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Mutational Analysis of Acute-Phase Response Factor/Stat3 Activation and Dimerization

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Signal transducer and transcription (STAT) factors are activated by tyrosine phosphorylation in response to a variety of cytokines, growth factors, and hormones. Tyrosine phosphorylation triggers dimerization and nuclear translocation of these transcription factors. In this study, the functional role of carboxy-terminal portions of the STAT family member acute-phase response factor/Stat3 in activation, dimerization, and transactivating potential was analyzed. We demonstrate that truncation of 55 carboxy-terminal amino acids causes constitutive activation of Stat3 in COS-7 cells, as is known for the Stat3 isoform Stat3β. By the use of deletion and point mutants, it is shown that both carboxy- and amino-terminal portions of Stat3 are involved in this phenomenon. Dimerization of Stat3 was blocked by point mutations affecting residues both in the vicinity of the tyrosine phosphorylation site (Y705) and more distant from this site, suggesting that multiple interactions are involved in dimer formation. Furthermore, by reporter gene assays we demonstrate that carboxy-terminally truncated Stat3 proteins are incapable of transactivating an interleukin-6-responsive promoter in COS-7 cells. In HepG2 hepatoma cells, however, these truncated Stat3 forms transmit signals from the interleukin-6 signal transducer gp130 equally well as does full-length Stat3. We conclude that, dependent on the cell type, different mechanisms allow Stat3 to regulate target gene transcription either with or without involvement of its putative carboxy-terminal transactivation domain.

Acute-phase response factor (APRF) was originally identified and characterized by us as a transcription factor rapidly activated in response to interleukin-6 (IL-6) and IL-6-related cytokines (36, 37). APRF was then demonstrated to be a member of the signal transducer and transcription (STAT) factor family and is now called Stat3 (1, 40). In addition to IL-6-type cytokines, i.e., IL-11, oncostatin M, leukemia inhibitory factor, ciliary neurotrophic factor, and cardiotrophin-1, other cytokines and hormones have been shown to activate APRF (Stat3). Among them are alpha interferon, gamma interferon (in murine cells), IL-2, IL-5, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), CSF-1, growth hormone, prolactin, epidermal growth factor, and others (2–5, 7, 12, 18, 22, 25, 26). As is true for other STAT family members, inactive Stat3 is a cytoplasmic protein that associates with the intracellular parts of cytokine receptors upon ligand binding (20). The factors recruited in this way are tyrosine phosphorylated, most probably by the receptor-associated JAK protein tyrosine kinases (20). The STAT factors then dissociate from the receptor to form dimers (31). Subsequently, STAT dimers translocate to the nucleus, where they bind to regulatory DNA elements of target genes.

The specificity of STAT factor activation and function is controlled at various levels. Tyrosine motifs within the cytokine receptor cytoplasmic parts that are phosphorylated after activation of the receptors represent specific docking sites for the STATs. As we have shown recently, the specific interaction of Stat3 and Stat1 with such tyrosine motifs within the gp130 subunit of the IL-6 receptor is determined by their SH2 domains (11, 15). Similarly, SH2 domains govern the specific activation of Stat1 and Stat2 by alpha interferon and gamma interferon and of Stat6 by IL-4 (14, 29).

A second level of specificity represents the formation of STAT homo- versus heterodimers. The alpha interferon-induced ISGF-3 complex contains Stat1-Stat2 heterodimers, while gamma interferon recruits mainly Stat1 homodimers (9, 28, 32). The concomitant activation of Stat1 and Stat3 by IL-6 and other cytokines gives rise to Stat1 and Stat3 homodimers as well as Stat1-Stat3 heterodimers (40). Recent evidence indicated the existence of Stat2-Stat3 heterodimers (12). In contrast, Stat4, Stat5, and Stat6 have not yet been reported to form heterodimers. In the nucleus, different STAT factors exhibit partially overlapping but distinct DNA-binding preferences. Further complexity is implemented by the interaction of STAT factors with other transcription factors, resulting in altered DNA-binding specificities or cooperative effects on transcriptional regulation.

For several STAT factors, the existence of isoforms that exhibit different functional properties has been documented. Stat1 mRNA occurs in two differentially spliced forms coding for the isoforms Stat1α and Stat1β, where Stat1β represents a carboxy-terminally truncated form of Stat1α (28). For Stat5, at least four isoforms originating from two distinct genes have been observed. Two laboratories have recently reported the existence of an alternatively spliced Stat3 mRNA that codes for a truncated protein, Stat3β, in which the 55 carboxy-terminal amino acids of Stat3 (now Stat3α) are replaced by a short, unrelated sequence of 7 residues (3, 27). Stat3β was shown to dominantly inhibit transcriptional responses to IL-5 in a COS cell system (3). In contrast, Stat3β but not Stat3α was reported

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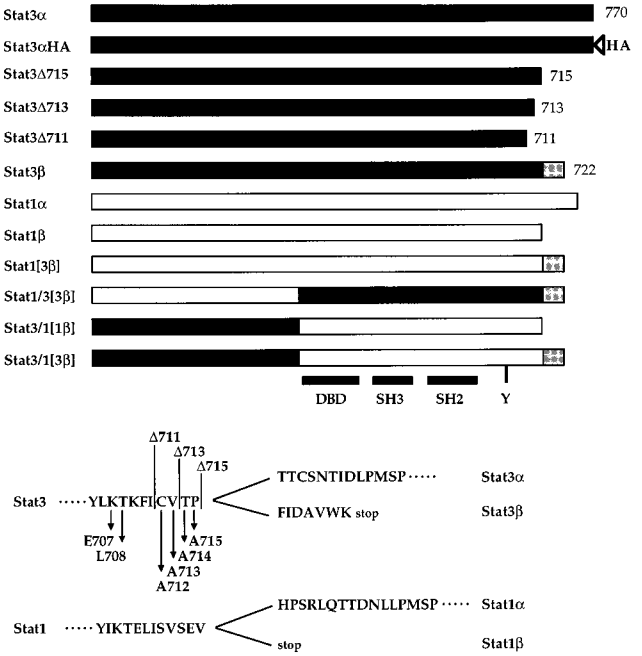

FIG. 1. Schematic representation of mutant and chimeric STAT factors used in this study. The Stat3 mutants and Stat1-Stat3 chimeras used are depicted graphically. Below is shown a comparison of carboxy-terminal Stat1 and Stat3 sequences. Deletion and point mutations introduced into Stat3 are indicated.

to transactivate the α_2-macroglobulin promoter in hepatoma cells when c-*jun* was overexpressed (27).

The structure-function relationship of STAT protein domains is far from being fully understood. A DNA-binding domain has been localized in the central part of the protein (17). The SH2 domain, in addition to being involved in receptor interaction, is thought to be responsible for STAT dimerization by mutual interaction with the phosphorylated tyrosine residues (31). Carboxy-terminal domains of Stat1α and Stat2 are reported to be important for the transactivating potential of these factors (19, 24).

By mutational analyses with a COS-7 expression system, we now demonstrate that in the context of Stat3 but not Stat1, carboxy-terminal truncation causes constitutive activation of the factor. This effect relies on the presence of amino-terminal domains of Stat3. In contrast, the amino-terminal part of Stat1 cannot support constitutive activation. We further report evidence that dimerization of Stat3 involves SH2 domain/phosphotyrosine recognition but requires additional interactions. Finally, we demonstrate that carboxy-terminal truncation of Stat3 does not ablate its transactivating potential in hepatoma cells.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from Boehringer Mannheim, radiochemicals were from Amersham, and oligonucleotides were synthesized by MWG-Biotech (Ebersberg, Germany). Recombinant human erythropoietin was a generous gift of Boehringer Mannheim (Germany). Stat1α and Stat3 antisera, as well as human Stat1α and murine Stat3 cDNAs, were kindly provided by Christian Schindler (Columbia University, New York, N.Y.) and James E. Darnell, Jr. (Rockefeller University, New York, N.Y.). Antiserum to the influenza virus hemagglutinin epitope HA was purchased from BAbCO (Richmond, California); antibodies to phosphotyrosine (RC20) and to tyrosine-phosphorylated Stat3 were from Transduction and New England Biolabs, respectively. A phosphopeptide corresponding to the tyrosine 767 motif of gp130 and a nonphosphorylated control peptide were synthesized as previously described (11).

Construction of expression vectors. A series of expression vectors coding for truncated Stat3 proteins and various Stat3-Stat1 chimeric proteins was constructed (Fig. 1). For construction of the STAT deletion mutants, murine Stat3 and human Stat1α cDNAs cloned into pBluescript vectors (Stratagene) were changed by introducing unique *Sal*I sites 3′ of the stop codons and unique *Bam*HI sites into Stat3 codons 697 to 699 and Stat1 codons 693 to 695 and introducing a unique *Xba*I site into Stat3 codons 716 to 717 as described previously (15). The Stat3 deletion and point mutants as well as Stat3β and Stat3βYF cDNAs were then constructed by inserting synthetic oligonucleotides coding for the respective sequences between the *Bam*HI and *Xba*I sites. The thus mutated cDNAs were subcloned into a pSVL expression vector (Pharmacia) which had been modified by eliminating the unique *Sal*I site in the vector backbone and instead introducing a *Sal*I site into the polylinker region.

An equivalent strategy was used to construct a Stat1β cDNA. Chimera Stat1[3β] was obtained by inserting an oligonucleotide coding for the carboxy-terminal 7 amino acids of Stat3β into the unique *Xba*I site of Stat1. The other Stat1-Stat3 chimeric molecules shown in Fig. 1 were constructed by swapping the respective parts of the Stat1 or Stat3 cDNAs by using the unique *Sph*I restriction site that is common to both cDNAs at codons 325 and 329, respectively. Stat3αHA, a Stat3α carrying the influenza virus HA epitope at the carboxy terminus, was generated by introducing a sequence coding for the epitope into the Stat3α cDNA by PCR.

Construction of the expression vector Eg coding for the erythropoietin (Epo) receptor-gp130 chimera has been described previously (11, 15).

Cell culture and transient transfection. COS-7 cells (ATCC CRL 1651) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were transfected by electroporation with a double pulse, 99 μs each, at 1.5 kV with an ElectroSquareporator (BTX, San Diego, Calif.). Approximately 2×10^6 cells were cotransfected in 0.8 ml of medium with 10 and 20 μg of receptor and STAT protein expression vector DNA, respectively. The cells were then grown to confluency (48 to 72 h), stimulated by incubation with human recombinant Epo (7 U/ml) in medium for 15 min where indicated, and harvested.

EMSA. Extraction of nuclear proteins and an electrophoretic mobility shift assay (EMSA) were carried out as described previously (15). Briefly, DNA binding of STAT factors was analyzed by EMSA with a double-stranded ^{32}P-labelled m67SIE oligonucleotide derived from the *sis*-inducible element (SIE) of the c-*fos* promoter region (35). After nuclear extracts were incubated with the probe, protein-DNA complexes were separated for 4 h by electrophoresis on a 4.5% polyacrylamide gel containing 7.5% glycerol in 0.25× Tris-borate-EDTA (TBE) buffer. The gels were fixed for 30 min in 10% acetic acid–10% methanol in water, dried, and autoradiographed.

For supershift analysis of STAT-DNA complexes, nuclear extracts were incubated in the gel shift cocktail with antisera at a final dilution of 1:20 at room temperature for 30 min. The m67SIE oligonucleotide probe was then added, and the EMSA was performed.

For phosphopeptide competition assays, peptide was added to nuclear extracts and incubated in the gel shift cocktail for 20 min at room temperature prior to addition of oligonucleotide probe and EMSA analysis.

Immunoprecipitation and immunoblot analysis. Cells were lysed and immunoprecipitated as described previously (20), and equal amounts of cellular protein were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Proteins were then transferred to polyvinylidene difluoride membranes with a semidry electroblotting apparatus, and antigens were detected by incubation with the appropriate primary and horseradish-peroxidase coupled secondary antibodies. The membranes were developed with an enhanced chemiluminescence kit (Amersham).

Transient transfection of HepG2 cells and reporter gene analysis. Human hepatoma HepG2 cells were grown and transient transfection by the calcium phosphate coprecipitation method, cell lysis, and chloramphenicol acetyltransferase (CAT) assays were performed as described previously (39). All transfections were done at least in triplicate. All CAT activity values were normalized to transfection efficiency monitored by cotransfecting a β-galactosidase expression vector (pCH110; Pharmacia). Construction of reporter constructs pα_2M-214CAT and pAPREtkCAT was described previously (39). Construction of plasmid pACT-359CAT containing the promoter region of the human α_1-antichymotrypsin gene fused to the CAT gene will be reported elsewhere (30).

RESULTS

Truncation by up to 55 carboxy-terminal amino acids does not impair Stat3 activation and DNA binding. To define the domains of the STAT proteins required for their activation through cytokine receptors, we constructed a series of expression vectors coding for Stat3 deletion and point mutants and for chimeric Stat3-Stat1 proteins (Fig. 1). The mutant STAT proteins were expressed in COS-7 cells by transient transfection. To activate these proteins, a hybrid receptor, Eg, that consists of the extracellular part of the Epo receptor and transmembrane and cytoplasmic parts of the IL-6 signal transducer gp130 was coexpressed as reported previously (11, 15). DNA

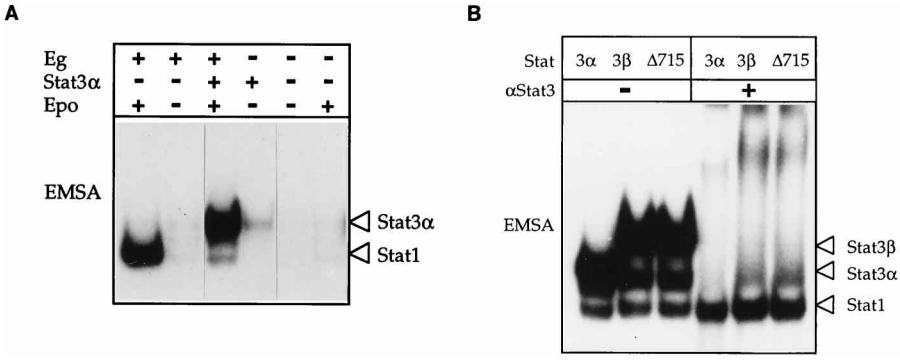

FIG. 2. Activation of full-length and truncated Stat3 forms through chimeric Epo receptor-gp130. (A) COS-7 cells were transiently transfected with expression vectors coding for the hybrid receptor Eg, consisting of the Epo receptor extracellular and the gp130 transmembrane and cytoplasmic parts, and for Stat3α as indicated. After stimulation with Epo (7 U/ml) for 15 min, nuclear extracts were prepared and analyzed by EMSA with the m67SIE probe. (B) COS-7 cells cotransfected with expression vectors coding for Eg and either Stat3α, Stat3β, or the truncation mutant Stat3Δ715 were stimulated with Epo as above. Nuclear extracts were analyzed by EMSA without (left panel) or with (right panel) prior incubation with antiserum to Stat3 (αStat3).

binding of Stat3α was induced by Epo in COS-7 cells coexpressing wild-type Stat3α and Eg, as demonstrated by EMSA analysis (Fig. 2A). As a probe, the high-affinity mutant m67SIE of the c-*fos* promoter *sis*-induced element was used in these experiments. Control experiments demonstrated that Stat3α activation depended largely on stimulation through Eg. Little DNA binding of Stat3α was observed in the absence of Epo. Similarly, in the absence of Eg, Epo did not activate STAT factors in these cells (Fig. 2A). When Stat3α was not overexpressed, endogenous Stat1 was the predominant target of signalling through Eg. This is due to low endogenous levels of Stat3 in COS-7 cells as reported previously (15).

We first coexpressed either Stat3α, the Stat3β isoform, or Stat3Δ715, a truncated Stat3 protein lacking the carboxy-terminal 55 amino acids of Stat3α, together with the hybrid receptor Eg in COS-7 cells. All three proteins yielded strong protein-DNA complexes upon EMSA analysis of nuclear extracts from Epo-stimulated cells (Fig. 2B). Preincubation with antiserum to Stat3 eliminated these bands, proving that they were formed by the Stat3 proteins (Fig. 2B, right panel). As previously reported by Caldenhoven et al. (3), the protein-DNA complexes formed with Stat3β were found to exhibit a significantly lower electrophoretic mobility compared to Stat3α complexes. Since it was also observed with mutant Stat3Δ715 (Fig. 2B), this phenomenon is not due to the unique carboxy-terminal 7 amino acids of Stat3β. The EMSA band pattern observed in Fig. 2B also indicates that not only Stat3α but also Stat3β and Stat3Δ715 formed heterodimers with Stat1.

Carboxy-terminal truncation causes constitutive activation of Stat3 in COS-7 cells. It has been reported that expression of Stat3β in COS-7 cells causes the formation of protein-DNA complexes even in the absence of external stimuli (3, 27). To evaluate whether this holds true with our system as well, we compared nuclear extracts from Epo-stimulated cells coexpressing Stat3β and the hybrid receptor Eg with cells that expressed solely Stat3β. In fact, Stat3β-DNA complexes were formed to the same levels with both extracts while activation of endogenous Stat1 was observed in Epo-stimulated cells only (Fig. 3A). Interestingly, mutant Stat3Δ715 was also found to bind DNA in the absence of Eg and Epo. Therefore, the constitutive activation observed with Stat3β does not rely on its unique carboxy terminus but is solely the result of truncation.

To verify that constitutive DNA binding of truncated Stat3 proteins was accompanied by constitutive tyrosine phosphorylation, anti-phosphotyrosine immunoblot analyses of cell lysates were performed. As shown in Fig. 3B, Stat3β was tyrosine phosphorylated already in the absence of stimulation through Eg. Similarly, mutant Stat3Δ715 was found to be equally tyrosine phosphorylated in Epo-treated and untreated cells while Stat3α tyrosine phosphorylation depended on cytokine stimulation (Fig. 3B). Furthermore, a Stat3β point mutant, Stat3βYF, in which the tyrosine phosphorylation site (Tyr 705) was replaced by phenylalanine did not form protein-DNA complexes (Fig. 3A) although it was expressed to the same level as wild-type Stat3β was (Fig. 3C). This indicates that phosphorylation of tyrosine 705 is required not only for inducible DNA binding of the Stat3α protein but also for constitutive activation of truncated forms.

Constitutive activation of truncated Stat3 forms is not due to enhanced dimer stability. Several mechanisms can be envisaged to explain the constitutive activation of truncated Stat3 proteins. Since Stat3β was originally identified by a two-hybrid approach with c-*jun* as bait (27), it seemed possible that an association with *jun* family members was involved in constitutive activation. In fact, the lower electrophoretic mobility of DNA-protein complexes formed by Stat3β and StatΔ715 (Fig. 3) suggested the possibility that other proteins were part of these complexes. However, antibodies to *jun* family members failed to supershift or eliminate these complexes, even upon overexpression of *jun* proteins (data not shown), indicating that *jun* factors do not participate in the formation of Stat3β-DNA complexes. It therefore seems unlikely that the lower mobility of Stat3β- and Stat3Δ715-DNA complexes is based on the presence of an additional protein(s). Rather, conformational changes of the truncated Stat3 forms may underlie this phenomenon.

Another possible mechanism for constitutive activation of truncated Stat3 proteins would be an enhanced stability of the dimers formed, resulting in reduced accessibility of the phosphotyrosine for phosphatases. This possibility was tested by a phosphopeptide competition assay. As shown previously, DNA binding of Stat3 can be inhibited by preincubation of nuclear extracts with phosphopeptides comprising specific phosphotyrosine motifs that act as docking sites for Stat3 within the cytoplasmic region of gp130 (11). It is believed that by binding to the SH2 domain, these peptides lead to a dissociation of Stat3 dimers and thereby inhibit DNA binding. DNA binding of both Stat3α and Stat3β was inhibited upon incubation of increasing concentrations of a phosphopeptide encompassing the tyrosine 767 motif of gp130 (Fig. 4A). When the same

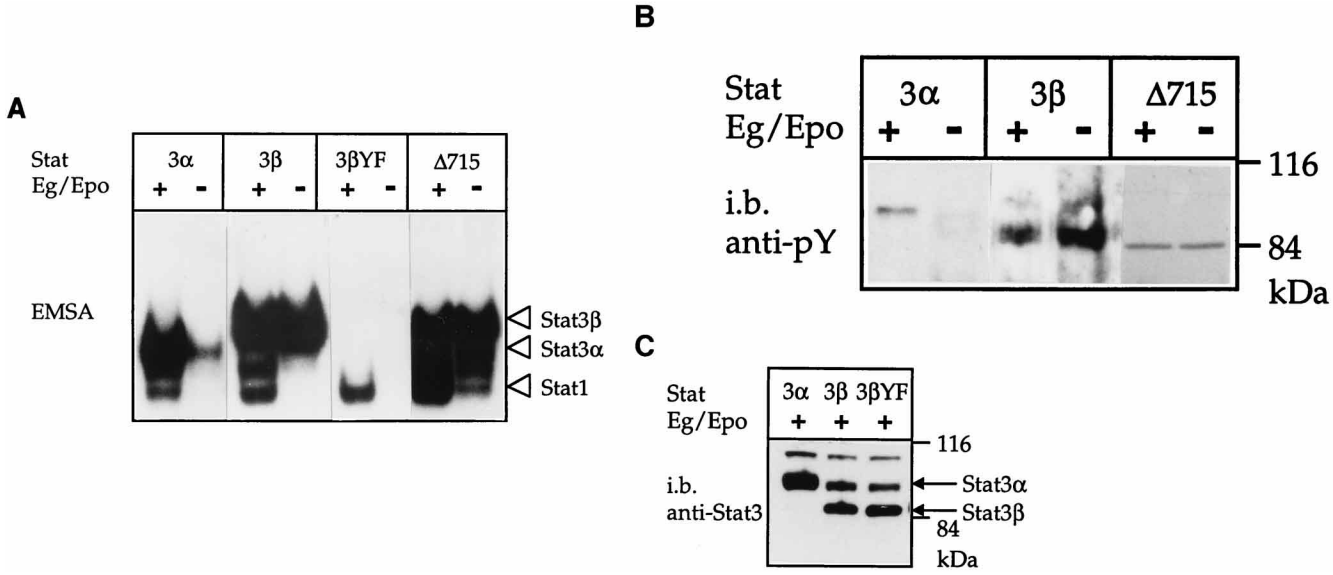

FIG. 3. Constitutive activation of carboxy-terminally truncated Stat3 mutants in COS-7 cells. (A) COS-7 cells were cotransfected with vectors coding for Eg and either Stat3α, Stat3β, Stat3βYF in which tyrosine 705 is replaced by phenylalanine, or Stat3Δ715, as indicated. Nuclear extracts of cells either untreated or stimulated with Epo for 15 min were analyzed by EMSA with the m67SIE probe. (B) Tyrosine phosphorylation of Stat3α, Stat3β, and Stat3Δ715 was analyzed by immunoprecipitation of cell lysates with antiserum to Stat3 and subsequent immunoblotting with antibodies to phosphotyrosine. (C) Equal expression of Stat3β and Stat3βYF was proven by immunoblotting of extracts with antiserum to Stat3. The membranes were developed by enhanced chemiluminescence.

peptide was used in a nonphosphorylated form, the EMSA signals were not reduced (data not shown), demonstrating the specificity of this effect. Quantitative analysis of the Stat3α and Stat3β signals obtained revealed that both factors were equally well competed by the peptide (Fig. 4B). Therefore, dimers formed by Stat3β do not appear to be more stable than Stat3α dimers.

Constitutive activation of truncated Stat3 proteins requires amino-terminal parts of Stat3. To further elucidate the basis of constitutive DNA binding of both Stat3β and Stat3Δ715, we extended our studies to Stat1. A truncated form of Stat1α is known as Stat1β. Sequence comparison between Stat3 and Stat1 molecules reveals that truncation of Stat1β occurs at almost the same position as in the deletion mutant Stat3Δ715 and in Stat3β (Fig. 1). To evaluate whether Stat1β is also constitutively activated under our experimental conditions, we coexpressed either Stat1α or Stat1β together with the hybrid receptor Eg in COS-7 cells. In both cases, an inducible formation of DNA-protein complexes was observed in EMSA experiments (Fig. 5A). Since rather high levels of Stat1α are present in COS-7 cells endogenously, we needed to discriminate between endogenous Stat1α and ectopically expressed Stat1β. For this purpose, we preincubated the nuclear extracts with an antiserum raised against the carboxy terminus of Stat1α. This antiserum recognizes Stat1α but not Stat1β. As expected, Stat1α-DNA complexes were completely supershifted while Stat1β expression yielded complexes which were not affected by the antiserum (Fig. 5A, lower panel). The appearance of these Stat1β complexes depended largely on Epo stimulation. This finding demonstrates that in contrast to Stat3, truncation of Stat1 does not yield constitutive activation.

Similarly, when the carboxy-terminal 7 amino acids of Stat3β were fused to Stat1β (mutant Stat1[3β]), DNA binding was still inducible through Eg (Fig. 5A). This further corroborates the notion that the alternate carboxy-terminus of Stat3β is not involved in constitutive activation.

To determine which parts of Stat3β define its potential of constitutive activation, we constructed chimeric Stat1-Stat3

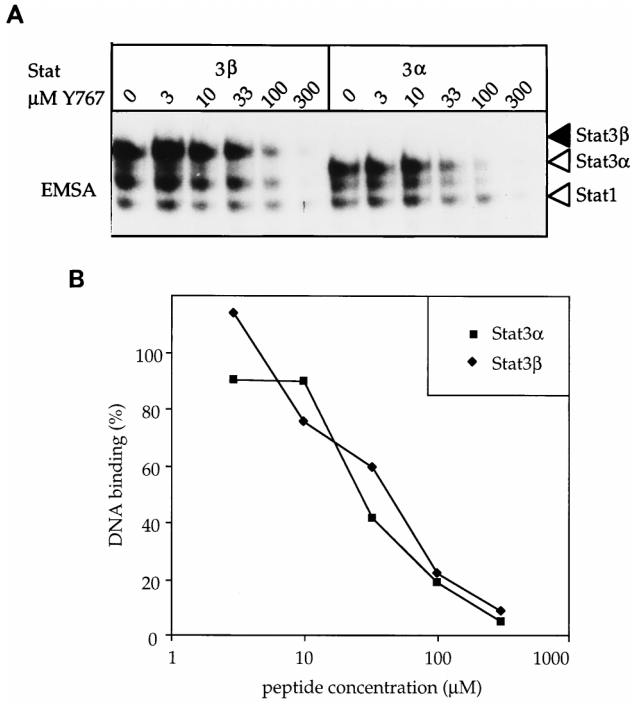

FIG. 4. Phosphopeptide competition analysis of Stat3α and Stat3β. (A) Stat3α and Stat3β were activated by coexpression with Eg and stimulation with Epo in COS-7 cells. Nuclear extracts were preincubated with different concentrations of the phosphopeptide TVVHSGpYRHQVPSV that corresponds to the sequence surrounding tyrosine 767 of gp130. Binding of Stat3α and Stat3β to the m67SIE DNA probe was then analyzed by EMSA. (B) The EMSA bands corresponding to DNA-protein complexes formed by Stat3α and Stat3β were quantitated with a PhosphorImager. The signals obtained for bands without phosphopeptide preincubation were set to 100%.

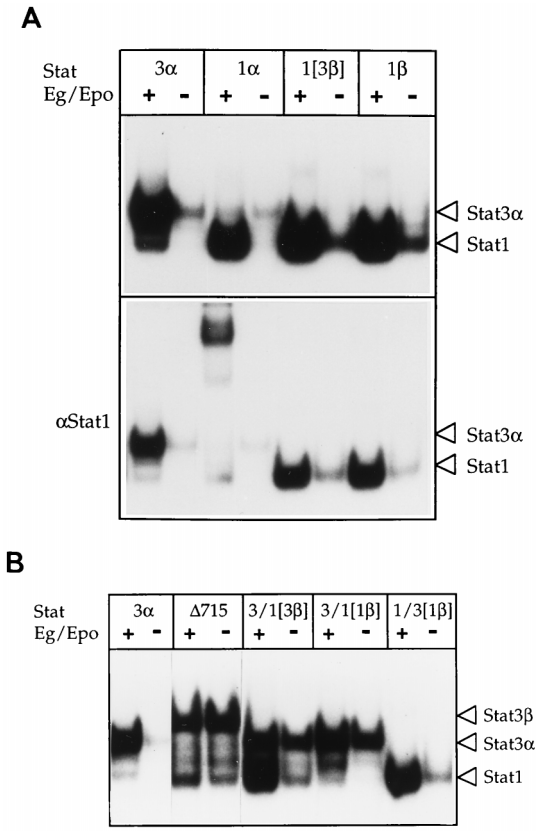

FIG. 5. The Stat3 amino terminus is required for constitutive activation. (A) Stat3α, Stat1α, Stat1β, and Stat1[3β] were expressed together with or without Eg in COS-7 cells. After stimulation with Epo where indicated, nuclear extracts were prepared and analyzed by EMSA either without (upper panel) or with (lower panel) preincubation with an antiserum raised to the carboxy-terminal portion of Stat1α. This antiserum specifically recognizes Stat1α but not Stat1β (28). (B) Stat3α, Stat3Δ715, and the chimeric STAT proteins Stat3-1[1β], Stat3-1[3β], and Stat1-3[3β] were expressed and stimulated through Epo and coexpressed Eg in COS-7 cells where indicated. Nuclear extracts were analyzed by EMSA.

molecules and expressed them in COS-7 cells. Surprisingly, a chimera (Stat1-3[3β]) consisting of the amino-terminal half of Stat1 and the carboxy-terminal half of Stat3β did not exhibit constitutive activation (Fig. 5B). However, the opposite chimeras Stat3-1[1β] and Stat3-1[3β] that combined the amino-terminal portion of Stat3 with truncated carboxy termini of Stat1 proved to be constitutively active (Fig. 5B).

In conclusion, constitutive activation of Stat3 proteins in COS-7 cells is due to carboxy-terminal truncation and requires the presence of amino-terminal Stat3 domains.

Further carboxy-terminal truncation yields defective Stat3 mutants. To gain further insight into Stat3 activation, we constructed expression vectors coding for deletion mutants Stat3Δ713 and Stat3Δ711, which lack 57 and 59 carboxy-terminal amino acids of Stat3α, respectively (Fig. 1). This further truncation resulted in a complete loss of DNA-binding ability (Fig. 6). To be able to directly compare the expression levels and the extent of tyrosine phosphorylation of the mutants tested, immunoblot analyses with either antiserum to Stat3 or an antibody specifically recognizing only tyrosine-phosphorylated Stat3 were performed, and all three Stat3 mutants were expressed at comparable levels (Fig. 6). Interestingly, both Stat3Δ713 and Stat3Δ711, although unable to bind DNA, were found to be tyrosine phosphorylated in response to Epo. In fact, a similar level of tyrosine phosphorylation was observed for all three mutants (Fig. 6). However, in contrast to the constitutive phosphorylation of Stat3Δ715, the defective mutants Stat3Δ713 and Stat3Δ711 were found to be inducibly phosphorylated.

We conclude from these findings that mutants Stat3Δ713 and Stat3Δ711 are still able to associate with gp130, where they become phosphorylated by gp130-associated JAK kinases. However, Stat3Δ715 represents the shortest carboxy-terminally truncated form of Stat3 that retains its ability to form protein-DNA complexes.

Mutation of valine 713 or threonine 714 impairs the formation of a DNA-binding status of Stat3. In mutants Stat3Δ713 and Stat3Δ711, truncation may disturb the overall conformation of the proteins and thereby impair DNA binding. To exclude such a rather nonspecific effect, we constructed mutants with alanine-scan mutations through the deleted region. As shown in Fig. 7, replacing either cysteine 711 or proline 715 by alanine did not affect the DNA binding of Stat3Δ715. However, when valine 712 or threonine 713 was mutated, the DNA-binding ability was completely lost. This result demonstrates that these residues are specifically required to allow the formation of an active, DNA-binding conformation. Expression and tyrosine phosphorylation of the alanine mutants was analyzed by immunoblotting as above. All were found to be equally expressed and tyrosine phosphorylated (Fig. 7). Therefore, as with mutants Stat3Δ713 and Stat3Δ711, mutants Stat3Δ715A713 and Stat3Δ715A714 were tyrosine phosphorylated but lacked DNA-binding potential. It is interesting that in contrast to the inducible tyrosine phosphorylation of Stat3Δ713 and Stat3Δ711, both defective alanine mutants were constitutively phosphorylated, as were the active ones.

We used mutants Stat3Δ715A712 and Stat3Δ715A713 (i.e., one functional and one defective mutant) to evaluate the fraction of constitutively activated Stat3 proteins that is tyrosine phosphorylated in comparison to Stat3α. COS-7 cells expressing either these mutants or Stat3α were lysed and immunoprecipitated with either antiserum to Stat3 or antibodies specific

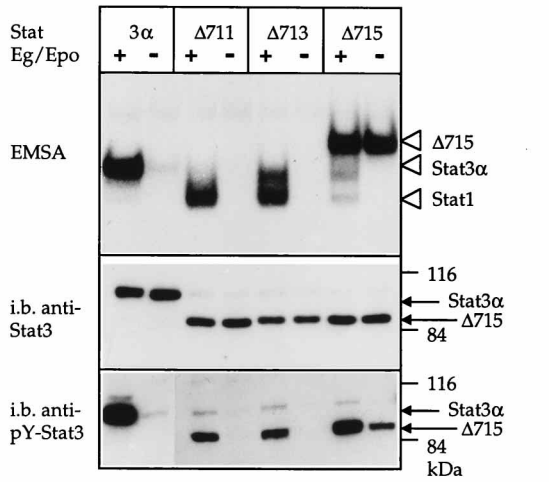

FIG. 6. Activation of carboxy-terminally truncated Stat3 mutants in COS-7 cells. COS-7 cells were cotransfected with vectors coding for Eg and either Stat3α or the Stat3Δ711, Stat3Δ713, or Stat3Δ715 deletion mutants where indicated. Nuclear extracts of cells either untreated or stimulated with Epo for 15 min were analyzed by EMSA with the m67SIE probe (upper panel). Expression and tyrosine phosphorylation of the Stat3 proteins was analyzed by immunoblotting (i.b.) with antiserum to Stat3 (anti-Stat3) (middle panel) or an antibody specifically recognizing tyrosine-phosphorylated Stat3 (anti-pY-Stat3) (lower panel).

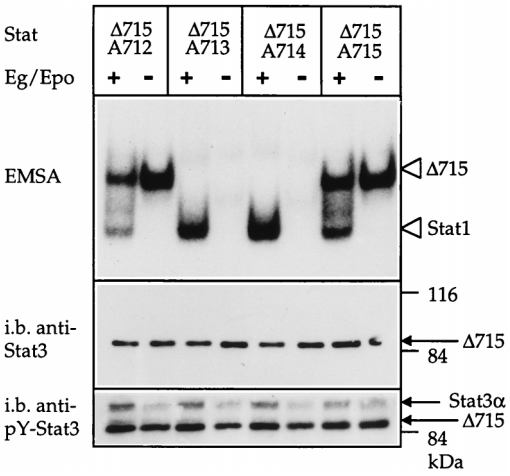

FIG. 7. Analysis of Stat3 point mutants in COS-7 cells. The Stat3 mutants indicated were expressed in COS-7 cells with or without coexpression of Eg. After stimulation with Epo, where indicated, DNA binding was analyzed by EMSA and expression and tyrosine phosphorylation were analyzed by immunoblotting (i.b.) with antiserum to Stat3 or antibodies to tyrosine-phosphorylated Stat3.

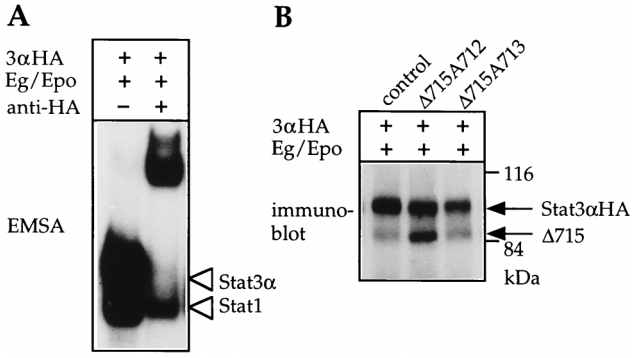

FIG. 9. Analysis of Stat3 dimerization by coprecipitation with HA-tagged Stat3. (A) HA-tagged Stat3α (Stat3αHA) was coexpressed with Eg in COS-7 cells. Nuclear extracts from Epo-treated cells were then analyzed by EMSA with or without prior incubation with HA antiserum. (B) Stat3αHA and Eg were coexpressed with either Stat3Δ715A712, Stat3Δ715A713, or a vector control in COS-7 cells. After stimulation with Epo, cell lysates were immunoprecipitated with HA antiserum and the precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis and blotted to polyvinylidene difluoride membranes. The membranes were developed by using an antiserum to Stat3 and enhanced chemiluminescence.

for tyrosine-phosphorylated Stat3. The total and phosphorylated protein levels were then measured by immunoblotting with anti-Stat3. The result shows that even with the constitutive activated mutants, only a fraction of the protein is tyrosine phosphorylated, comparable to the fraction of Stat3α phosphorylated after stimulation via gp130 (Fig. 8).

The lack of DNA binding of a Stat3 point mutant is caused by its inability to dimerize. The DNA-binding domain of the STATs has been localized to a central portion of the proteins (17). Therefore, it seems unlikely that truncation or point mutation of Stat3 residues 713 and 714 directly affects DNA binding. A defect in nuclear translocation theoretically could also explain the results obtained. However, we found that in the COS-7 cell system, all mutants (even nonfunctional ones like Stat3βYF) show a high incidence of nuclear translocation, possibly due to the high expression levels in these cells (data not shown). Alternatively, residues 713 and 714 may be required for Stat3 dimerization, which in turn is believed to be a prerequisite for DNA binding. To test this possibility, we established an assay to directly measure dimerization of the Stat3 mutants. A Stat3 molecule, Stat3αHA, tagged with an influenza virus HA epitope (HA or "flu" tag) was used to coprecipitate different Stat3 mutants. Stat3αHA was efficiently activated through Eg in transfected COS-7 cells, and DNA-protein complexes formed by Stat3αHA were completely supershifted by an antiserum to the HA epitope (Fig. 9A). No supershift was observed with wild-type Stat3α lacking the epitope (data not shown). This indicates that Stat3αHA was functionally expressed and specifically recognized by antibodies to HA. We next coexpressed either Stat3Δ715A712 or Stat3Δ715A713 together with Stat3α HA and Eg in COS-7 cells. Immunoprecipitation with HA antiserum coprecipitated Stat3Δ715A712 but not Stat3Δ715A713 (Fig. 9B), although both mutants were equally expressed (compare Fig. 7). We conclude that mutation of valine 713 into alanine rendered Stat3 unable to dimerize and that this defect in turn caused the inability of the mutant to bind DNA.

Stat3α point mutants suggest multiple contacts involved in Stat3 dimerization. To prove that the importance for dimerization of amino acids defined above holds true not only in the context of the Stat3Δ715 truncation mutant, we introduced two of these mutations into full-length Stat3α. As shown in Fig. 10,

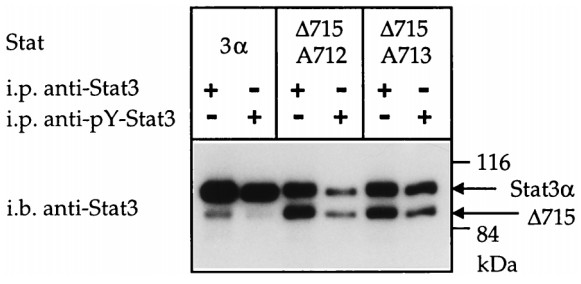

FIG. 8. Semiquantitative analysis of the tyrosine-phosphorylated Stat3 protein fraction. COS-7 cells transfected with expression vectors for the Eg hybrid receptor and either Stat3a, Stat3Δ715A712, or Stat3Δ715A713 were stimulated with Epo for 15 min, lysed, and immunoprecipitated (i.p.) with either antiserum to total Stat3 (anti-Stat3) or antibodies specific for tyrosine-phosphorylated Stat3 (anti-pY-Stat3). Precipitates were analyzed by immunoblotting (i.b.) with anti-Stat3.

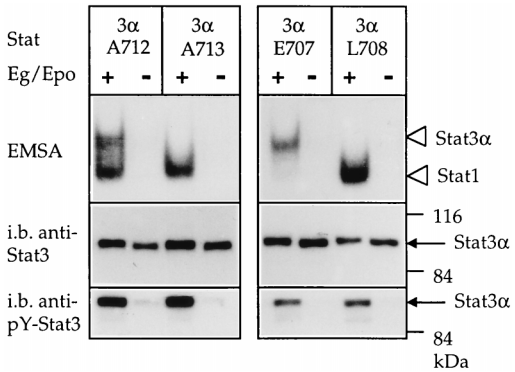

FIG. 10. Stat3α point mutants define residues required for Stat3 dimerization. The Stat3α mutants indicated were expressed in COS-7 cells with or without coexpression of Eg. After stimulation with Epo, where indicated, DNA binding was analyzed by EMSA and expression and tyrosine phosphorylation were analyzed by immunoblotting (i.b.) with antiserum to Stat3 or antibodies to tyrosine-phosphorylated Stat3.

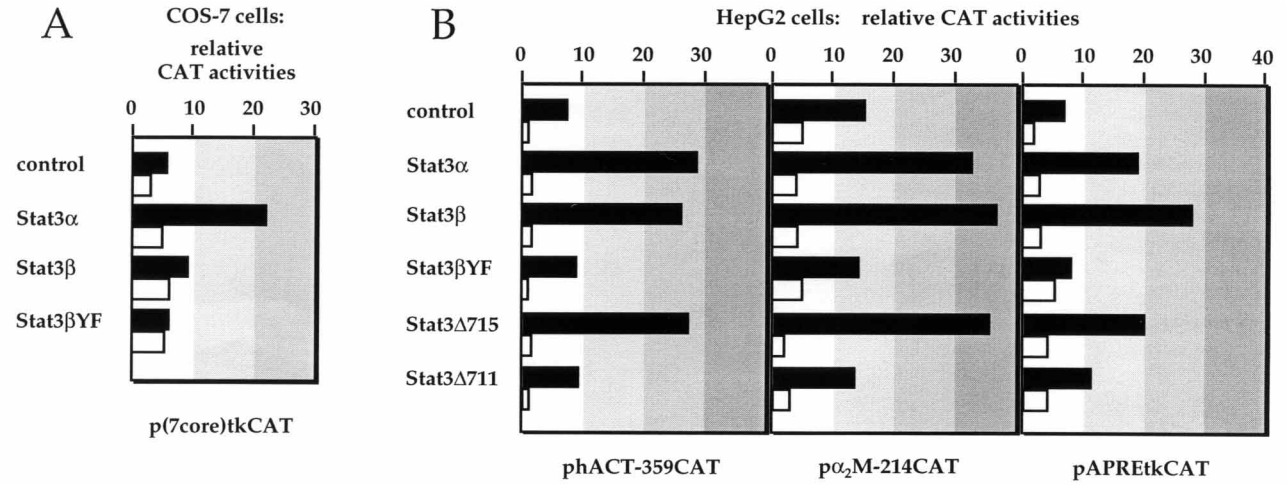

FIG. 11. Truncated Stat3 proteins are transcriptionally inactive in COS-7 but active in HepG2 human hepatoma cells. (A) COS-7 cells were transiently transfected with a reporter construct, p7coretkCAT, that contains seven copies of the proximal STAT-binding site of the α_2-macroglobulin promoter fused to the tk promoter and the CAT gene (8 μg plasmid DNA), with a β-galactosidase expression vector (pCH110; 2 μg), and with expression vectors for the EpoR-gp130 hybrid receptor Eg (6 μg) and the Stat3 proteins (4 μg) as indicated. The cells were incubated without (open bars) or with (solid bars) Epo (7 U/ml) for 20 h prior to harvesting. CAT and β-galactosidase activities were measured in cell lysates. All assays were done at least in triplicate. (B) HepG2 cells were transfected with CAT reporter constructs, β-galactosidase expression vector pCH110, and expression vectors for the EpoR-gp130 hybrid receptor Eg and the Stat3 proteins as above. The phACT-359CAT, pα_2M-214CAT, and pAPREtkCAT contain 359 bp of the promoter region of the human α_1-antichymotrypsin gene, 214 bp of the rat α_2-macroglobulin gene promoter, and the IL-6 response element (APRE) of the latter promoter fused to the tk promoter, respectively. Stimulation of the cells and CAT and β-galactosidase measurements were done as for panel A.

mutation of cysteine 712 did not impair Stat3 DNA-binding activity as judged from EMSA, whereas the exchange of valine 713 by alanine rendered the factor inactive. Again, expression and tyrosine phosphorylation of both mutants were equal. This result corroborates the importance of valine 713 for Stat3 dimerization.

Dimerization of STAT factors has been shown to involve the mutual binding of SH2 domains to phosphorylated tyrosine residues (31). Binding of the Stat3 SH2 domain to gp130 phosphotyrosine motifs has been shown to rely mainly on a glutamine residue at position +3 relative to the phosphotyrosines in gp130 (11, 33). However, valine 713 and threonine 714, which we identified above as being critical for Stat3 dimerization, are located 8 and 9 residues carboxy-terminal of phosphotyrosine 705, respectively. The importance of these residues suggests either that the interaction between the SH2 domains and phosphotyrosine motifs in STAT dimers occurs in a configuration different from that involved in STAT-receptor association or that additional contacts, possibly involving another protein domain(s), are implicated in Stat3 dimerization.

To evaluate whether "conventional" SH2-domain–phosphotyrosine motif binding is also important in Stat3 dimerization, we mutated lysine 707 and threonine 708, which are located in the vicinity of tyrosine 705, into glutamate and leucine, respectively. Although lysine 707 is conserved among Stat1, Stat2, Stat3, and Stat5, its mutation did not ablate the activation and DNA binding of Stat3 (Fig. 10). However, replacing threonine 708 by a lipophilic leucine residue completely eliminated Stat3-DNA complex formation. Since this hydrophilic residue is located at the critical position +3 relative to tyrosine 705, this finding suggests that disruption of SH2-domain/phosphopeptide recognition also interferes with dimerization.

Carboxy-terminally truncated Stat3 proteins are transcriptionally inactive in COS-7 cells but active in HepG2 human hepatoma cells. Stat3β has been reported to be incapable of transactivating target genes of the IL-5 pathway in COS-7 cells and even to inhibit the action of Stat3α in a dominant-negative fashion (3). Similarly, a carboxy-terminally truncated Stat3 mutant blocked the IL-6-induced differentiation of M1 promyelocytic cells (21). We therefore investigated whether truncated Stat3 forms are also defective in transactivating the promoters of IL-6 target genes. The IL-6-responsive promoters of acute-phase protein genes were found to be inactive in COS-7 cells. Therefore, for transient-transfection experiments with these cells, we used a construct, p7coretkCAT, that contained seven copies of the proximal STAT-binding site (the so-called core site) of the rat α_2-macroglobulin promoter, fused to the herpes simplex virus thymidine kinase (tk) promoter and the CAT gene. This reporter construct responded about twofold to stimulation through the chimeric Epo receptor-gp130 protein Eg. Upon cotransfection of an Stat3α expression vector, this response increased to about fivefold (Fig. 11A). COS-7 cells responded poorly to stimulation by IL-6, probably due to small numbers of the endogenous IL-6 receptor α (IL-6Rα) subunit. Concomitant addition of IL-6 and the agonistic soluble IL-6Rα to the medium, however, yielded results similar to those observed with Eg (data not shown). Therefore, the p7coretkCAT reporter construct is activated by both the endogenous gp130 and the hybrid receptor Eg in COS-7 cells, and the responses are increased by overexpression of Stat3α in both cases. In contrast, when Stat3β was coexpressed, no enhanced response of the p7coretkCAT reporter was observed (Fig. 11A). We conclude that in accordance with the results reported by Caldenhoven et al. for the IL-5 pathway (3), the carboxy-terminal part of Stat3α is required for the gp130-mediated transcriptional regulation of target genes.

Stat3 was originally characterized by us as a transcription factor that is intimately involved in the IL-6-dependent expression of acute-phase protein genes in hepatocytes and hepatoma cells (36, 37, 39). We therefore next evaluated whether carboxy-terminal truncations of Stat3 similarly affect its role in this cellular system. The human hepatoma cell line HepG2 had previously been used by us for transient-transfection experiments with IL-6-responsive promoters. However, overexpression of Stat3α in HepG2 cells only slightly increased the response of acute-phase promoter–CAT reporter constructs to

stimulation with IL-6 (30). This is probably due to the comparably high endogenous levels of Stat3α in these cells. In contrast, when the hybrid receptor Eg was coexpressed with reporter vectors in HepG2 cells, a significantly increased response to Epo stimulation upon Stat3α overexpression was observed (see below). We assume that in the case of IL-6-stimulation, the magnitude of the response is limited by the number of endogenous IL-6Rα molecules, while in the presence of large numbers of ectopically expressed hybrid Eg receptors, the number of Stat3 molecules becomes rate limiting for the transcriptional response.

We used this system to monitor the transcriptional activity of various Stat3 mutants in HepG2 cells. First, the promoter of the human gene for the acute-phase plasma protein α_1-antichymotrypsin was studied. The IL-6-responsive element of this promoter has previously been localized by us and was shown to contain two adjacent Stat3-binding sites. Induction of this promoter by IL-6 specifically relies on binding of Stat3, but not other STAT factors, to this element (30). Surprisingly, both Stat3β and Stat3Δ715 exhibited the same positive transcriptional effect on the α_1-antichymotrypsin promoter as did Stat3α (Fig. 11B). In contrast, mutants Stat3βYF and Stat3Δ711, which were shown above to be incapable of DNA binding due to impaired tyrosine phosphorylation and dimerization, respectively, were both inactive. Truncated Stat3 proteins are therefore, in contrast to the situation in COS-7 cells, able to transactivate a target promoter in HepG2 cells. In spite of their constitutive activation in COS-7 cells, these factors act in a stimulation-dependent manner in the hepatoma cells (Fig. 11B). We have reported previously, however, that STAT tyrosine phosphorylation alone is not sufficient to trigger transactivation of IL-6 target promoters but that concomitant activation of an as yet unknown protein serine kinase that is sensitive to inhibition by the isoquinoline derivative H7 is also required (19a). Therefore, the inducible transcriptional activity of truncated Stat3 proteins might reflect the requirement of this additional pathway for target gene induction.

To evaluate whether the activity of truncated Stat3 forms in HepG2 cells is specifically observed with the α_1-antichymotrypsin promoter or whether this is a more general effect, the studies were extended to the promoter of the rat α_2-macroglobulin gene. With a reporter containing 214 bp of the promoter region of this gene, equivalent results were obtained (Fig. 11B). Again, truncated Stat3 forms that retained their ability to dimerize and bind DNA were as active as full-length Stat3α, while nonfunctional mutants were not. Furthermore, a reporter construct, pAPREtkCAT, that contains the isolated IL-6-responsive element (APRE) of the rat α_2-macroglobulin promoter fused to the tk promoter yielded a comparable pattern (Fig. 11B). These findings suggests that the promoter context is not responsible for the transcriptional activity of truncated Stat3 proteins in HepG2 cells. In conclusion, our data demonstrate that in contrast to COS-7 cells, the transactivating potential of Stat3 does not rely on its carboxy-terminal domains in hepatoma cells.

DISCUSSION

STAT factors occur in various isoforms generated by alternative splicing. The biological functions of the various isoforms, however, are as yet poorly understood. It was found that the carboxy-terminally truncated version of Stat1, Stat1β, lacks the ability to transactivate genes on its own in gamma interferon signalling. However in a heterodimeric complex with Stat2, it is able to support gene induction in response to alpha interferon (24). Short Stat5 isoforms were recently reported to be transcriptionally inactive and even to repress transactivation by the long isoforms in a dominant negative fashion (23). Similarly, Caldenhoven et al. (3) demonstrated that Stat3β represses the IL-5-induced transactivation of the ICAM-1 promoter by Stat3α in COS cells. In this study, we used a mutational approach to analyze structure-function relationships of the Stat3 carboxy-terminal region adjacent to the tyrosine phosphorylation site. The data presented allow us to draw a number of conclusions with respect to molecular mechanisms of Stat3 dimerization, activation, and function.

Constitutive activation of truncated Stat3 proteins in COS cells. We observed that carboxy-terminally truncated Stat3 proteins are constitutively activated when expressed in COS-7 cells. For Stat3β, this observation has been reported previously by Caldenhoven et al. (3). We show here that constitutive activation does not require the unique Stat3β carboxy terminus but depends solely on the truncation of carboxy-terminal 55 amino acids of Stat3α. Furthermore, our data demonstrate that constitutive DNA binding of Stat3 mutants is accompanied by and requires constitutive tyrosine phosphorylation.

For Stat5, it has recently been shown that carboxy-terminal truncation causes a decreased inactivation rate of this protein (23). An equivalent mechanism may be responsible for the constitutive activation of truncated Stat3 forms as well. One possible explanation is a reduced access of tyrosine phosphatases to the phosphorylated tyrosine residue, causing an imbalance in phosphorylation and dephosphorylation kinetics. In fact, even with the constitutively activated mutants, only a fraction of the protein was tyrosine phosphorylated (Fig. 8). Furthermore, the tyrosine phosphatase inhibitor vanadate is known to cause stimulus-independent STAT activation (6), suggesting that a low but constant rate of phosphorylation occurs that is normally counteracted by phosphatases.

A reduced accessibility of the phosphotyrosine could be caused by enhanced dimer stability of truncated Stat3 forms. However, our data obtained by phosphopeptide competition assays show that, at least in vitro, Stat3α and Stat3β dimers exhibit equal stabilities. This does not completely rule out, however, that the kinetic constants of dimer association and dissociation are altered upon truncation. Alternatively, the deleted carboxy-terminal region may harbor a phosphatase association site or, conversely, an inhibitory domain that decreases the phosphorylation rate.

However, our data imply quite a complex nature for the mechanisms involved. It is shown that truncation mutants Stat3Δ713 and Stat3Δ711 are unable to bind DNA but still can be tyrosine phosphorylated. However, in contrast to Stat3Δ715 and Stat3β, these mutants are not constitutively but inducibly phosphorylated. Astonishingly, Stat3Δ715 point mutants that were unable to dimerize (Stat3Δ715A713 and Stat3Δ715A714) were still constitutively tyrosine phosphorylated. These findings indicate that subtle changes within the Stat3 portion adjacent to the tyrosine phosphorylation site can influence the activation-deactivation balance. This view is supported by recent results from our laboratory demonstrating that certain point mutations in the putative dimerization surface of Stat3 can also cause constitutive activation (16).

Furthermore, by using chimeric STAT proteins, it is shown here that in addition to the carboxy-terminal region, the Stat3 amino-terminal parts play a role in constitutive activation whereas the respective part of Stat1 cannot support it. As yet, the functions of this part of STAT proteins are largely unknown. Secondary-structure predictions suggested the existence of extended α-helical portions, including a potential leucine zipper (8). Our data now demonstrate that the amino-terminal portion of Stat3 is involved in the control of activa-

tion-inactivation processes. Similar observations were recently reported by Shuai et al., who demonstrated that mutations in the amino-terminal part of Stat1 inhibited tyrosine dephosphorylation (32a). The molecular basis of this is unclear, but one might speculate that amino-terminal domains make direct contacts with the carboxy-terminal domains involved in dimerization.

An interesting discrepancy exists between our results obtained in COS-7 cells and data reported by Minami et al. for the myeloid leukemia cell line M1 (21). In these cells, the Stat3Δ715 truncation mutant is inducibly tyrosine phosphorylated, in contrast to the constitutive phosphorylation observed by us in COS-7 cells. Therefore, additional cellular factors may contribute to the activation characteristics of STAT isoforms, and whether a truncated STAT protein is activated in an inducible, constitutive, or prolonged manner is likely to depend on the cellular environment. As a consequence, the function of different STAT isoforms may vary with cell type.

Stat3 dimerization studies. Another aspect of our data is the identification of amino acids required for dimerization of Stat3. We show that mutation of threonine 708 (position +3 with respect to the Stat3 tyrosine phosphorylation site) impairs dimerization. In view of our previous observation that position +3 relative to the tyrosine is crucial for the interaction of Stat3 with gp130 phosphotyrosine motifs, this result is in accordance with a mutual SH2-domain–phosphotyrosine motif interaction in Stat3 dimers. However, mutation of amino acids 713 and 714 (valine and threonine, respectively) also blocked Stat3 dimerization. So far, no other SH2 domain is known to make contacts with residues that distant from the phosphotyrosine. Without structural data available, it cannot be ruled out that STAT dimerization uses a rather unconventional SH2-domain–phosphopeptide topography. However, these findings may rather indicate that additional contacts, possibly involving other protein domains, contribute to dimer stabilization. In fact, recognition of STAT phosphotyrosine motifs by their SH2 domains has been demonstrated to occur with low affinity (13). Therefore, additional interactions, possibly involving the residues identified in this report, may be required to stabilize the dimer. Further analyses are needed to reveal the nature of such contacts.

Transactivation by truncated Stat3 proteins in hepatoma cells. IL-6 is known to be the major regulator of the rat α_2-macroglobulin gene as well as other acute-phase genes during an acute-phase response (10). Schaefer et al. have reported a cooperative action of Stat3β and c-*jun* on the promoter of this gene (27). However, whether Stat3β can, like Stat3α, transduce the stimulatory signal from IL-6-type cytokines to that promoter has not been investigated. In COS cells, Stat3β represses the induction of the ICAM-1 promoter in a dominant-negative fashion (3). Furthermore, the truncated Stat3Δ715 acts as a dominant-negative regulator of IL-6-induced differentiation of M1 myeloid leukemia cells (21). Wen et al. demonstrated the importance of phosphorylation of serine 727 for maximal transcriptional activity of Stat3 in COS cells (38). This serine residue is not present in Stat3β and Stat3Δ715. Taken together, these data underscored the importance of the Stat3α carboxy terminus for transactivation of target genes.

In the context of that background, our result that both Stat3β and Stat3Δ715 are as active as Stat3α in transactivating the rat α_2-macroglobulin and human α_1-antichymotrypsin promoters in response to stimulation through gp130 is quite challenging. The fact that we observed positive transcriptional effects of the truncated factors even with a reporter containing the isolated APRE of the α_2-macroglobulin gene fused to a control promoter indicates that the promoter context is not important for this effect. It must be concluded, therefore, that the mechanism by which Stat3 transactivates target genes in hepatoma cells differs significantly from that in COS or M1 cells. Cell-type-specific factors, e.g., other transcription factors or coactivators, may be involved by cooperating with Stat3. Several other transcription factors have been reported to interact with STAT proteins. Recently, for example, Stat5 has been shown to physically and functionally interact with the glucocorticoid receptor (34). As mentioned above, Stat3β was originally identified by its association with c-*jun* (27). In spite of the documented cooperative action of Stat3β and c-*jun* on the α_2-macroglobulin promoter, however, it seems unlikely that the positive transcriptional effect of truncated Stat3 proteins in response to cytokine stimulation can be explained by this interaction. First, interaction between c-*jun* and Stat3 was restricted to the Stat3β isoform but did not occur with Stat3α (27). Second, the α_2-macroglobulin promoter *jun*-binding site is not present in the reporter construct containing the isolated APRE, nor does the α_1-antichymotrypsin promoter region used contain any obvious potential AP-1 site.

It is intriguing that the transcriptional activity of the truncated Stat3 forms in HepG2 cells is inducible, although these proteins were shown above to be constitutively tyrosine phosphorylated in COS-7 cells. Due to the comparably high endogenous levels of Stat3 in HepG2 cells, we could not determine whether the ectopically expressed Stat3 proteins were constitutively or inducibly tyrosine phosphorylated in these cells. However, it is to be emphasized that according to our previously reported results, tyrosine phosphorylation of STAT proteins is not sufficient for target gene induction (19a). An additional pathway involving an as yet unknown protein serine kinase is also required. Inhibition of this kinase by H7 blocks IL-6-dependent gene induction. Interestingly, a serine residue in Stat3 that is known to be phosphorylated through this pathway is not present in the carboxy-terminally truncated Stat3 forms. Preliminary results from our laboratory demonstrate, however, that the transactivating action of truncated Stat3 proteins is also H7 sensitive (30). This indicates that the H7-sensitive pathway has additional targets that are also involved in target gene regulation by IL-6.

In conclusion, IL-6-induced transcription of target genes in hepatoma cells through Stat3 uses a mechanism that does not rely on the potential carboxy-terminal transactivation domain of Stat3 and, in that respect, differs principally from other cell types. The identification of additional, cell-type-specific proteins involved will be the subject of future studies.

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