (Olive *et al*., 1994). Interactions between these two unrelated transcription families resulted in formation of a composite protein-DNA

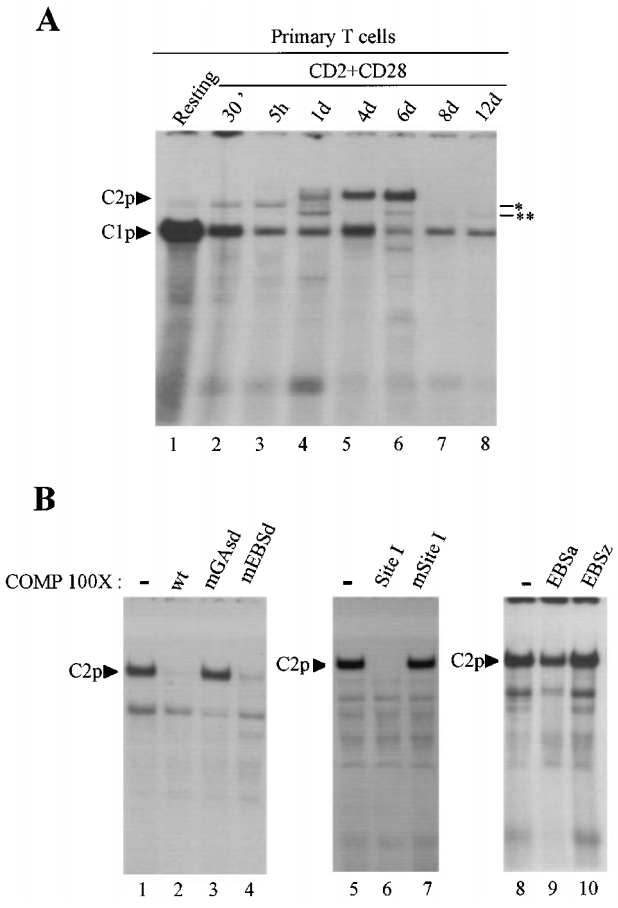

Figure 6 Time course analysis of GASd/EBSd in vitro occupancy by nuclear proteins from resting and CD2+CD28-stimulated human primary T cells. (**a**) EMSAs were performed with the GASd/EBSd probe and with nuclear extracts from human T lymphocytes unstimulated (lane 1) or CD2+CD28 stimulated during 30 min (lane 2), 5 h (lane 3), 1 day (lane 4), 4 days (lane 5), 6 days (lane 6), 8 days (lane 7) or 12 days (lane 8). Stars, on the right of the panel, indicated minor protein-DNA complexes not further characterized. (**b**) Competition assays were performed with nuclear extracts of primary T cells CD2+CD28-stimulated for 6 days (lanes 1–10) without (lanes 1, 5 and 8) or with a 100X excess of unlabeled competitors: wild-type GASd/EBSd (lane 2, wt); mutated in the GASd site (lane 3, mGASd); mutated in the EBSd site (lane 4, mEBSd); wild-type site I (lane 6, Site I), mutant site I (lane 7, mSite I). To address the putative involvement of Ets-related proteins in the complexes revealed by the GASd/EBSd probe, competitions with 100X excess of either a high affinity (lane 9, EBSa) or mutant (lane 10, EBSz) EBS binding sites were performed. Filled arrowheads indicated the position of the specific protein-DNA C1p and C2p complexes

complex revealed by EMSA with GAS-specific oligonucleotidic probes derived from IL-2Rα and FcγRI genes, and in the cooperative transactivation of trimeric GASd/EBSd reporter gene constructs.

We previously reported that Elf-1 a lymphoid Ets family member can serve as a transcriptional repressor of IL-2rE/PRRIII In Kit225 leukemic T cells (Lecine *et al*., 1996). Our conclusions were based upon transient overexpression of an Elf-1 expression vector in presence of IL-2 alone. In the light of our new observations, we wondered whether this ectopic factor might act so because of the absence of the additional stimulation of PMA and ionomycin. However, we failed to observe any significant cooperation between Stat5 and Elf-1 in transient cotransfection in IL-2+PMA+ionomycin-treated Kit225 T cells (data not shown). The dramatic decrease of the constitutive complex C1p observed in resting T lymphocytes stimulated by CD2+CD28 suggests that this Ets-related protein-DNA complex plays a negative role in IL-2Rα gene expression regulation. The C1p complex was mostly composed of GABPα/β, an Ets-related heterotetramer (Brown and McKnight, 1992; Thompson *et al*., 1991) that could act as a repressor or as an activator depending on the promoter context (Genuario and Perry, 1996; Schaeffer *et al*., 1998). Altogether these observations suggest that GABP could play a role in primary T cells, yet to be confirmed.

The identification of the Stat5-Ets DNA binding complex with various GAS-specific oligonucleotidic probes derived from IL-2Rα and FcγRI genes, containing or not an identified EBS consensus, demonstrated that this composite complex was not a peculiarity of the human GASd/EBSd motif. Two recent studies have underscored the requirement for Stat5 tetramerization mediated by the two PRRIII GAS motifs for efficient promoter recruitment and transcriptional activation (John *et al*., 1999; Meyer *et al*., 1997). Although the experiments presented here were not designed to address the respective role of the two GAS elements present within human PRRIII, some of our results differ significantly from those recently reported. *In vivo* genomic footprinting clearly evidenced an inducible occupancy solely within the GASd/EBSd site, whereas the GASp/GATA site was constitutively occupied (Lecine *et al*., 1996). This argues against an IL-2 inducible cooperative binding on both sites but does not preclude their functional interaction. Moreover, we confirmed our previous report (Lecine *et al*., 1996) and observed efficient binding of two GAS-specific complexes on monomeric GAS-specific motif using both tumoral and primary human T cell extracts, whereas John and collaborators (John *et al*., 1999) did not report any significant Stat5 binding with a monomeric GASc probe (almost identical of our GASd/EBSd probe). We did not elucidate this discrepancy but we suspect some differences in EMSA protocols. This interpretation is reinforced by the fact that the authors reported only a weak *bona fide* Stat5 binding in IL-2-stimulated PBL but did not detect the faster migrating EBS-specific and slower Stat5-Ets composite complexes.

Presence of Stat5 in the C2p inducible complex is in line with one of its roles evidenced in mice defective for Stat5 expression (Feldman *et al*., 1997; Imada *et al*., 1998; Moriggl *et al*., 1999; Nakajima *et al*., 1997; Teglund *et al*., 1998; Udy *et al*., 1997). The phenotype of peripheral T cells from Stat5a/b-deficient mice, opposed to single knock-out, revealed a crucial and interchangeable role for these proteins in IL-2 signaling (Moriggl *et al*., 1999). Mature T cells of these mice exhibited a dramatic decrease in proliferative response triggered by anti-CD3 and IL-2. Furthermore, the expression of CD25/IL-2Rα on lymphocytes from Stat5a/b-deficient mice was not maintained in the presence of IL-2, consistent with a role for the Stat5 proteins in CD25 expression in CD4$^+$ lymphocytes.

In agreement with a role in IL-2-mediated lymphocyte survival (Zamorano *et al*., 1998), the Stat5-Ets complex was predominant in long-term proliferating primary T cells. Ets-1 presence is consistent with its

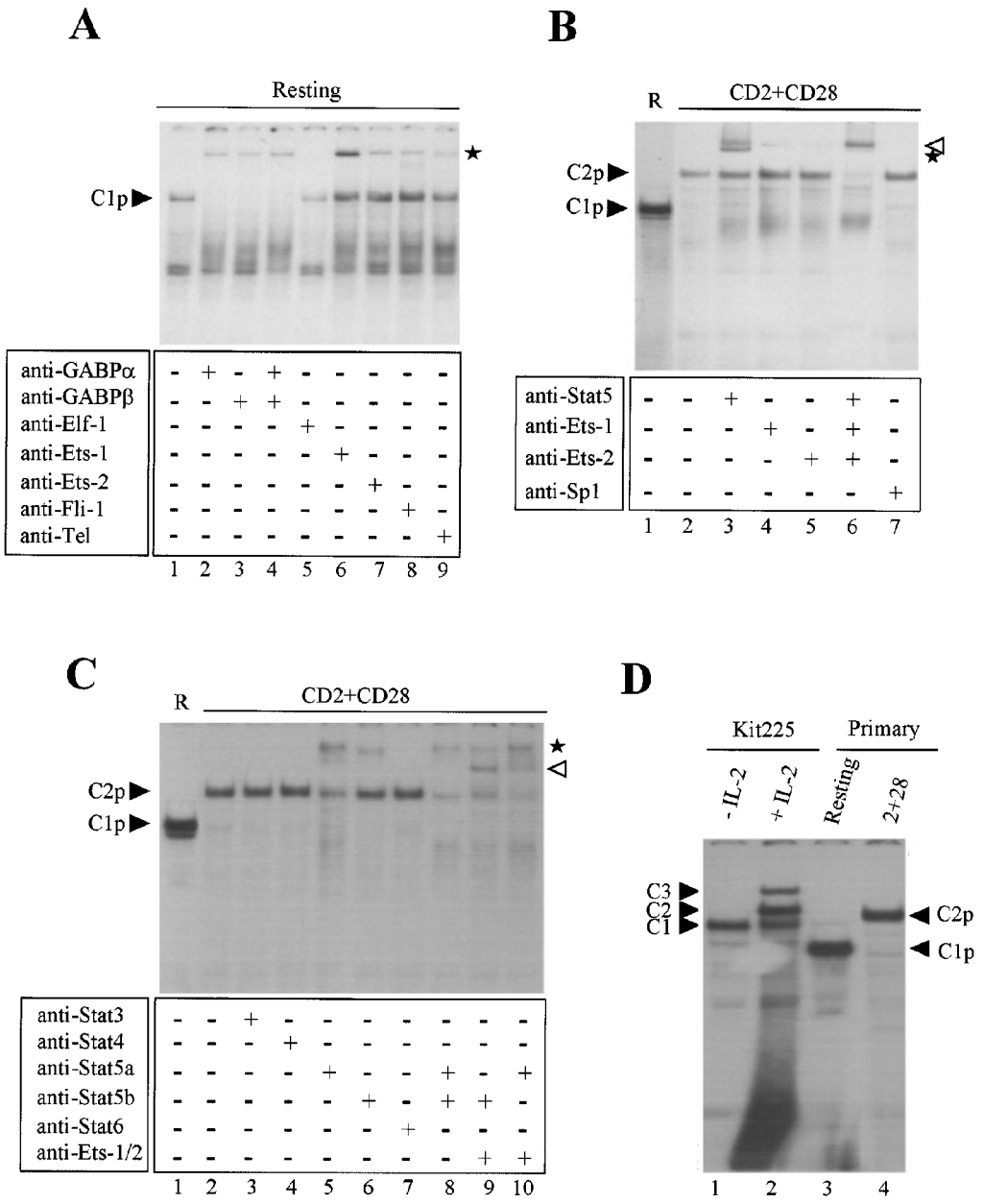

Figure 7 Characterization of the major constitutive and CD2+CD28-inducible complexes specific for the GASd/EBSd element in human primary T cells. (**a**) Supershift and inhibition of binding assays performed with nuclear extract from resting primary T cells and by addition of various antisera directed against the following Ets family members: GABPα (lane 2), GABPβ (lane 3), alone or in combination (lane 4), Elf-1 (lane 5), Ets-1 (lane 6), Ets-2 (lane 7), Fli-1 (lane 8) or Tel (lane 9). (**b**) Supershift and inhibition of binding analysis performed with primary T cells nuclear extracts 6 days after CD2+CD28 costimulation and antisera directed against Stat5 C17 (lane 3), Ets-1 (lane 4), Ets-2 (lane 5), in combination (lane 6) and Sp1, as negative control (lane 7). (**c**) Supershift and inhibition of binding analysis performed with primary T cells nuclear extracts 6 days after CD2+CD28 costimulation and antisera directed against Stat3 (lane 3), Stat4 (lane 4), Stat5a (lane 5), Stat5b (lane 6), Stat6 (lane 7), Stat5a and b (lane 8), Stat5b and Ets-1/2 (lane 9) and Stat5a and Ets-1/2 (lane 10). (**d**) Comigration patterns of the protein-DNA binding complexes in leukemic Kit225 T cells and primary T lymphocytes. EMSAs were performed with the GASd/EBSd probe and nuclear extracts from Kit225 IL-2 starved for 48 h (lane 1) or IL-2 stimulated for 1 h (lane 2) or from human primary T lymphocytes resting (lane 3) or 6 days after CD2+CD28 costimulation (lane 4). Filled arrowheads, position of the specific protein-DNA complex; open arrowheads, position of the complexes supershifted by the specific antisera. Stars indicate the positions of complexes due to nonspecific DNA-binding activities contributed by the sera

regulatory role in T cell proliferation and apoptosis (Muthusamy *et al*., 1995), whereas Ets-2 does not appear essential for lymphoid cells (Yamamoto *et al*., 1998). Ets-1 expression during activation of normal mature T cells is in agreement with the late appearance of the Stat5-Ets complex. Ets-1 mRNA is expressed at high levels in quiescent cells, efficiently down regulated after activation and reinduced to the levels observed in resting cells 3 days after PMA+ionomycin stimulation (Bhat *et al*., 1989).

Activation of resting T lymphocytes requires at least two signals: one provided by engagement of the T cell antigen receptor complex (TcR/CD3) with antigen associated with MHC and the second by costimulatory molecules, such as CD28 (Cantrell, 1996; Ward, 1996). In Kit225 T cells, transcriptional activity, but not formation, of Stat-Ets complex required a triple combination of stimuli (IL-2, PMA and ionomycin). These observations suggest integration between different T cell signaling pathways, mediated by the

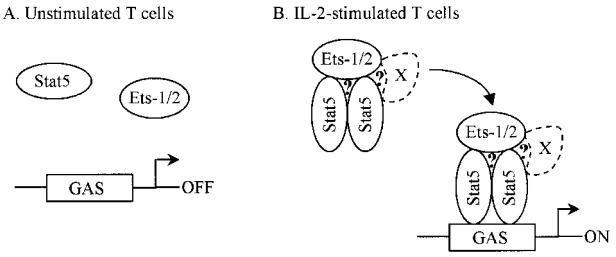

Figure 8 Two steps model of IL-2-induced Stat5-Ets interaction in T lymphocytes. IL-2 induces formation of a complex associating Stat5, Ets-1 and Ets-2 proteins in absence of their cognate DNA binding sites. This complex then binds to GAS-related regulatory elements. The requirement of a third partner (X), related or not to Stat5 and Ets families, cannot be excluded

formation of Stat5 and Ets-1/2 protein complex in solution that can then form higher order oligomeric complexes on DNA. IL-2 activates Stat5 via activation of protein tyrosine kinases Jak1 and Jak3 associated with receptor components (Ihle *et al*., 1997; Lin and Leonard, 1997) whereas T cell activation results in calcium-dependent serine phosphorylation and activation of Ets-1/2 proteins (Bassuk and Leiden, 1997; Pognonec *et al*., 1988). Several studies have placed Ets-1 and Ets-2 in the Ras/MAPK signaling pathway (Coffer *et al*., 1994; Miyazaki and Taniguchi, 1996; Rabault *et al*., 1996; Yang *et al*., 1996). Our GST-pull down assays evidenced that the C-terminal region of Ets-1, containing the Ets domain, can interact with IL-2-activated Stat5b. The reciprocal experiment performed with GST-Stat5 recombinant proteins failed to reveal either Ets-1 or Ets-2 (data not shown), suggesting that Stat-Ets interaction requires IL-2-induced Stat5 dimerization (Figure 8). Further experiments will determine whether activation of the different pathways required for Stat5-Ets complex formation and transactivation depends only on IL-2 engagement on its receptor, or could integrate CD3/TcR, CD28 and IL-2R signaling. Finally, the present report does not address the question of the IL-2 specificity and it will be pertinent to determine whether such complexes might be activated by other cytokines.

The cross-talk of Stat with several Ets proteins highlights an additional mechanism of gene regulation, involving physical association of Stat5a/b with a family of structurally distinct transcription factors in the absence of their cognate DNA binding sites (Figure 8). This molecular mechanism might synergize their individual biological activities and act in long-term maintenance of T cell proliferation and/or survival.

Materials and methods

Cell culture

The IL-2-dependent human T cell Kit225 (Hori *et al*., 1987) was maintained in RPMI 1650 with 10% fetal calf serum (FCS) containing 2 mM L-glutamine and 1 nM recombinant IL-2 (Chiron). The cells were arrested in a quiescent state by washing them twice in PBS, and culturing in RPMI 1650 10% FCS without IL-2 for 20 h (for transient transfection assays) and for 2 days (for EMSAs, affinity purification of DNA-binding proteins GST-pull down and immunoprecipitations). T cell purification from human peripheral blood and activation were performed as described (Costello *et al*., 1993). Primary T cells were maintained in RPMI 1650 10% FCS. Stimulations were performed with the following monoclonal antibodies (mAbs) used in combination at saturating concentrations. Anti-CD2 mAb 39C1.5 (rat IgG_{2a}) and 6F10.3 (mouse IgG_1) were used as purified mAbs at 10 μg/ml each. Anti-CD28 248 (mouse IgM) was obtained from Dr A Moretta (Cancer Institute, Genova, Italy) and was used as ascitic fluid (1/200 dilution). T cell activation was controlled by proliferation assays and CD25/IL-2Rα expression.

EMSA, affinity purification of DNA-binding protein, GST-pull down, immunoprecipitation and immunoblotting

Nuclear extracts (Costello *et al*., 1993) and whole cell extracts (Lecine *et al*., 1996) of Kit225 and primary T cells were prepared as described. Kit225 cells were IL-2-starved for 2 days and stimulated by IL-2 during 1 h. Synthetic oligonucleotidic probes were labeled with γ-^{32}P ATP. Name, sequence and origin of oligonucleotides used in EMSAs are indicated in Table 1. Binding reactions, gel separation and detection were performed as described (Lecine *et al*., 1996) using 4 μg of nuclear extract. For competition experiments, unlabeled double-stranded oligonucleotides were pre-incubated with cell extracts for 10 min at RT prior to addition of the probe. Immunoassays were performed by incubating the binding reactions 30 min at 4°C with antisera directed against Ets1/2 (#8), Fli-1 (#61), Tel (#69), Ets-1 (#49), Ets-2 (#36) (Bailly *et al*., 1994; Pognonec *et al*., 1988), GABPα, GABPβ (de la Brousse *et al*., 1994), Stat3, Stat4, Stat6 (Transduction Laboratories), Elf-1, Sp1, Stat5a, Stat5b, Stat5 C17 which recognizes both Stat5a and Stat5b (Santa Cruz Biotechnology), as indicated in Figure legends. DNA binding proteins were isolated from whole cell extracts as described (Beadling *et al*., 1994). For coimmunoprecipitation, 1 ml of whole cell extracts (10^7 cells) were incubated 1 h at 4°C with 5 μl of antiserum. Immune complexes were precipitated with protein A-Sepharose (Pharmacia) as described (Kahn-Perles *et al*., 1997). For GST-pull down assays, 1 ml whole cell extracts (10^7 cells) were incubated 2 h at 4°C with GST-recombinant proteins as shown in Figure 3 legend coupled to glutathione sepharose 4B resin (Pharmacia), followed by three washes with 0.1% NP-40 lysis buffer. For immunoblot analysis, after separation by SDS–PAGE and transfer to PVDF membranes, proteins were detected with various antisera: Stat5b-specific antiserum (R&D Systems), anti-c-Rel polyclonal serum (Kahn-Perles *et al*., 1997), anti-Ets-1 (#41), -Ets-2 (#38) or -Ets-1/2 (#8).

Plasmids and mutagenesis

Wild-type or mutants pTK4-CAT/(GASd/EBSd) × 3 plasmids were constructed by multimerizing three copies of the following oligonucleotidic sequences containing the GASd/EBSd element from the human IL-2Rα gene and cloning into the *Sac*I and *Hind*III sites in the minimal promoter reporter gene vector pTK4-CAT (Lecine *et al*., 1996): TC<u>TTCTAGGAA</u>GTAT for wild-type GASd/EBSd (3 × G/E), TCTTCT**CC**GAAGTAT for mutant EBSd (3 × G/mE), TC**GGCTAGGAA**GTAT for mutant GASd (3 × mG/E) and TC**GGCTCCGAA**GTAT for double mutant GASd/EBSd (3 × mG/mE) (GAS motif is underlined and EBS motif is doubly underlined; substituted nucleotides are in boldface). The choice for the mutations was based on previous studies in which either factor binding or functional activity of the particular binding site was destroyed (Beadling *et al*., 1996; Wasylyk *et al*., 1993). Wild-type or mutants pTK4-CAT/(EBS) × 5 plasmids were constructed by multimerizing five copies of the following oligonucleotidic sequences containing functional or mutated EBS element (Sieweke *et al*., 1998) and cloning into the *Sac*I and *Hind*III sites in the minimal promoter reporter gene vector pTK4-CAT: TCGAGC<u>AGGAAGT</u>TTCG for wild-type EBS (5 × EBS), TCGAGC-

ACCAAGTTTCG for mutant EBS (5 × mEBS) (EBS motif is doubly underlined and substituted nucleotides for its disruption are in boldface). Expression vectors for mStat5b (pXM/Stat5b), Ets-1 and Ets-2 were previously described (Boulukos *et al*., 1989; Gegonne *et al*., 1993; Liu *et al*., 1995). The GST-Ets constructs were kindly provided by M Sieweke and used in GST-pull down assays as described (Sieweke *et al*., 1996).

Transient transfections and CAT assays

IL-2-starved Kit225 cells (1.5×10^7) were electrotransfected in a BioRad Gene Pulser (330 V, 960 μFD) with 20 μg of wild type or mutant pTK4-CAT/(GASd/EBSd) × 3 constructs. Amounts of expression vector used in transfections are indicated in Figure legends. Following transfection, cells were either maintained for 12–16 h in RPMI 1650 10% FCS without IL-2 or with 20 nM IL-2, PMA (20 ng/ml) and ionomycin (1 μg/ml). Cell lysis and chloramphenicol acetyltransferase (CAT) assays were performed with the CAT enzyme-linked immunosorbent assay kit (Boehringer Mannheim) according to the manufacturer's instructions with 50 μg of cell extracts.

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