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A Single Amino Acid in the DNA Binding Regions of STAT5A and STAT5B Confers Distinct DNA Binding Specificities*

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STAT5A and STAT5B are two highly related transcription factors encoded by two distinct genes. STAT5A and STAT5B are activated by a broad range of cytokines and growth factors. Although they can be differentially activated, the functional difference between these two molecules relative to their structure is not known. Here we demonstrated that STAT5A and STAT5B homodimers have distinct DNA binding preferences. Chimeric STAT5 molecules allowed us to identify a region between amino acid 420 and 545 responsible for the DNA binding specificity. This region is located in the previously characterized DNA binding region of STAT proteins. Sequence comparison between STAT5A and STAT5B from different species showed a difference of 5 amino acids in the region 420–545 between STAT5A and STAT5B. Substitution of these amino acids demonstrated that a glycine residue at position 433 in STAT5B and a glutamic residue at a similar position in STAT5A determined the DNA binding specificity. These data indicate that STAT5A and STAT5B homodimers may have distinct function and probably regulate the expression of common as well as distinct genes.

Cytokines activate intracellular signaling pathways during growth and differentiation responses. One of the signal transduction pathways activated by these ligands involves the family of STAT (signal transducer and activator of transcription) proteins. STAT proteins are latent transcription factors that transmit signals from activated receptors to the nucleus (1, 2). All STAT members contain a SH2 domain that allows their selective recruitment to the activated receptor kinase complexes (3–5). After phosphorylation on a single tyrosine residue present in the COOH part of the protein (6, 7), STAT proteins dimerize (homodimerize and/or heterodimerize) through an interaction between the SH2 domain of one STAT and the phosphotyrosine residue of another STAT molecule (8). Dimers of STAT migrate to the nucleus, bind to specific DNA sequences, and activate or repress the transcription of target genes. Functional domains involved in DNA binding and activation of transcription have been defined. The DNA binding region is located in the center of the STAT proteins (from amino acids 350 to 500), and the transactivation domain is present at their COOH-terminal end (9–13). Isoforms of STAT proteins that lack the COOH-terminal transactivation domain still bind to DNA but do not induce transcriptional activation of responsive genes (14). In addition to tyrosine phosphorylation, STAT proteins are phosphorylated on serine residues. This phosphorylation has been shown to modulate transcriptional activity and the DNA binding of STAT proteins (15–17). The NH_2-terminal region has been found to be required for the cooperative binding of STAT dimers (18, 19). STAT5 also forms tetramers on the IL-2[1] receptor enhancer element that contains two STAT binding sites (20, 21). Similar observations were reported for the *cis* gene and for the hepatic serine protease inhibitor 2.1 (spi2.1) gene promoter elements (22, 23).

At present seven mammalian STAT proteins that display a highly specific role in innate and acquired immunity have now been isolated: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6. STAT5, originally identified in the mammary gland of lactating animals, confers prolactin response in mammary epithelial cells (24). In addition to prolactin, STAT5 is activated by a broad range of cytokines including growth hormone, erythropoietin, thrombopoietin, IL-3, GM-CSF, and IL-2 (25–29). Constitutive activation of STAT5 has been observed in cells transformed by oncogenes like v-*mpl*, v-*abl*, *bcr-abl*, and also in hematopoietic cells from leukemic patients (28, 30–32). The role of STAT5 in cell proliferation and differentiation as well as in suppression of apoptosis has been examined in different cell lines, but conflicting results emerged from these studies. Use of cytokine receptor mutants unable to activate STAT5 and of a dominant-negative form of STAT5 indicated that STAT5 activation was required for erythropoietin or IL-3-induced cell proliferation while other studies suggested that STAT5 was not essential for this effect (33–37). The same approaches have been used to demonstrate a role of STAT5 in erythroid differentiation and in the suppression of apoptosis in T cells induced by IL-2 (38–40).

STAT5A and STAT5B are two highly related proteins that share 96% homology and are encoded by separate genes on chromosome 11, tightly linked to STAT3 (41–44). STAT5A−/− knock out mice have a defect in mammary gland development and lactation while phenotypes associated with the loss of growth hormone responses were observed in STAT5B−/− mice (45, 46). In STAT5A−/− mice, proliferation of macrophages was found to be reduced in response to GM-CSF (47).

Although STAT5A and STAT5B can be differentially activated (48), the specific function of these two proteins has not yet been determined. In this report, we analyzed the DNA binding properties of STAT5A and STAT5B homodimers.

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[1] The abbreviations used are: IL, interleukin; GM-CSF, granulocyte macrophage-colony stimulating factor; APRE, acute phase response element.

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TABLE I
Sequences of the DNA probes

The STAT sequences are shown in **boldface.**

β-Casein(−150)	5′-CCCCAGAAT**TTCTTGGGAA**AGAAAATAGAA-3′
β-Casein(−105)	5′-AGAT**TTCTAGGAA**TTCAATCC-3′
APRE (α2-*macroglobulin*)	5′-GATCC**TTCTGGGAA**TTCCTA-3′
SIEm67(c-*fos*)	5′-CAT**TTCCCGTA**AATC-3′
IRF-1	5′-GATCCAT**TTCCCCGAA**ATGA-3′
Iε	5′-GTCAAC**TTCCCAAGAA**CAGAA-3′
Cis2	5′-GCTGC**TTCCGGGAA**GGGCT-3′
Bcl-x	5′-GCAT**TTCGGAGAA**GACGGGG-3′

STAT5A or STAT5B and the prolactin receptor expression vectors were transfected in COS cells. After stimulation with prolactin, DNA binding activities of STAT5A and STAT5B were compared on STAT sequences known to bind distinct members of the STAT family. We found that STAT5A and STAT5B had distinct DNA binding specificities. By using chimeric STAT5A/STAT5B molecules, we next identified that the regions responsible for this property were located between amino acids 420 and 545 in the DNA binding regions of STAT5A and STAT5B. Comparison of the sequences of these regions showed a 5-amino acid difference between STAT5A and STAT5B. Substitution of these amino acids demonstrated that a glycine residue at position 433 in STAT5B and a glutamic acid residue at a similar position in STAT5A were crucial for the differential DNA binding of STAT5A and STAT5B.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—COS7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 2 mM glutamine. COS7 cells were transfected with 2 μg of STAT5 and 3 μg of prolactin receptor expression vectors using the calcium phosphate method as described (24). Cells were stimulated 1 h with ovin prolactin (1 μg/ml) (Sigma).

Plasmids—The expression vectors for m-STAT5A (pXM-STAT5A), m-STAT5B (pXM-STAT5B), and the long form of the murine prolactin receptor have been described previously (43). The chimeric constructs STAT5AB1 and STAT5BA1 were generated by ligation of a *Sal*I-*Xho*I fragment (amino acids 1 to 545) of pXM-STAT5A into the *Sal*I-*Xho*I sites of pXM-STAT5B and vice versa. For the constructs STAT5AB2 and STAT5BA2 a fragment *Bam*HI-*Xho*I (from amino acids 180 to 545) from STAT5B cDNA was cloned into *Bam*HI-*Xho*I sites of pXM-STAT5A and vice versa. The other chimeric constructs, STAT5AB3, STAT5BA3, and A, B, C, D, E, F were generated by ligation with polymerase chain reaction-generated fragments. A first set of polymerase chain reactions was used to create chimeric junctions or mutations in STAT5AB3, STAT5BA3, A, B, C, D, E, F with the respective following oligonucleotides and their overlapping antisense: 5′-CTCAGCGCCCACTTCAGAAACATG-3′, 5′-CGAATCAAGAGGGCCGACCGCCGT-3′, 5′-AAGTTCACGGTCCTGTTTGACTCA-3′, 5′-TTCAGCGTCGGTAGCAACGAGCTG-3′, 5′-GGCAGCCAGGACCACAATGCCACA-3′, 5′-CGCCTGGGGGCAGAGTCAGTAACG-3′, 5′-GGCGTGGTGCAGGGTCGGTGACG-3′.

The final polymerase chain reactions were performed with an upper primer that contains the *Bam*HI site (with the following oligonucleotide: 5′-GCCTGCGGATCCAAGCTCAG-3′) and a lower primer containing the restriction site *Xho*I (with the following oligonucleotide: 5′-GTTGTAGTCCCTCGAGGTGGTT-3′). These polymerase chain reaction products were digested with *Bam*HI and *Xho*I and cloned in the corresponding vector (see Fig. 3). The sequences of the chimeric constructs have been verified.

Antibodies and Immunoblotting Analysis—The antiserum against STAT5 was generated in rabbit using a glutathione *S*-transferase fusion protein containing amino acids position 350–480 of human STAT5A as an antigen (12). The fragment was produced in bacteria as a glutathione *S*-transferase fusion protein. An anti-Stat5 antibody that recognizes the SH2 domain of STAT5A and STAT5B was also used (Transduction Laboratories). The phosphotyrosine antibody (4G10) was purchased from UBI. Immunoreactive bands were visualized using an *epi*-chemiluminescence Western blotting system (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared by suspending cell pellets in a buffer containing 10 mM HEPES pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 2 μg/ml pepstatin, 1 mM dithiothreitol, and 100 μM sodium ortho-vanadate. After 15 min on ice, Nonidet P-40 was added (final concentration: 0.6%). Lysates were centrifuged for 1 min at 4 °C and 14000 rpm. The pellets were extracted with a buffer containing 20 mM HEPES, pH 7.9, 25% glycerol, 0,4 NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 100 μM ortho-vanadate, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 2 μg/ml pepstatin. The samples were vigorously rocked for 20 min at 4 °C and centrifuged for 10 min at 4 °C and 14000 rpm. The supernatants were recovered for the band shift or Western blotting experiments. Equal amounts of proteins as judged by expression and tyrosine phosphorylation of the STAT5 molecules in Western blot were used in the band shift experiments. The protocol for the band shift assays has been described elsewhere (24). The oligonucleotides used in the band shift experiments were end-labeled with polynucleotide kinase to a specific activity of 8000 cpm/fmol.

RESULTS

DNA Binding Properties of STAT5A and STAT5B—It is generally admitted that the core DNA sequence recognized by STAT5 proteins contains the motif TTC(NNN)GAA (26). To determine whether STAT5A and STAT5B homodimers preferred different STAT binding sites, we analyzed their DNA binding properties on 8 oligonucleotide probes that bind distinct STAT members (including STAT1, STAT3, STAT6, STAT4) (Table I). COS cells were transfected with expression vectors for STAT5A or STAT5B and the prolactin receptor. Tyrosine phosphorylation and activation of STAT5A and STAT5B by prolactin in transfected COS cells have already been reported and proved to be a useful system to study their DNA binding properties (43). Nuclear extracts were prepared in the absence or presence of prolactin and assayed in band shift experiments (Fig. 1). Differences were clearly observed among these probes. Seven probes were bound by STAT5B homodimers although with different affinities. Strong DNA binding activities were observed with the β-casein (−105), APRE, IRF-1, and Bcl-x probes (*lanes 4*, *12*, *16*, and *28*) whereas β-casein(−150), Iε, and Cis2 probes gave weak and intermediate signals (*lanes 8*, *20*, and *24*). In the case of STAT5A homodimers, a strong DNA binding activity was only observed with the β-casein(−105) (*lane 2*). IRF-1, Cis2, and Bcl-x probes were also bound by STAT5A but with an overall lower affinity as judged by the intensity of the binding signal and with a lower mobility (*lanes 14*, *22*, and *26*). No DNA binding of STAT5A homodimers was observed with β-casein(−150), APRE, or Iε probes (*lanes 6*, *10*, and *18*). The SIEm67 probe did not bind to STAT5A or STAT5B homodimers (data not shown). Thus the two closely related STAT5A and STAT5B differ in their ability to recognize natural STAT binding sites.

Expression and activation of STAT5A and STAT5B proteins from COS cell extracts were analyzed in immunoblot with an anti-phosphotyrosine antibody (Fig. 2). STAT5A and STAT5B were indifferently tyrosine-phosphorylated in response to prolactin (*lanes 2* and *4*). These tyrosine-phosphorylated bands were not detected in COS cells transfected with the prolactin receptor expression vector alone (data not shown). Reprobing

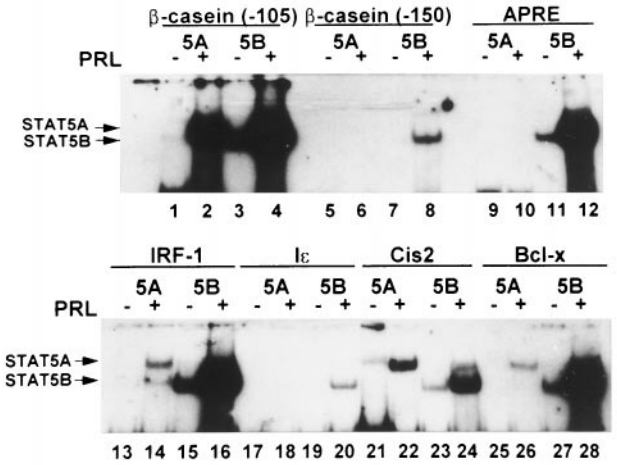

FIG. 1. **Differential DNA binding of STAT5A and STAT5B to STAT sequences.** COS cells were transfected with the expression vectors for the prolactin receptor, STAT5A, and STAT5B. Nuclear extracts were prepared from non-stimulated cells (*lanes 1*, *3*, *5*, *7*, *9*, *11*, *13*, *15*, *17*, *19*, *21*, *23*, *25*, and *27*) or cells treated for 1 h with prolactin (*lanes 2*, *4*, *6*, *8*, *10*, *12*, *14*, *16*, *18*, *20*, *22*, *24*, *26*, and *28*). Band shift experiments were carried out with the indicated radioactive DNA probes.

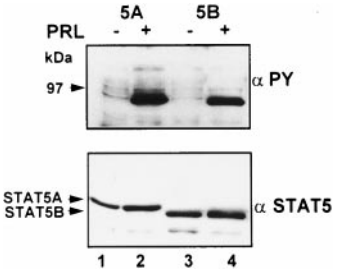

FIG. 2. **Expression and tyrosine phosphorylation of STAT5A and STAT5B.** The proteins from nuclear extracts of transfected COS cells were separated by SDS-gel electrophoresis, blotted, and probed with a phosphotyrosine specific antibody (4G10, *upper panel*). The membranes were reprobed with a polyclonal rabbit antiserum specific for STAT5A and STAT5B (*lower panel*).

the membranes with an anti-STAT5 antibody that recognizes STAT5A and STAT5B evidenced a similar level of expression of both proteins. Thus, the differential DNA binding activity of STAT5A and STAT5B did not reflect a differential expression or tyrosine phosphorylation of the two proteins.

Regions of STAT5A and STAT5B Comprising Amino Acids 420 and 545 Confer DNA Binding Specificity—We proceeded to use the distinct DNA binding specificities of STAT5A and STAT5B to the APRE element to identify protein regions required for this differential DNA binding. The DNA binding regions of STAT1, STAT3, and STAT6 have been identified in the NH_2-terminal part of these proteins, and amino acids between residues 400 and 500 determine the DNA binding site specificity (9, 10). To identify the protein region that discriminates between STAT5A and STAT5B DNA binding activities, gene fusions were constructed that encode STAT5 proteins containing the NH_2-terminal region of STAT5A fused to STAT5B or vice versa (Fig. 3). STAT5A, STAT5B, and chimeras were transfected with the prolactin receptor expression vector in COS cells. Nuclear extracts were prepared in the absence or presence of prolactin and assayed in band shift experiments with the APRE probe (Fig. 4*A*). A chimeric STAT5 protein containing the first 545 amino acids of STAT5A and the COOH terminus of STAT5B (AB) did not bind to the APRE probe behaving like STAT5A (*lanes 2* and *8*), and the complementary chimera with the NH_2-terminal 545 amino acids of STAT5B

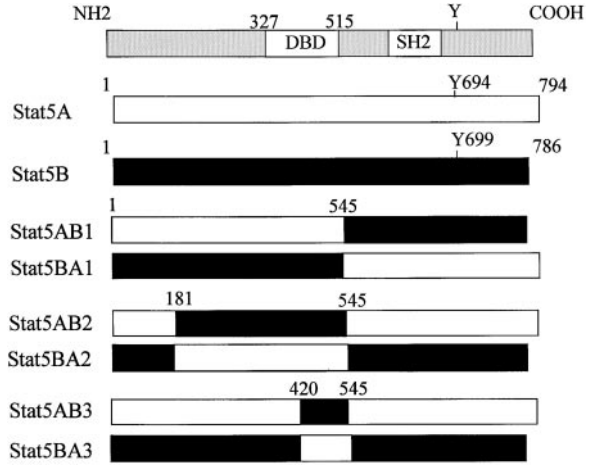

FIG. 3. **Diagrammatic representation of the STAT5A/STAT5B chimeras.** The DNA binding domain (*DBD*) and the SH2 domain in the structure of a STAT protein are indicated by *open boxes*. The *numbers* above the *boxes* refer to the position of the DNA binding domain in the sequence. STAT5A and STAT5B are represented by *open* and *solid boxes,* respectively. The *numbers* above the chimeras indicated the amino acids involved in the fusion between STAT5A and STAT5B.

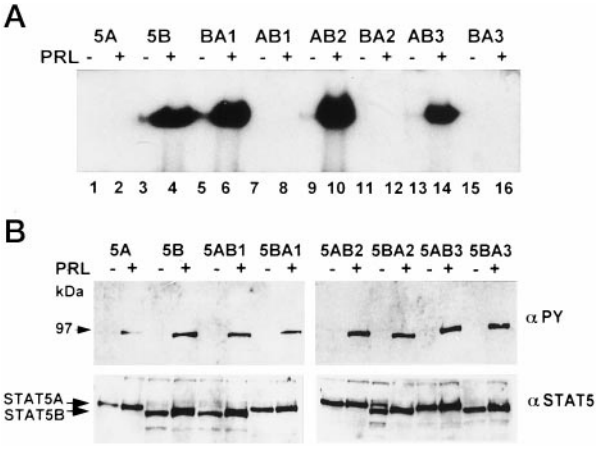

FIG. 4. **DNA binding properties of the chimeric STAT5 molecules.** *A*, DNA binding of the chimeras: COS cells were transfected with the various STAT5 constructs and the prolactin receptor expression vector. Nuclear extracts were prepared from unstimulated (*lanes 1*, *3*, *5*, *7*, *9*, *11*, *13*, and *15*) or prolactin-stimulated cells (*lanes 2*, *4*, *6*, *8*, *10*, *12*, *14*, and *16*) and assayed in a band shift experiment with the APRE oligonucleotide as probe. *B*, expression and tyrosine phosphorylation of the STAT5 chimeras: Western blot analysis were performed with nuclear extracts from transfected COS cells with an anti-phosphotyrosine antibody (4G10) (*upper panel*). The blot was stripped and reprobed with an anti-STAT5 antibody (*lower panel*).

fused to the COOH terminus of STAT5A (5BA) had the recognition property of STAT5B, that is, it bound well to APRE probe (*lanes 4* and *6*). Thus, the differential DNA binding activity of STAT5A and STAT5B was localized in the amino-terminal 545 amino acids. Additional chimeras were constructed containing the region from 182 to 545 of STAT5A substituted in STAT5B (BA2) and vice versa (AB2) and analyzed. Like STAT5A and the chimera AB, the recombinant molecule BA2 did not recognize the APRE probe (*lane 12*) whereas the complementary chimera AB2, like STAT5B, did (*lane 10*). To further dissect this region we finally replaced the region from 420 to 545 of STAT5B by the same region in STAT5A (BA3). This recombinant molecule failed to bind to the APRE probe (*lane 16*). The complementary chimera in which the 420–545 amino acid sequence of STAT5A was replaced by the same region of STAT5B (AB3) bound well to the APRE probe. Analysis of expression and tyrosine phosphorylation of

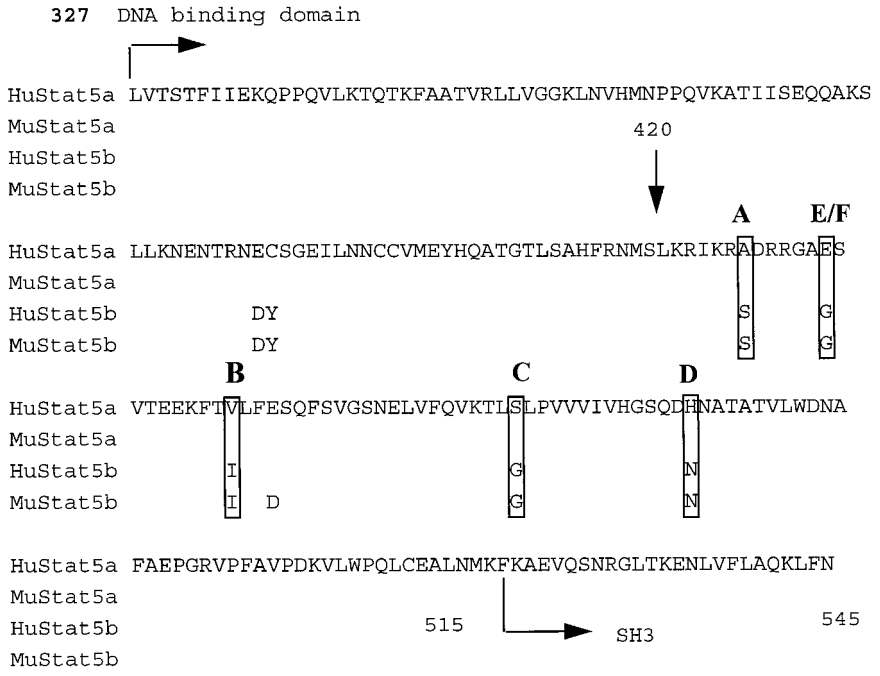

FIG. 5. **Sequence comparison of the regions between amino acids 420 and 545 of STAT5A and STAT5B.** Sequence alignment of the region 420–545 between STAT5A and STAT5B from human and mouse origin is shown. Amino acid changes between the sequences are *boxed*. The *letters* above the sequences indicated the substitutions made in STAT5B (*A*, *E*, *B*, *C*, and *D*) and in STAT5A (*F*).

the different chimeric molecules by Western blot with an anti-phosphotyrosine antibody (4G10) and an anti-STAT5 antibody showed that the levels of expression and activation of all chimeras were similar (Fig. 4*B*). We concluded that the amino acids that determine the DNA binding specificity of STAT5A and STAT5B lie in this 125-amino acid segment between residues 420 and 545.

A Single Amino Acid Substitution in the DNA Binding Region of STAT5A and STAT5B Determines the DNA Binding Specificity—Sequence alignment of the region 420–545 of STAT5A and STAT5B from human and mouse species showed a conserved divergence of 5 amino acids between the two molecules (Fig. 5). We suspected that one or more of these amino acids were crucial for the differential DNA binding of STAT5A and STAT5B to the APRE probe. The 5 amino acids present in the region 420–545 of STAT5B were individually replaced by the amino acid residues present at identical positions in STAT5A. These 5 STAT5B mutants were transfected with prolactin receptor expression vector in COS cells, and nuclear extracts prepared from prolactin-stimulated or nonstimulated cells were tested on the APRE probe (Fig. 6*A*). Like with STAT5A, no signal was detected with the mutant E after prolactin stimulation (*lanes 2* and *8*) indicating that the substitution Gly → Glu (residue 433 in STAT5B) dramatically affected DNA binding of STAT5B on the APRE probe whereas the 4 other substitutions corresponding to mutants A, B, C, and D had weak or no effect (*lanes 6*, *10*, *12*, and *14*). Levels of expression and tyrosine phosphorylation of STAT5B and the STAT5B mutants as judged by Western blot analysis were found to be similar (Fig. 6*B*). Thus the glycine residue at position 433 is important for the binding of STAT5B to the APRE probe. To ascertain whether this residue might be crucial for the observed effect, we substituted the glutamic acid residue at position 433 in STAT5A by the glycine residue. This STAT5A mutant (mutant F) was transiently expressed in COS cells (Fig. 7*A*). Mutant F and STAT5B bound to the APRE probe (*lanes 4* and *8*) indicating that substitution Glu → Gly in mutant F allowed the recovery of DNA binding of STAT5A to the APRE probe. Western blot analysis with anti-phosphotyrosine and anti-STAT5 antibodies revealed that STAT5A, STAT5B, F (STAT5A(E → G)), and E (STAT5B(G → E)) were expressed and activated by prolactin. Level of expression and activation of mutant F was even lower (Fig. 7*B*). We concluded that a glutamic acid residue in STAT5A at position 433 and a glycine residue in STAT5B at a similar position determine the DNA binding specificities of STAT5A and STAT5B.

DISCUSSION

STAT5A and STAT5B are two highly related proteins that share 96% homology. These proteins differ at their COOH terminus, a region highly variable among other STAT proteins that is thought to be involved in transcriptional activation. Despite their homology, a recent report has shown that STAT5A and STAT5B can be differentially activated suggesting that they might have distinct functions. However, no evidence has yet been presented that defines functional differences in relation to the structure of these two STAT proteins. In this report we showed that STAT5A and STAT5B homodimers have distinct DNA binding specificities. We found that the APRE, the β-casein(−150), and the Iε elements are bound by STAT5B homodimers but not by STAT5A homodimers while Stat5A bound well but STAT5B bound with a higher affinity to the β-casein(−105), the STAT5 optimum sequence. This did not reflect a change in the expression or tyrosine phosphorylation of both proteins in response to prolactin. STAT5A and STAT5B also have different affinities for the three STAT5 binding sites of the β-lactoglobulin gene promoter (49). In COS cells transfected with a β-casein-luciferase construct STAT5B but not STAT5A stimulates the basal activity of the promoter in the absence of prolactin (43). Similar observations were done with a multimerized STAT5 response element reporter construct in hematopoietic cells (12). It is possible that the respective levels of STAT5A and STAT5B homodimers may determine the selective expression of genes that are regulated by both factors. Interestingly, STAT5B bound to the β-casein(−105) and the β-casein(−150) sequences. Occupancy of both sites was observed in extracts from lactating mammary gland but not in extracts from glands of pregnant mice (50) suggesting that activation of different STAT dimers might contribute to the β-casein gene transcription. Changes in the levels of STAT5A and STAT5B homodimers during mammary epithelial cell differentiation may be responsible for this selective gene activa-

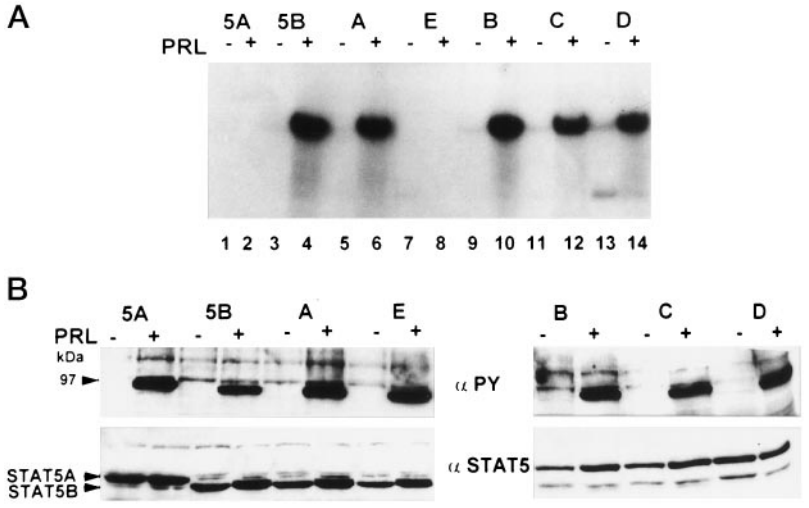

FIG. 6. **DNA binding of the point-mutated STAT5B molecules.** *A*, COS cells were transfected with the indicated STAT5 constructs and the prolactin expression vector. Nuclear extracts were prepared from unstimulated (*lanes 1*, *3*, *5*, *7*, *9*, *11*, and *13*) or prolactin-stimulated cells (*lanes 2*, *4*, *6*, *8*, *10*, *12*, and *14*) and assayed in a band shift experiment with the APRE oligonucleotide as probe. *B*, expression and tyrosine phosphorylation of the point-mutated STAT5 molecules. Immunoblot analysis of the proteins contained in nuclear extracts was performed with an anti-phosphotyrosine antibody (4G10) (*upper panel*). The blot was stripped and reprobed with an anti-STAT5 antibody (*lower panel*).

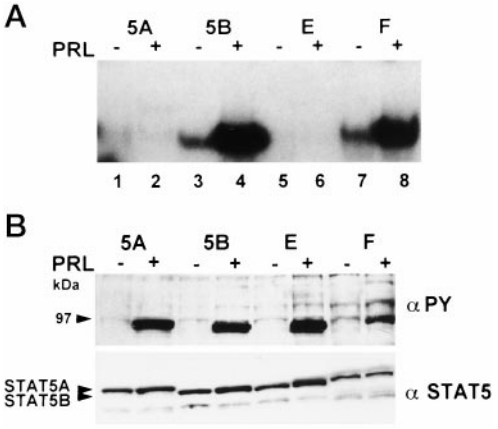

FIG. 7. **Differential DNA binding of STAT5A, STAT5B, and the mutants E and F.** *A*, DNA binding of the mutants F (STAT5A(E → G)) and E (STAT5B(G → E)). COS cells were transfected with the indicated STAT5 constructs and the prolactin expression vector. Nuclear extracts were prepared from unstimulated (*lanes 1*, *3*, *5*, and *7*) or prolactin-stimulated cells (*lanes 2*, *4*, *6*, and *8*) and assayed in a band shift experiment with the APRE oligonucleotide as probe. *B*, expression and tyrosine phosphorylation of the mutants F (STAT5A(E → G)) and E (STAT5B(G → E)). Western blot analysis of the proteins contained in nuclear extracts was performed with an anti-phosphotyrosine antibody (4G10) (*upper panel*). The blot was stripped and reprobed with an anti-STAT5 antibody (*lower panel*).

tion (51). STAT5A and STAT5B bound also to the Cis and Bcl-x elements with different affinities, and expression of both genes is regulated by STAT5 in hematopoietic cells (52).[2] In STAT5A−/− mice, regulation of Cis gene expression by GM-CSF in macrophage is affected (47). However, we don't know yet if these genes are differentially regulated by STAT5A and STAT5B

The most striking difference in the DNA binding properties of STAT5A and STAT5B was observed with the APRE binding site. The core sequences of the APRE and the β-casein(−105) are very similar. Only one nucleotide differs between the two core sequences. It is probable that this minor change dramatically affects binding of STAT5A although the core sequences of IRF-1, Cis, and Bcl-x, which are less related, still bound to STAT5A. It is also possible that nucleotides in the flanking sequences might be important for binding to the APRE probe. Nucleotides adjacent to the core element have been shown to modulate STAT DNA binding (53). APRE (acute phase response element) originally had been described as an IL-6 response element in the α_2-macroglobulin gene promoter that interacts with STAT3 and STAT5B but not STAT5A in rat liver after induction of an acute phase response despite the expression and activation of STAT5A (54, 55). These data are in agreement with our observation that STAT5A did not bind to the APRE element. However, DNA binding of STAT5A to the APRE element was observed in ovarian granulosa cells after induction with prolactin (55). The probe used in this study contained two copies of the APRE element. It is possible that the presence of two STAT sequences allowed cooperative binding of two STAT5A dimers. DNA binding of STAT5A as a tetramer on a duplicated sequence which did not bind or weakly bound these elements individually were observed for the CD25 gene, the *cis* gene, and the spi2.1 gene (21–23). We recently showed that purified active STAT5A binds as a tetramer on such duplicated sites in the Cis gene promoter while STAT5B binds preferentially as a dimer (22). Thus it is reasonable to think that STAT5A also forms a tetramer when the APRE site is duplicated.

Weak DNA binding of STAT5B was observed on the Iε and the β-casein(−150) probes which did not contain the core sequence TTCNNNGAA but did contain TTCNNNNGAA.

Spacing between TTC and GAA has been reported to control specific binding of different STAT members (56). It has been demonstrated that STAT6 binds preferentially to such 4 base-spacing elements. β-Casein and Iε gene expression are known to be regulated by IL-4, a cytokine that specifically induces activation of STAT6 (57, 58). Thus, STAT5B can bind to STAT6 specific elements. However, no evidence has been provided that STAT5B can regulate the expression of STAT6 target genes.

We used chimeric STAT5A/STAT5B molecules to identify the regions that confer DNA binding specificity to STAT5A and STAT5B. This region located between amino acids 420 and 545 of STAT5A and STAT5B is contained in the DNA binding region previously characterized for STAT1, STAT3, and STAT6 (9, 10). Within these 125 amino acids, STAT5A and STAT5B differ only by 5 amino acids. We showed that a glycine residue at position 433 is crucial for the DNA binding of STAT5B and of the STAT5A(E → G) mutant to the APRE element while a glutamic acid residue at the same position dramatically affected the DNA binding of STAT5A and of the STAT5B(G → E) mutant. Interestingly, these two amino acids are located close to the motif VTEE conserved in the DNA binding regions of all STAT members and found to be crucial for DNA binding of STAT proteins. Mutations in the VTEE sequence (EE → AA)

[2] S. Dumon, S. Santos, F. Debierre-Grockiego, V. Gouilleux-Gruart, L. Cocault, V. Boucheron, P. Mollat, S. Gisselbrecht, and F. Gouilleux, unpublished results.

dramatically reduced DNA binding of STAT3 whereas the same mutations in STAT5 increased its DNA binding activity (9, 59). It is possible that these neighboring residues might influence the secondary structure of the VTEE sequence increasing or decreasing the DNA binding affinity. The crystal structures of the DNA complexes of STAT1 and STAT3 homodimers have been determined. Interestingly, a glutamic acid residue at position 421 close to the VTEE sequence of STAT1 and the valine residue of the VTEE sequence of STAT3 make contact with DNA (60, 61). Thus it is probable that the same regions of STAT5A and STAT5B are required for this interaction. Crystallographic structure of the DNA binding regions of STAT5A and STAT5B will help to define important amino acids that are involved in contact with DNA.

One important question that results from the present study is the identification of genes that are specifically regulated by STAT5A and STAT5B. Inactivation of STAT5A and STAT5B genes demonstrated the essential role of the two STAT5 proteins in the physiological responses to growth hormone or prolactin (62). Regulation of some liver proteins like MUP (major urinary protein), the testosterone 15α-hydroxylase (CYP2A4), and testosterone 16α-hydroxylase (CYP2D9) that are expressed in a sexually dimorphic pattern was impaired in STAT5B−/− but not in STAT5A−/− mice (62). Level of serum IGF-1 was also reduced in STAT5B−/− but not in STAT5A−/− mice. It is possible that the genes encoding these proteins are under the specific control of STAT5B. Characterization of the promoter regions of these genes and the use of specific dominant-negative forms of STAT5B will help to identify the specific target genes of STAT5B. This study supports a functional difference between STAT5A and STAT5B and increases the number of potential target genes of STAT5 that can be regulated commonly or specifically by STAT5A and STAT5B.

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