Ser727-dependent transcriptional activation by association of p300 with STAT3 upon IL-6 stimulation

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Abstract Activation of the signal transducer and activator of transcription 3 (STAT3) in response to interleukin-6 (IL-6) type cytokines involves both phosphorylation of Tyr705, which enables dimerization, nuclear translocation and DNA binding, as well as ser727 phosphorylation. Here, we describe that the 65 C-terminal amino acids of STAT3 can function as an independent transcription activation domain (TAD), particularly when a negative charge is introduced at position 727 by mutation of the serine residue into aspartate. The strong transcriptional activity of the C-terminal STAT3 Ser727Asp TAD is coupled to a constitutive association with the co-activator p300. In HepG2 cells, p300 associates with STAT3 upon IL-6 stimulation, and overexpression of p300 enhances the transcriptional activity of STAT3α, but not of STAT3β or STAT3 Ser727Ala. We conclude that Ser727 phosphorylation in the C-terminal region of STAT3 is required for transactivation by association with p300.

Key words: Interleukin-6; Signal transducer and activator of transcription 3; Ser727 phosphorylation; Transactivation; p300

1. Introduction

The signal transducer and activator of transcription (STAT) family of transcription factors is activated in response to a variety of extracellular ligands [1,2]. Currently, seven mammalian STATs have been identified which all become phosphorylated on a tyrosine residue in response to cell-surface receptor activation allowing STAT dimerization, nuclear translocation and transcription activation [1,2]. All STATs have crucial physiological roles during embryogenesis, development, hematopoiesis and immune responses, while each STAT serves a highly specific function [3,4]. Isoforms of at least STAT1 and STAT3 exist, designated as STAT1β and STAT3β, respectively, which both lack a C-terminal region due to differential splicing [5–7]. STAT1β and STAT3β have strongly reduced transcriptional activities as compared to wild type STAT1α and STAT3α, indicating the C-terminal region serves as an important transactivation domain (TAD) [5,6]. Indeed, phosphorylation of a specific serine residue located in the TAD, Ser727 in both STAT1 and STAT3, is required to achieve maximal transcriptional activity [8,9].

Most transcription factors function in multi-protein enhanceosomes to initiate gene transcription. Interactions of STATs have been described with hormone receptors, mini-chromosome maintenance proteins, members of the AP-1 family and members of the interferon (IFN) regulatory factor family [10–15]. Also, interactions between some STATs and CREB binding protein (CBP)/p300 have been described [16–18]. The p300 and CBP transcriptional co-activators are important regulators of many cellular processes. They contain histone acetyltransferase (HAT) domains, are involved in chromatin remodeling [19], and interact with a wide range of DNA binding transcription factors, including p53, E2F, AP-1, nuclear receptors, MyoD, and the p65 subunit of nuclear factor κB [20,21]. Specifically, p300/CBP has been reported to interact with STAT1, where both the N-terminal as well as the C-terminal region of STAT1 appear to be involved in its association with p300/CBP [18]. Also, p300/CBP interactions with STAT5 have been described [22].

However, it is unclear to what extend such interactions are of importance for optimal STAT3 transactivation. The experiments presented here were designed to study the role of Ser727 phosphorylation in relation to association with other proteins. We report that the 65 C-terminal amino acids of STAT3 can serve as an independent TAD. p300/CBP associates with STAT3 in response to interleukin-6 (IL-6) stimulation, whereas it is constitutively associated with the C-terminal region of the STAT3 Ser727Asp mutant. Furthermore, we demonstrate that overexpression of p300/CBP enhances STAT3α, but not STAT3β or STAT3 Ser727Ala, transcriptional activities.

2. Materials and methods

2.1. Cell culture and antibodies

HepG2 and COS7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (Integro B.V., Zaandam, The Netherlands). Cells were stimulated with 25 ng/ml human recombinant IL-6 [23]. Antibodies against STAT3 (C-20) and p300 (C-20) (Santa Cruz) were used in dilutions of 1:4000. The antibodies against the DNA binding domain of GAL4 were obtained from Upstate Technology and were used in a dilution of 1:1000.

2.2. Reporters, expression vectors and plasmid constructions

The pIRE-ti-LUC reporter was made by inserting a synthetic oligonucleotide (5′-ctagcaggTTTCCGGGAAAgcacagcttaggTTTCCGGGAAAgcac-3′) containing two copies of the IL-6 response element

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Abbreviations: IL-6, interleukin-6; STAT3, signal transducer and activator of transcription 3; TAD, transcription activation domain; CBP, CREB binding protein

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(IRE) of the ICAM-1 promoter in the *Nhe*I site of the pGL3ti minimal promoter luciferase construct as described previously [24]. The UAS-GAL4-luciferase reporter contains five copies of the GAL4 binding site in front of the luciferase gene. Furthermore, the following eukaryotic expression vectors were used: pSG5-STAT3α, encoding human STAT3α (generous gift from Dr. P. Coffer, Department of Pulmonary Diseases, AZU, Utrecht, The Netherlands); pSG5-STAT3β encoding human STAT3β (generous gift from Dr. R. de Groot, Department of Pulmonary Diseases, AZU, Utrecht, The Netherlands) [5]; and pSG5-STAT3 Ser727Ala, encoding the Ser727Ala mutant of STAT3 [25]. The mutant pSG5-STAT3 Ser727Asp was cloned by site-directed mutagenesis and the sequence was confirmed by sequencing. The expression vector pCMV-p300 was obtained from Dr. D.M. Livingston. pGAD424-STAT3α (502–771), pGAD424-STAT3α (599–771), pGAD424-STAT3α (705–771), pGAD424-STAT3 Ser727Asp (502–771), pGAD424-STAT3 Ser727Asp (599–771), and pGAD424-STAT3 Ser727Asp (705–771) were constructed by PCR using pSG5-STAT3α or pSG5-STAT3 Ser727Asp as templates and the following primers: (502) 5′-GAGAATTCGACCAAGTGGCCGAGG-3′, (599) 5′-GAGAATTCAGCACTAAGCCCCCAG-3′, (705) 5′-GAGAATTCCTGAAGACCAAGTTTATCTGTGTG-3′, all containing an *Eco*RI site in the flanking sequence, and (reverse) 5′-GCGAGCTCTCACATGGGGGAGGTAG-3′ which contains an *Sac*I site in the flanking sequence. PCR products were cut with *Eco*RI and *Sac*I and the fragments were cloned into the *Eco*RI and *Sac*I sites of pGAD424.

2.3. Transient transfection assays

HepG2 cells were seeded at 3×10^5 cells per well in 6-well plates (Costar), and 24 h later cells were transfected with 10 μg plasmid DNA using the calcium phosphate co-precipitation method [26]. Transfection mixtures consisted of a mixture of 2.5 μg reporter, 2.5 μg pDM2-LacZ as a control to determine transfection efficiency, and 2.5 μg expression plasmids unless stated otherwise in Section 3. When necessary, pUC18 was added to the transfection mixture to obtain a total of 10 μg of DNA. Cells were incubated with precipitate for 24 h, washed with phosphate-buffered saline (PBS), and stimulated for an additional 24 h. Cells were collected in 200 μl reporter lysis buffer (Promega) and subjected to the assays for luciferase and β-galactosidase as previously described [25]. Experiments were performed in triplicate and representative examples of at least three independent experiments are shown. Standard deviations were calculated using Sigmaplot (Jandel Corp.).

2.4. Western blotting and immunoprecipitations

A total of 1×10^7 cells were lysed on ice in lysis buffer (100 mM NaCl pH 7.4, 2 mM EGTA, 1 mM dithiothreitol, 1 mM Na_2VO_3 (*ortho*), 1% Triton X-100, 10% glycerol, 10 μg/ml leupeptin, and 0.4 mM PMSF). Protein concentrations were determined (Bio-Rad). Whole-cell extracts were boiled for 5 min in the presence of Laemmli sample buffer prior to separation on 12.5% sodium dodecyl sulfate (SDS)–polyacrylamide gels. The proteins were transferred to a nitrocellulose filter (Millipore) in Tris-glycine buffer at 100 V for 1.5 h using an electroblotter (Pharmacia). Membranes were blocked with PBS buffer containing 5% non-fat milk prior to incubation with antibodies. Binding of each antibody was detected by chemiluminescence using ECL according to the manufacturer's recommendations (Amersham Corp.). For immunoprecipitations, whole-cell lysates were incubated with 1 μg of antibodies, precipitated with Protein-A Sepharose beads (Pharmacia), and washed three times with lysis buffer. The precipitates were boiled for 5 min in Laemmli sample buffer and subjected to 12.5% SDS–polyacrylamide gel electrophoresis.

3. Results

3.1. The role of the C-terminus in STAT3 transactivation

In order to study the functional role of the C-terminus in STAT3 transactivation, STAT3α, STAT3β and the point mutants STAT3 Ser727Ala and STAT3 Ser727Asp were transiently overexpressed in COS7 cells which express low levels of endogenous STAT3. As depicted in Fig. 1A, overexpression of STAT3α resulted in an approximately 4-fold induction of reporter activation in response to IL-6, while overexpression

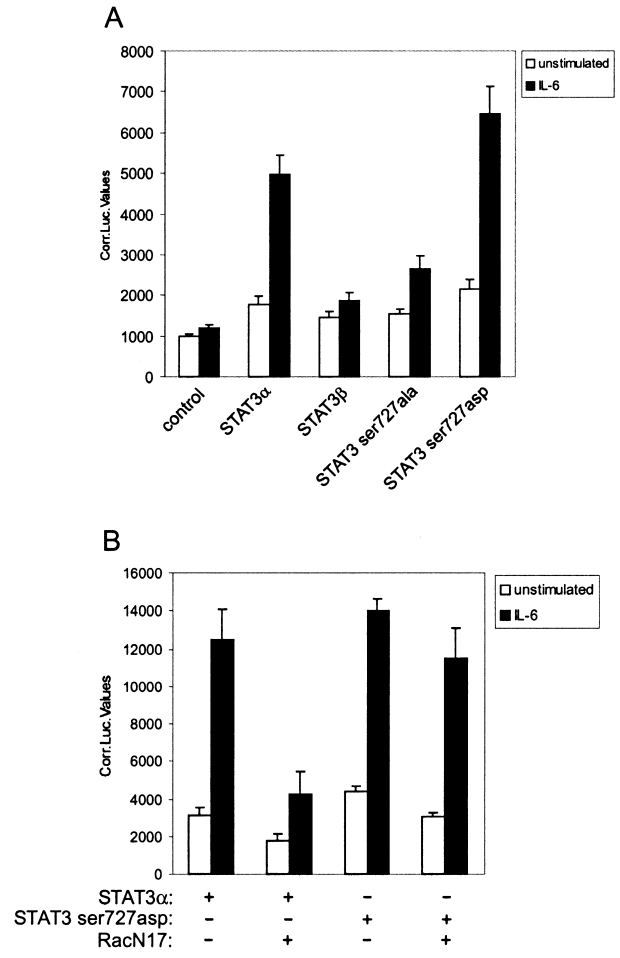

Fig. 1. The C-terminal region of STAT3 is required to achieve maximal transactivation. A: COS7 cells were transiently transfected with the IRE-LUC reporter, together with expression vectors for STAT3α, STAT3β, STAT3 Ser727Ala or STAT3 Ser727Asp as indicated. Cells were stimulated with 25 ng/ml IL-6 for 24 h prior to harvest and luciferase and LacZ assays. Experiments were performed in triplicate and a representative example of three independent experiment is shown. B: HepG2 cells were transiently transfected with the IRE-LUC reporter together with expression vectors for STAT3α, STAT3 Ser727Asp and dominant negative RacN17 as indicated. Cells were stimulated with 25 ng/ml IL-6 for 24 h prior to harvest and luciferase and LacZ assays.

of STAT3β did not significantly induce reporter activation. Overexpression of STAT3 Ser727Ala resulted in a 1.5-fold induction of reporter activation in response to IL-6, while STAT3 Ser727Asp enhanced reporter activation to higher levels than wild type. These data confirm that the C-terminal region is important for maximal STAT3 transcriptional activity and suggest that phosphorylation of the serine residue 727 serves to provide a negative charge on the molecule.

Recently, we have demonstrated that IL-6 induced STAT3 Ser727 phosphorylation and that transactivation requires activation of the GTPase Rac-1 [25]. To test whether the STAT3 Ser727Asp mutant has become independent of IL-6-induced Rac-1 activity, STAT3α or STAT3 Ser727Asp were transiently overexpressed in HepG2 cells together with dominant negative RacN17. As depicted in Fig. 1B, overexpression of dominant negative RacN17 severely impaired IL-6-induced activation of STAT3α, whereas STAT3 Ser727Asp transactivation remained unaffected. These data indicate that IL-6-in-

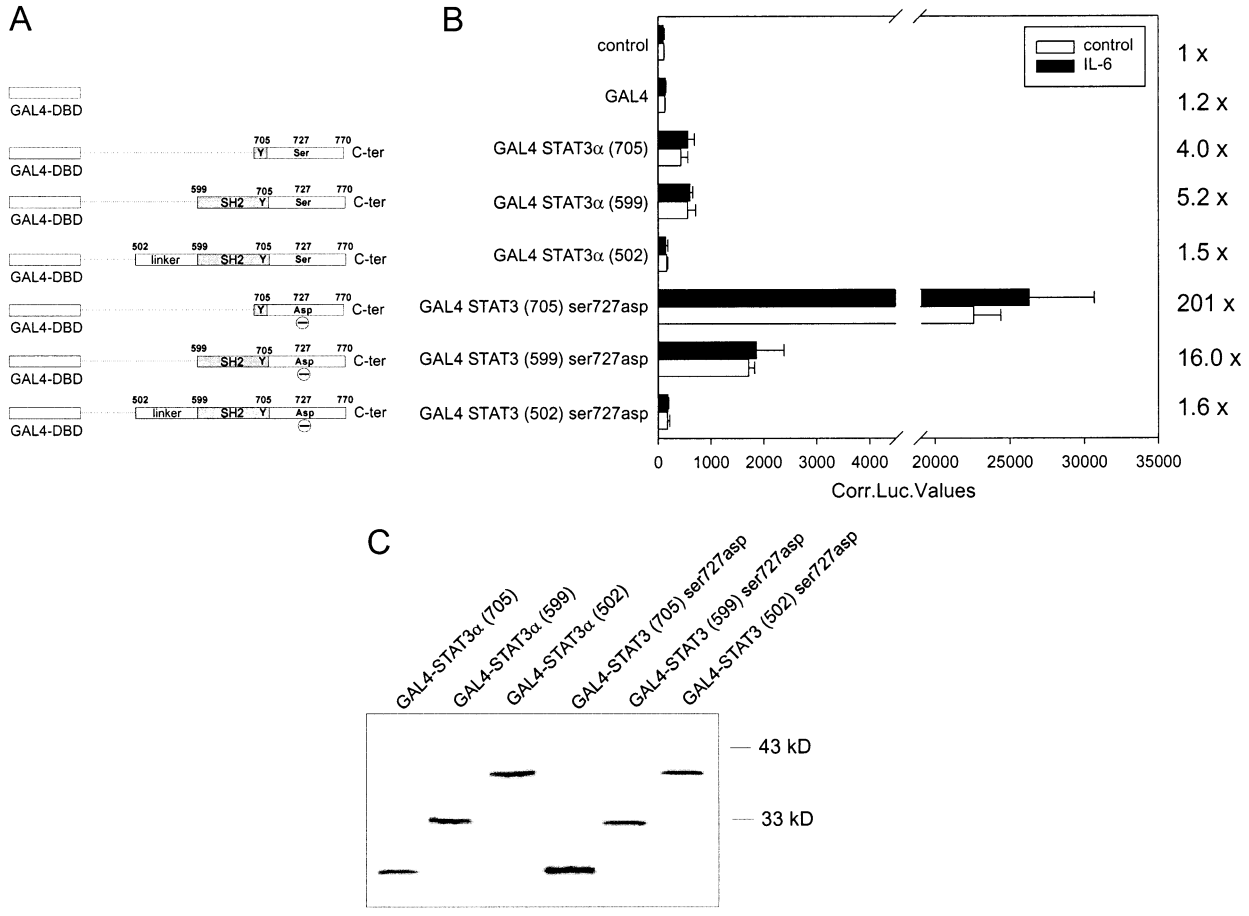

Fig. 2. Defining the minimal STAT3 transactivation domain. A: Schematic view of the GAL4-STAT3 chimeras used in this study: C-terminal fragments of STAT3α or STAT3 Ser727Asp (regions 502–770, 599–770 and 705–770) were fused to the DNA binding domain of GAL4. B: HepG2 cells were transiently transfected with the UAS-LUC reporter together with expression vectors for the GAL4-STAT3 chimeras as indicated. Cells were stimulated with 25 ng/ml IL-6 for 24 h prior to harvest and luciferase and LacZ assays. C: GAL4-STAT3 chimeras were overexpressed in HepG2 cells and total cell lysates were Western blotted using antibodies against the DNA binding region of GAL4.

duced activation of the STAT3 Ser727Asp mutant is independent of activation of the Rac-1 signal transduction cascade.

3.2. Defining the minimal STAT3 transactivation domain

In order to identify the minimal STAT3 transactivation domain, a series of GAL4-STAT3 chimeras were constructed in which C-terminal fragments of either STAT3α or STAT3 Ser727Asp were fused to the DNA binding domain of GAL4 (Fig. 2A). Transcriptional activities of chimeras were tested on the UAS-luciferase reporter by transient transfection in HepG2 cells. Overexpression of GAL4-STAT3α (705) enhanced reporter activation 4-fold over basal levels, comparable to the transcriptional activities of GAL4-STAT3α (599) (Fig. 2B). In contrast, GAL4-STAT3α (502) did not drive reporter activation. Surprisingly, in each case IL-6 did not further enhance transcription activation, which may suggest that STAT3 dimerization is a prerequisite for Ser727 phosphorylation. GAL4-STAT3 (705) Ser727Asp strongly enhanced reporter activation approximately 200-fold over basal levels, while GAL4-STAT3 (599) Ser727Asp induced a 16-fold induction of reporter activation. GAL4-STAT3 (502) Ser727-Asp did not induce activation of the reporter. Again, IL-6 did not further enhance transcriptional activities of the GAL4-STAT3 Ser727Asp chimeras. All proteins were expressed to comparable levels (Fig. 2C). These data indicate that the C-terminal 65 amino acids of STAT3 can serve as a minimal TAD, particularly if a negative charge is introduced at position 727.

3.3. The minimal TAD of STAT3 Ser727Asp is constitutively associated with p300

Next, it was investigated whether p300 was associated with the GAL4-STAT3 chimeras thus driving the transcriptional activities. GAL4-STAT3 (705) Ser727Asp, GAL4-STAT3 (599) Ser727Asp and GAL4 were overexpressed in HepG2 cells, and cells were either stimulated with IL-6 for 15 min or left unstimulated. The GAL4-STAT3 chimeras were immunoprecipitated from total cell extracts by using anti-GAL4 antibodies and immunoprecipitates were blotted against p300. Comparable amounts of GAL4 or GAL4-STAT3 chimeras were immunoprecipitated, while p300 co-immunoprecipitated with GAL4-STAT3 (705) Ser727Asp, and to a lesser degree with GAL4-STAT3 (599) Ser727Asp (Fig. 3, lanes 1–4). IL-6 stimulation did not influence the amount of co-immunoprecipitated p300. p300 did not co-immunoprecipitate with GAL4 and no a-specific binding of p300 to the beads was observed (Fig. 3, lanes 5–7). Also, no significant p300 association was observed with GAL4-STAT3α (705) or GAL4-STAT3α (599) (data not shown). These data correlate

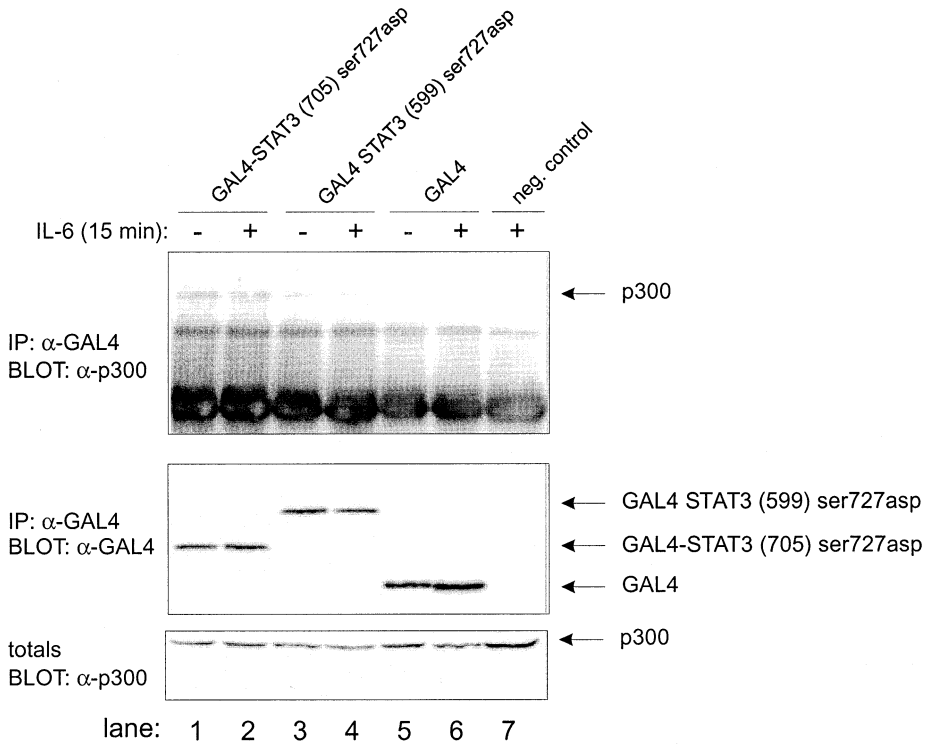

Fig. 3. The minimal TAD of STAT3 Ser727Asp is constitutively associated with p300. GAL4, GAL4-STAT3 (705) Ser727Asp and GAL4-STAT3 (599) Ser727Asp were overexpressed in HepG2 cells, cells were either stimulated with 25 ng/ml IL-6 for 15 min or left unstimulated and lysates were prepared as described in Section 2. Immunoprecipitations were performed using anti-GAL4 antibodies and precipitates were blotted against p300 and GAL4. Also, the endogenous levels of p300 are shown in each sample as a loading control. As a negative control, lysates were incubated with beads alone in the absence of anti-GAL4 antibody.

well with the levels of transcriptional activity of the GAL4-STAT3 chimeras.

3.4. p300 associates with STAT3 upon IL-6 stimulation

To determine whether p300 also associates with STAT3 in vivo, HepG2 cells were stimulated with IL-6 or left unstimulated and nuclear extracts were prepared. Endogenous p300 was immunoprecipitated, and immunoprecipitates were blotted against STAT3. As depicted in Fig. 4, STAT3 translocated to the nucleus upon IL-6 stimulation (lower panel), and associated with p300 (upper panel). No a-specific association of STAT3 with the beads was observed (data not shown). These data indicate that STAT3 associates with p300 in vivo in the nucleus upon IL-6 stimulation.

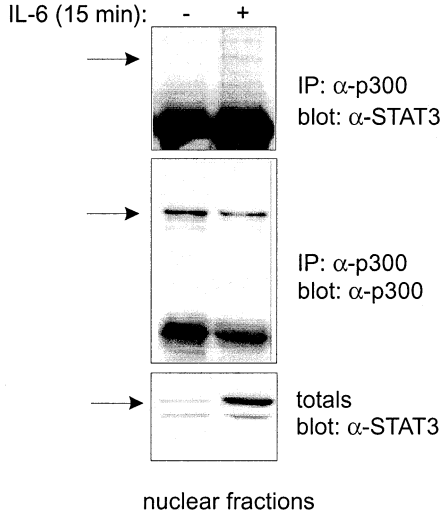

Fig. 4. p300 associates with STAT3 in vivo upon IL-6 stimulation. HepG2 cells were stimulated with 25 ng/ml IL-6 for 15 min or left unstimulated, nuclear extracts were prepared as described in Section 2 and p300 was immunoprecipitated using anti-p300 antibody. Immunoprecipitates were blotted against STAT3 and p300. As a loading control, total levels of STAT3 expression are shown.

3.5. The effects of p300 overexpression on STAT3 transcriptional activity

Since p300 associated with STAT3 in vivo, the functionality of this interaction was studied on a STAT3 responsive IRE-luciferase reporter in COS7 cells. Increasing amounts of p300 were overexpressed which strongly enhanced STAT3α transactivation at low concentrations, but inhibited STAT3α transactivation at high concentrations (Fig. 5A). Similar patterns of reporter activation were observed in the presence of overexpressed STAT3 Ser727Asp (Fig. 5B). In contrast, in the presence of overexpressed STAT3β or STAT3 Ser727Ala, IL-6 did not significantly enhance reporter activation, and overexpression of p300 did not alter STAT3 transcriptional activities (Fig. 5C,D). These data indicate that p300 specifically affects transcriptional activities via interaction with the Ser727 residue in the C-terminal region of STAT3.

4. Discussion

STAT1, STAT3 and STAT4 all share a conserved stretch of amino acids in their C-terminal region (LPMSP) in which both the leucine as well as the serine residues are important

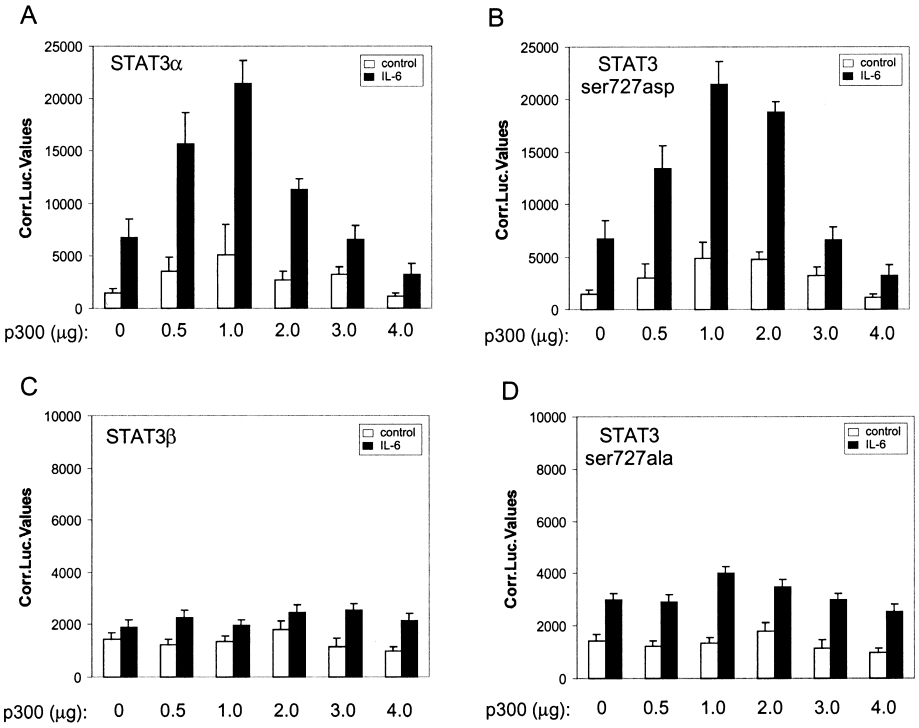

Fig. 5. The effects of p300 overexpression of STAT3 transactivation. COS7 cells were transiently transfected with the IRE-LUC reporter, together with expression vectors for STAT3α (A), STAT3 Ser727Asp (B), STAT3β (C) or STAT3 Ser727Ala (D) as indicated. Increasing amounts of p300 were overexpressed as indicated in each experiment. Cells were stimulated with 25 ng/ml IL-6 for 24 h prior to harvest and luciferase and LacZ assays.

to achieve maximal transactivational activity [2,8,27]. Upon cytokine or growth factor stimulation, the serine residue becomes phosphorylated, and it has been well documented that this phosphorylation event is critical in order to initiate high levels of gene transcription in various cellular settings [28,29]. Isoforms of STAT1 and STAT3 exist, designated as STAT1β and STAT3β, which lack a C-terminal region that contains the LPMSP motif due to splicing events, and these isoforms display reduced transcriptional activities [5,6]. These studies suggested that the C-terminal regions of STAT1 and STAT3 serve as a transcription activation domain (TAD). However, the mechanisms underlying these observations are not well defined.

Here, we confirm that Ser727 phosphorylation of the C-terminal region of STAT3 is required to achieve maximal transcriptional activity in response to IL-6 stimulation. In the presence of overexpressed STAT3β or STAT3 Ser727Ala, IL-6 only induced low reporter activation as compared to STAT3α as determined with IRE-luciferase reporter assays in COS7 cells. When the STAT3 Ser727Asp mutant was overexpressed, IL-6 stimulation was still required to induce STAT3 Tyr705 phosphorylation and dimerization, while the IL-6-induced transactivation was not significantly different from wild type. This indicates that a negative charge at position 727 does not significantly affect IL-6-induced Tyr705 phosphorylation or nuclear import. gp130-mediated STAT3 activation involves STAT3 Tyr705 phosphorylation via JAK tyrosine kinases, while the IL-6-induced STAT3 Ser727 phosphorylation involves activation of a signal transduction cascade that is comprised of the gp130-associated guanine nucleotide exchange factor Vav, the small GTPase Rac-1 and the kinases MEKK-1 and SEK-1/MKK-4 [25]. Here, we demonstrate that IL-6-induced activation of the STAT3 Ser727Asp mutant is independent of the Rac-1-mediated signal transduction cascade, indicating that the role of this signal transduction pathway is to provide STAT3 with a negative charge at position 727.

The 65 C-terminal amino acids of STAT3 can serve as an independent TAD, since fusion of this domain to the DNA binding region of GAL4 strongly enhanced reporter activation, particularly when a negative charge was introduced at position 727. The strong transcriptional activity of GAL4-STAT3 (705) Ser727Asp is coupled to a strong and constitutive association with the co-activator p300, suggesting that p300 might serve as a bridging factor between STAT3 and the basal transcription machinery. These data also indicate that dimerization of STAT3 is not required for its association with p300. p300 associated to a lesser extend with GAL4-STAT3 (599) Ser727Asp, while no p300 association was detected with the TAD domain regions of STAT3α which correlated well with their transcriptional activities, respectively. Surprisingly, the longest C-terminal region of STAT3 (502–770) did not induce reporter activation nor associated with p300, even when Ser727 was mutated into aspartate. Possibly, steric effects account for these observations or alternatively, it is conceivable that the linker region of STAT3 contains motifs that negatively regulate STAT3 transactivation. Further experiments are required to resolve these issues. Furthermore, it is intriguing that the transcriptional activity of the C-terminal TAD of STAT3α is not further enhanced upon IL-6 stimulation. These data suggest that a STAT3 monomer is not phosphorylated on Ser727, but that IL-6-induced Ser727 phosphorylation requires a STAT3 dimer. Similar mechanisms have been described for the transcription factor AP-1,

which is comprised of homo- or heterodimers of c-Fos, c-Jun or other Jun family members, for which dimerization is a prerequisite for serine phosphorylation by JNK-1 [30]. Whether similar mechanisms also apply for STAT3 activation requires further experiments, but comparable observations published by Zhang et al. have demonstrated that the transactivation of GAL4-STAT1 monomers is not further enhanced upon IFN-γ stimulation [14].

In vivo, p300 associates with STAT3 upon IL-6 stimulation, suggesting that both nuclear translocation and serine phosphorylation are required to enable STAT3–p300 complex formation. Since overexpression of p300 enhances both STAT3α as well as STAT3 Ser727Asp transcriptional activities, we conclude that the Ser727 phosphorylation in the C-terminus is required for the p300 driven transcriptional activity of STAT3. This is also further supported by the finding that the transactivation of STAT3β or STAT3 Ser727Ala is not enhanced by p300 overexpression. Similar mechanisms have been described for c-Jun and p53, where phosphorylation of serine residues is a prerequisite for association with p300 and high transcriptional activities [31,32]. Unexpectedly, high concentrations of p300 reduced STAT3 transactivation. Possibly, squelching effects account for these observations.

An important issue concerns the mechanism by which p300 enhances STAT3 transactivation. Since p300 contains a HAT domain which is required for the cooperative transcriptional activity with c-Jun and p53 [31,32], it is conceivable that HAT activity is also required for its cooperativity with STAT3. However, we can exclude the possibility that p300 directly acetylates STAT3, since no acetylation of STAT3 was observed in response to IL-6 using a pan-Acetyl antibody in Western blotting experiments (J.J. Schuringa et al., unpublished observations). Alternatively, it is plausible that p300 enhances STAT3 transactivation by functioning as a bridging factor coupling DNA-bound, Ser727 phosphorylated STAT3 to the basic transcription machinery via association of p300 with TBP (TATA binding protein) [33–35]. Taken together, our data provide a mechanism via which the C-terminal TAD of STAT3 enables high levels of gene transcription by association with p300.

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