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CONVERGENCE OF PKC AND JAK-STAT SIGNALING ON TRANSCRIPTION FACTOR GATA-4

Running title: GATA-STAT interaction

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ABSTRACT

Angiotensin II (AII), a potent vasoactive hormone, acts on numerous organs via G-protein-coupled receptors and elicits cell-specific responses. At the level of the heart, AII stimulation alters gene transcription and leads to cardiomyocyte hypertrophy. Numerous intracellular signaling pathways are activated in this process; however, which of these directly link receptor activation to transcriptional regulation remains undefined. We used the ANF gene (NPPA) as marker to elucidate the signaling cascades involved in AII transcriptional responses. We show that ANF transcription is activated directly by the AII type 1 receptor and precedes the development of myocyte hypertrophy. This response maps to a STAT and GATA binding sites and the two elements transcriptionally cooperate to mediate signaling through JAK-STAT and PKC-GATA-4 pathways. PKC phosphorylation enhances GATA-4 DNA binding activity and STAT-1 functionally and physically interacts with GATA-4 to synergistically activate AII and other growth factor-inducible promoters. Moreover, GATA factors are able to recruit STAT proteins to target promoters via GATA binding sites which are sufficient to support synergy. Thus, STAT proteins can act as growth factor inducible coactivators of tissue-specific transcription factors. Interactions between STAT and GATA proteins may provide a general paradigm to understand cell specificity of cytokines and growth factors signaling.

INTRODUCTION

Hormones and growth factors acting through cell surface receptors activate multiple signaling cascades leading to diverse biological responses that depend largely on the cellular context. Considerable understanding has been achieved regarding the mechanisms that couple receptor activation to cytoplasmic effectors. However, the mechanisms by which specific outcomes are generated from common signaling molecules remain incompletely understood. The discovery of complex interconnections between different signaling pathways, combined with the observation that similar cytoplasmic events are associated with, or relay distinct biological effects, has lead to the suggestion that specificity may be achieved at the level of target genes (4,69).

G-protein-coupled receptors (GPCR) constitute the largest family of transmembrane receptors in mammals (77). The AII type 1 receptor (AT1R), which transduces the biologic effects of AII, is one of the most extensively studied GPCR (18) and drugs that target AT1R are widely used for the treatment of cardiovascular diseases like hypertension and cardiac hypertrophy (17). AT1Rs activate a plethora of signaling cascades including MAPK, P13K, PKC, JAK-STAT and calcineurin resulting in apoptosis, proliferation, hypertrophy or differentiation depending on the cell type and developmental stage (35). At the level of the nucleus, AT1R activation has been shown to alter expression of some ubiquitous as well as tissue-specific transcription factors. They include: the immediate early genes, c-fos, c-jun and egr1 [reviewed in (8)], and in smooth muscle and adrenal cells, tissue-restricted transcription factors like the homeobox factors MHOX and DAX-1 (27,52) and the zinc finger proteins KLF5 and SF-1 (52,65). AII also enhances nuclear accumulation of STAT family members [reviewed

in (9)], NFκB (59) and NFAT-3 (72). However, the exact role of these factors in mediating AII actions remains largely controversial.

At the level of the heart, AT1R activation causes myocyte hypertrophy and apoptosis (55), and is associated with upregulation of c-jun, c-fos and the cardiac-specific atrial natriuretic factor (ANF) gene which is induced during hypertrophy (55,74) AII also activates several STAT family members which have themselves been implicated in cardioprotection, apoptosis and hypertrophy through mechanisms and effectors that remain to be elucidated [reviewed in (9)]. Similarly, the various signaling pathways activated by AII have all been implicated in cardiac hypertrophy, apoptosis, or both (1,24,46). Their importance in mediating specific AII effects as well as their involvement in AII regulation of cardiac genes has not been firmly elucidated.

STAT (signal transducers and activators of transcription) proteins are evolutionary conserved transcription factors that reside in the cytoplasm until they are activated by tyrosine phosphorylation which leads to their dimerization and nuclear accumulations. Initially identified as the targets of interferon, the STAT family in mammals now comprises seven members that are activated by cytokine and growth factor signaling through receptor coupling to the Janus kinase (JAK) family of proteins [reviewed in (40)]. STATs can also be activated by receptor tyrosine kinases, like epidermal growth factor receptor (EGF-R) and by some members of the GPCR family such as the AT1 receptor (9). STAT proteins play important roles in developmental decisions as well as in stress response and host defense [(40). Although most STAT proteins are widely expressed, gene targeting in mice revealed non-redundant functions for the different family members. For example, lack of STAT1 or STAT2 leads to

impaired response to interferon and hypersensitivity to infections, while lack of STAT4 and STAT6 is associated with impaired T cell differentiation. Interestingly, while loss of STAT3 causes early embryonic lethality, tissue-specific inactivation of the STAT3 gene revealed distinct and at times opposing effects in cell survival, proliferation and acute phase response, depending on the targeted tissue or organ. Similarly, inactivation of each of the two highly homologous STAT5 genes revealed hematopoietic defects, growth retardation (for STAT5a) and mammary gland development (in STAT5a null).

At the level of the heart, targeted overexpression of constitutively active STAT3 was shown to enhance vascular endothelial growth factor (VEGF) expression and causes hypervascularization (53) which would enhance cardiac adaptation to stress. Others found that overexpression of STAT3 leads to cardiac hypertrophy but also protects against drug-induced cardiotoxicity (37). More recently, mice with cardiac-specific inactivation of STAT6 were generated and found to have impaired response to pressure overload (29). In human, STAT3 is constitutively active in several tumors (10) and mutation of STAT1 lead to impaired bacterial immunity (20). The mechanisms by which STAT proteins can transduce such wide spectrum of cellular responses and biologic effects remain to be determined.

The activity of STAT proteins can be modulated by protein-protein interaction and a growing number of STAT-interacting proteins is being reported (66). In addition to STAT-interacting kinases and phosphatases, several transcription factors and coactivators/corepressors were shown to associate with and modulate STAT activity. They include CBP/p300 (31) and histone deacetylases (82) as well as inducible regulators such as c-jun (85) and members of the nuclear receptor family (22,67,71).

Finally, a role for tissue-specific transcription factors in STAT transcriptional regulation is emerging. In T cells, STAT proteins have been shown to physically and functionally interact with members of the ETs family of transcription factors over composite STAT-Ets DNA elements required for cytokine regulation of target genes (60,76). In hepatocytes, STAT3 was shown to cooperate with HFN1 in mediating transcriptional response to IL-6 during liver regeneration (38).

GATA proteins are tissue-specific transcription factors that play crucial roles in organogenesis. In mammals, the six members are divided into two subfamilies based on sequence homology and tissue distribution. GATA-1, -2 and -3 play essential roles in hematopoietic cell survival, proliferation and differentiation whereas GATA-4, 5 and -6 are key regulators of endodermal and cardiovascular development (56).

In the present study, we show that STAT and GATA proteins cooperatively mediate AII responsiveness of the ANF (NPPA) promoter through direct physical interaction. Moreover, we found that GATA-4 is essential for transcriptional activation by AII which enhances GATA-4 binding to DNA through PKC-mediated phosphorylation. In turn, GATA-4 is able to recruit activated STAT to target promoters suggesting that STAT proteins can act as inducible coactivators of the tissue-specific GATA transcription factors. This unravels a novel GATA-dependent mechanism for STAT action which could account for cell specificity of cytokine and growth factor action. Interaction with GATA-4 and activation of ANF may also explain some of the cardioprotective effects of STAT proteins.

MATERIALS AND METHODS

Materials. The following reagents were purchased from Calbiochem, USA: SB 203580, PD 98059, LY 294002, U-73122, GF 109203X, AG490. Except for the anti-GATA-4 antibody (14), the antibodies used were purchased from Santa Cruz: Pol II (N20), STAT1 (E23), STAT3 (C20), STAT5 (C17). The anti-GATA-4, HA and Flag antibodies have been described previously (14).

Plasmids and adenovirus vectors. The luciferase reporter constructs used and most GATA expression vectors were described previously (3,49). The SV-sport1-HA-GATA-4 plasmids were prepared based on the original rat GATA-4 cDNA (25). Heterologous promoters were generated by multimerizing the relevant oligonucleotides flanked by BamHI and BglII sites upstream of the minimal (-57 bp) ANF or BNP luciferase reporter as described previously (48). Expression vectors for rat AT1aR and its mutants were gifts from Dr Sadashiva S. Karnik (45). Erk1/2, expression vectors were previously described (43). The VEGF-luciferase reporter was a kind gift of Dr. Darren Richard. It contains the upstream sequence from the human VEGF gene (44). STATs cDNA vectors expressing wild-type proteins from Svsport1 were provided by Dr Juergen A Ripperger. HA-tagged wild-type and mutant STAT1α vectors were generated using PCR-mediated amplification and cloning in the CMV-driven pCGN plasmid. All constructs were confirmed by sequencing. The Ad-LacZ has been described previously (3) and the AII type 1a receptor adenovirus vector (Ad-AT1aR) was a gift of Walter G. Thomas (74). The virus was amplified and quantified as previously described (14).

Cell cultures, adenovirus infection and transient transfections. Myoblast C2C12 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 20% fetal bovine serum (FBS). Cotransfections were carried out using calcium phosphate 24 h after plating. At 16 h post-transfections, the medium was changed to serum free and supplemented with 0.1 μM AII for overnight stimulation. Cells were harvested and luciferase activity assays performed with a Berthold LB953 luminometer. The amount of reporter construct was kept at 1 μg per 20 mm dish and the total amount of DNA at 3 μg. One μg rat AT1a expression vector was used unless otherwise stated. Primary cardiomyocyte cultures were prepared from 4-day-old Sprague-Dawley rats and co-transfected as previously described with minor modifications (14). In brief, cardiomyocytes were cotransfected and treated with or without 100 nM AII in serum free for 24 h. For adenovirus infections, cardiomyocytes were infected with 2 plaque forming units (PFU) of Ad-AT1aR or the control Ad-LacZ per cardiomyocyte as previously described (14) and treated with or without 100 nM AII in serum free for 20 min or 48 h. When required, pharmacologic inhibitors were added to the cells 30 min prior to AII stimulation. Unless otherwise indicated, the data shown for transfections are the mean of at least three independent experiments carried out in duplicate and with different DNA preparation.

Chromatin immunoprecipitation assays and QPCR analysis. Chromatin immunoprecipitation assays (ChIP) were carried out using a modification of a previously described protocol (23). Essentially, 16 million cardiomyocytes per ChIP were plated at 25 500 cells/cm^2 in DMEM supplemented with 10% FBS and 10 μg/mL cytosine β-D-arabino-furanoside hydrochloride (Sigma). On day two, the media was changed for a

serum free media (SF) supplemented with 50 mM KCl. Cardiomyocytes were infected with 2 plaque forming units (PFU) of Ad-AT1aR per cardiomyocyte. The SF media was changed on day three and the cells were treated with 100 nM AII for two days. Cardiomyocytes were washed with ice cold phosphate buffered saline (PBS) and cross-linked with 1% formaldehyde at 4ºC for 15 min. Cross-linking was stopped by incubating the cells in a solution of 125 mM glycine for 5 min at 4ºC. Cells were harvested in PBS, centrifuged then resuspended and lysed in a hypotonic buffer for 5 min in ice. The lysate was centrifuged and the pellet containing the nuclei was resuspended in a buffer containing 300 mM NaCl and incubated for 30 min at 4ºC to extract free nuclear proteins. The pellet containing the cross-linked-chromatin (Ch) was resuspended and sonicated to achieve fragments of about 600 bp. Fragmented chromatin was pre-cleared with protein A/G PLUS-agarose (Santa Cruz Biotechnology) with a 1-2 h incubation with agitation at 4ºC. A small aliquot was saved as the input and the remaining was subdivided in equal fractions for ChIP. Immunoprecipitated chromatin was washed several times for 5 min at room temperature. DNA was purified with QIAquick PCR purification kit (Qiagen). Samples and input were analysed by real time quantitative PCR (QPCR). DNA template and 1 µM oligonucleotides were used at an annealing temperature of 58ºC using the Quantitect SYBR green PCR kit (Qiagen) in a MX4000 (STRATAGENE, La Jolla CA, USA). The primers used were: 5'-AAAGCGGTTTCATCCTCCAGGC-3' and 5'-ACAGGCTCTAAAGAATTCAGCTACACG-3' for the distal ANF region and 5'-GCCTTTGTCCGTCACTGTCT-3' and 5'-GAGCGCCCAGGAAGATAACC-3' for the proximal ANF promoter A comparative quantification was used and the input was set as the calibrator. The results are

expressed as the fold enrichment of GATA or STAT ChIP over the values obtained with the control IgG.

Northern blot and QPCR. Total RNA was prepared using TRIZOL reagent (Invitrogen Canada Inc., Burlington ON, Canada). Northern blots were performed as previously described (55). QPCR was performed on cDNAs obtained by reverse transcription of 2 µg of total RNA using Omniscript reverse transcriptase (Qiagen Inc., Mississauga, Canada). Two ng of cDNA template and 1 µM oligonucleotides were used. The oligonucleotides for mouse ANF are TGCCGGTAGAAGATGAGGTC (forward) and AGCAGCTGGATCTTCGTAGG (reverse). The oligonucleotides for mouse ribosomal protein S16 are ATCTCAAAGGCCCTGGTAGC (forward) and ACAAAGGTAAACCCCGATCC (reverse).

Electrophoretic mobility shift assays. Preparation of nuclear extracts from cell culture was as described previously (13,49). Nuclear proteins were extracted from hearts of human AII type I receptor (hAT1R) transgenic and non transgenic mice essentially as described (19). Briefly, the hearts were excised from mice and washed with PBS to remove blood, then broken into pieces in liquid nitrogen. Afterwards, the heart pieces were homogenized in solution A [0.6% NP40, 150 mM NaCl, 10 mM HEPES pH 7.9, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] at 4°C and followed by centrifugation for 30s at 2000 rpm. The supernatant was then saved and centrifuged for 5 min at 5000 rpm. The pellets obtained were suspended in solution B [25% glycerol, 20 mM HEPES pH 7.9, 420 mM NaCl, 1.2 mM MgCl2, 0.2 mM EDTA, 05 mM dithiothreitol (DTT), 0.5 mM PMSF, 2 mM benzamidine, 5 µg/ml aprotinin] at 4°C for 20 min and centrifuged 15s at full speed in a microcentrifuge. Extracts were then

aliquoted and stored at -80°C for future use. Protein concentration was determined using Biorad assays. Binding assays were performed at room temperature for 30 min in the presence of 500 ng of poly(dI-dC) in 120 mM KCl, 25 mM MgCl2, 20 mM Tris-Cl pH 7.9, 2 mM DTT, 2 mM EDTA, 8% Ficoll (for GATA-4 and SRF binding assays) or 5 mM HEPES pH 7.9, 100 mM NaCl, 0.25 mM EDTA, 0.5 mM DTT, and 5% glycerol (for STAT binding assay).

Western blots and co-immunoprecipitations. Western blots were performed using whole cell or nuclear extracts according to standard protocols. Polyclonal antibodies were used at a 1:1000 dilution and incubated with the membrane for 1 h at room temperature and then with peroxidase conjugated antibody for another 1 h at room temperature. The bands were revealed using ECL Plus standard protocol (Amersham Pharmacia Biotechnology). Co-immunoprecipitation of STAT1 and HA-GATA-4 or Flag-GATA-4 and HA-STAT1 were carried out using nuclear extracts of 293T cells overexpressing the relevant proteins, as described previously (49).

Kinase assays. The recombinant proteins GST-GATA-4 1-207, GST-GATA-4 329-440 and GST-GATA-4 329-440 with 419-420, SS->AA mutation were produced as previously described (15). Five µg of bacterially expressed protein was incubated with 20 ng of the purified catalytic subunit of protein kinase C (Calbiochem) in the reaction buffer [20 mM Tris (pH7.5), 12.5 mM $MgCl_2$, 0.5 mM EGTA, 50 µM ATP] at 30°C for 1 h. Thereafter, the proteins were resolved on 10% SDS-PAGE and exposed to X-Ray films.

RESULTS

The ANF promoter harbors multiple AII response elements. AII-induced cardiac hypertrophy results in upregulation of the ANF gene (55). However, since activation of ANF transcription is a hallmark for the genetic changes that accompany cardiac hypertrophy irrespective of etiology, we first tested whether ANF is a direct target for AII. Previously, we generated transgenic mice (TG) with myocardial specific expression of the AII type 1 receptor (AT1R); these mice develop progressive cardiac hypertrophy and remodeling (55). We analyzed the levels of ANF transcripts in the ventricles of young TG mice (50-60 days old) at a time when cardiac function was intact, and when there was no microscopic evidence of cardiomyocyte enlargement or apoptosis. We found that overexpression of AT1R was sufficient to increase ANF transcript levels by 3-fold (Fig. 1A). This increase could be recapitulated in primary cardiomyocyte cultures at the level of endogenous ANF mRNAs as well as transfected ANF promoter (Fig. 1B). Moreover, coexpression of the ANF-luc reporter with expression vectors encoding the rat AT1aR in some non-cardiac cells was sufficient to induce promoter activity following treatment with AII (Fig. 1C, and data not shown) indicating that AII-mediated transcriptional activation of ANF is not secondary to cardiomyocyte hypertrophy. The specificity of the response was confirmed by the use of the AT1R antagonist Losartan, which blocked ANF promoter activation (Fig 1B); additionnally, cotransfection of an expression vector encoding the AII type 2 receptor (AT2) or a dominant negative AT1 receptor mutant (N74D) failed to activate the ANF promoter in response to AII (Fig. 1C). It should also be noted that expression of AT1R alone or treatment with AII was not sufficient to elicit significant transcriptional effects

and promoter activation required addition of both receptor and ligand. Interestingly, although liganded AT1R could enhance ANF promoter activity in different cell lines, maximal response was achieved in muscle cells suggesting the involvement of local factors (data not shown). These results indicate that AT1R activation targets ANF transcription directly and that the ANF promoter harbors AII response element(s).

Next, we used mutational analysis to map the response elements. The ANF promoter contains numerous regulatory elements that confer tissue specificity and/or hormonal response (13,14,21,43,49). They include the composite GATA/SRE site that was shown to be targeted by endothelin 1 (49), *a bona fide* AP1 site (43) that was reported to be essential for *in vivo* response to pressure overload (79), a putative binding site for STAT proteins located around -675 bp and an A/T-rich region that was shown to bind MEF2 proteins (48). Deletion scanning revealed that two regions are required for maximal AII response; the -695 to -500 bp distal region whose deletion reduced AII response by 2-fold and the proximal region (-50 to -135 bp) whose deletion in the context of the -695 bp promoter resulted in the loss of 75% of the response (Fig. 2A and data not shown). Finer mutagenesis was then used; within the distal region, mutation of the AP1 site as well as the distal MEF-like element had no effect on promoter activation by the liganded-AT1R. In contrast, mutation or deletion of the putative STAT element resulted in a 50% decrease in AT1R response. Mutation of two high affinity GATA-4 binding sites also reduced promoter activation by 50% (Fig. 2A). Within the proximal promoter, mutation of either the GATA or the low affinity SRE elements also reduced hormone response. Together, these results show that, at the level of the ANF promoter, maximal AT1R responsiveness requires the STAT element

as well as the GATA sites. Moreover, cooperative interaction between these elements was suggested with the finding that an artificial promoter containing the STAT element upstream of the composite GATA/SRE site mediated a synergistic response to AT1R activation (Fig. 2B). The relative importance of the GATA and STAT elements in ANF promoter activation in response to AII was confirmed in neonatal cardiomyocytes. Consistent with the results obtained in C2C12 cells, deletion of the STAT element resulted in reduced AII responsiveness, but mutation of the GATA elements completely abrogated AII-dependent transcriptional activation (Fig. 2C). Thus, in cardiomyocytes GATA binding sites appear essential while the putative STAT element contributes quantitatively to AII response.

GATA binding over the ANF GATA site reflects GATA-4 interaction (14). To determine the nature of the binding over the putative STAT element, we carried out electrophoretic mobility shift assays using extracts prepared from mouse hearts and myoblast cultures. The sequence of all probes used is shown in Fig. 3D. First, we tested the ability of the putative ANF STAT element to effectively compete binding over a consensus STAT site; these experiments indicated that this region of the ANF promoter is able to displace binding over the well characterized STAT element of the LY-6E promoter, albeit with lower efficiency than the cold self probe (Fig. 3A). The ANF STAT element also bound transfected STAT1α (Fig 3B, left panel) as well as endogenous STAT proteins present in nuclear extracts from mice hearts (Fig 3B, middle panel); binding over the ANF probe was efficiently displaced by several well characterized STAT elements (Fig. 3B, middle panel). Interestingly, STAT binding was increased in TG mice extracts, suggesting that activation of STAT proteins may be an

early event in AT1R action in the heart (Fig. 3B, right panel). This was directly confirmed using Western blot analysis which revealed increased nuclear accumulation of STAT1 and 3 but no detectable change in the level of GATA-4(Fig. 3C).

Nuclear action of AII involves JAK-STAT and PKC-GATA-4 pathways. AT1R may activate STAT proteins via JAK2-dependent or independent pathways (16,63). On the other hand, contribution of the GATA element in AII response may be the result of activation of the MAPK cascade which could lead to phosphorylation and potentiation of GATA-4 activity as previously reported (15,41). To test the involvement of the various signaling cascades in AII regulation of transcription, we first checked the effect of various pharmacologic inhibitors on ANF promoter response to AII. As shown in Figure 4A, inhibition of JAK kinases with AG490, resulted in a 50% loss of promoter activation. Inhibition of p38 MAPK (with SB203580) or ERK (PD98059) did not block the response; in fact, ERK inhibition resulted in a modest but reproducible enhancement of AII responsiveness. Similarly, inhibition of the PI3 kinase pathway (LY294002) consistently resulted in 50-60% increase in AII-dependent promoter activation. In contrast, inhibition of phospholipase C (U-73122) or protein kinase C (GF109203T) virtually abolished ANF promoter responsiveness to AII. Thus, it appeared that two major signaling pathways, JAK-STAT and PKC were involved in linking AT1R to transcriptional regulation. We further extended these results with the use of a mutant AT1 receptor in which the YIPP motif (AA 319-322) required for JAK interaction was mutated (2). Although this mutant receptor (IIGG) could still mediate an AII transcriptional response, the maximal activation achieved was consistently 30-40% lower than wild-type receptor when the reporter used was driven by the intact -695ANF promoter; however, when the reporter

used contained a mutation in the STAT binding site, the mutant receptor ability to activate transcription was similar to that of the wild-type receptor (Fig. 4B). To ascertain the effect of the mutant AT1 receptor on STAT activation, we analyzed STAT1 levels in whole cell extracts from AII treated C2C12 cells transiently transfected with the intact or mutated receptor. STAT1 (p91 and p84) are the predominant STAT proteins in these cells. As shown in the inset of Fig. 4B, the level of endogenous pSTAT1, was lower in extracts prepared from cells transfected with the mutant receptor. Together, these data are consistent with an involvement of JAK2 in linking AT1R to STAT activation and transcriptional effects.

We also used genetic approaches to confirm the involvement of PKC in AII transcriptional effects. Coexpression of the catalytic subunit of PKCβ resulted in 2.5 enhancement of AII response whereas expression of constitutively active ERK1/2 had no effect on the level of ANF activation in response to AII (Fig. 4C). Finally, we tested which regulatory element(s) are targeted by PKC. As shown in Figure 4D, a minimal promoter driven by the ANF STAT element was activated 4-fold in response to AII and this response was not altered in presence of the PKC inhibitor; in contrast, the ability of the proximal promoter region, and more specifically the GATA element therein, to respond to AII was abrogated by pharmacologic inhibition of PKC. Together, these results suggest that two pathways JAK-STAT and PKC-GATA-4 mediate AT1R transcriptional response.

We further examined the effect of PKC on GATA-4. Bioinformatics assisted analysis of the GATA-4 protein revealed a putative PKC phosphorylation site within its C-terminal activation domain (AA 417-423); this motif is conserved across all species

(Fig. 5A). To test whether this or other domains of GATA-4 are phosphorylatable by PKC, we performed *in vitro* phosphorylation analysis on bacterially-expressed GST fusion proteins containing either the N- or C-terminal transactivation domains of GATA-4. As shown in Figure 5B, the recombinant protein containing the C-terminal region of GATA-4 (AA 329-443) was efficiently phosphorylated by the purified catalytic subunit of PKC. Mutation of the serine residues within the PKC recognition motif (S419,420A) dramatically decreased PKC phosphorylation suggesting that this site is the major PKC phosphorylation target on GATA-4. Next, the functional consequences of PKC phosphorylation on GATA-4 were assessed. Pharmacologic inhibition of PKC significantly decreased GATA-4 activation of the ANF promoter (Fig. 5C, right panel) and S419,420A mutation in GATA-4 reduced its transcriptional activity by over 50% (Fig. 5C, left panel). To elucidate the mechanisms by which PKC activates GATA-4, we analyzed the effect of the S419,420A mutation on nuclear protein accumulation and DNA binding properties. As shown in Figure 5D, although the S419,420A mutation was consistently expressed at slightly higher level (top panel), the ability of this mutant to interact with the ANF GATA binding site (bottom panel) was markedly decreased (2.5-3x, without correcting for protein expression). This result suggested that AII stimulation enhances GATA-4 activity, in part by increasing its DNA-binding capacity. This hypothesis was directly tested by analyzing the level and DNA-binding activity of endogenous GATA-4 in AII-stimulated cardiomyocytes. Treatment of neonate cardiomyocyte cultures with AII did not affect the level of nuclear GATA-4 protein (Fig. 5E, lower panel), a finding consistent with that obtained in extracts from the hearts of AT1R transgenics (Fig. 3C). Nevertheless, gel shift analysis revealed a 2.7 fold

increase in GATA binding activity that was confirmed to correspond to GATA-4 with antibody supershift assay (Fig. 5E). That nuclear signaling by AII involved GATA-4 was also confirmed by the ability of two dominant negative GATA-4 proteins to block AII response in cardiomyocytes while addition of the intact GATA-4 protein could substitute for AII stimulation (Fig. 5F). Together, the above results reveal that GATA-4 is an essential mediator of nuclear AII action and that AII activates GATA-4, in part, through PKC-mediated phosphorylation and enhancement of its DNA binding activity.

Synergistic activation of transcription by GATA-4 and STAT1. The data presented in Fig. 2B and Fig 4 above also suggested that transcriptional regulation of ANF by AII may involve cooperative interaction between STAT proteins and GATA factors. We tested this hypothesis using cotransfections and immunoprecipitation assays. First, we analyzed the effects of different STAT proteins on ANF promoter activity. STAT1α but not STAT 3 or STAT5b consistently activated the ANF promoter in cardiac and non-cardiac cells, and this effect was further potentiated in presence of AII (Fig. 6A). Next, we tested whether STAT1α and GATA-4 cooperate in transcriptional activation. As shown in Figure 6B, STAT1 α and GATA-4 synergistically activated the ANF promoter. Addition of STAT1α consistently enhanced GATA-4 transcriptional activation of this promoter by 5- to 6-fold ($n > 20$). In contrast, STAT3, at varying amounts, had no effect on GATA-4 activity. Interestingly, although STAT5b by itself did not activate the ANF promoter, it was able to cooperate with GATA-4 in transcriptional activation, though to a lower extent compared to STAT1α (Fig. 6B).

To better understand the mechanisms involved in STAT/GATA synergy, we carried out structure-function analysis of GATA-4 and STAT1α. The GATA-4 protein

contains two transcriptional activation domains flanking its two zinc finger DNA-binding domain. As shown in Figure 6C, removal of the first 129 AA, which decrease GATA-4 transcriptional activity reduced but did not abrogate synergy; deletion of the C-terminal activation domain significantly reduced synergy, indicating that intact GATA-4 transcriptional activity is required for functional interaction with STAT1. Consistent with this, the DNA binding domain (AA200-332) was unable to support synergy. Mutations in the second zinc finger – which abolish DNA binding – also interfered with STAT1 synergy (Fig. 6C) suggesting that GATA-4 binding to its site is necessary for functional interaction with STAT over the ANF promoter. Next, we tested whether the transactivation domains of STAT1 were required for synergy with GATA-4. Various STAT1 functional domains have been identified (12) and are schematically depicted in Figure 6D. Removal of the entire C-terminal transactivation domain significantly reduced but did not abolish synergy; however, removal of the N-terminal region which harbors a domain required for STAT dimer-dimer formation as well as a coiled-coiled domain involved in CBP/p300 interaction abrogated synergy (Fig. 6D). From this analysis, we conclude that the C-terminal activation domain of STAT1 is required for maximal synergy but that the N-terminal region is essential for functional interaction with GATA-4. The finding that the activation domains of both GATA-4 and STAT1 are needed for maximal synergy and, given that both proteins interact with the CBP/p300 coactivators (6,31) lead us to investigate whether GATA-4/STAT1 cooperativity involves co-recruitment of CBP. As shown in Figure 6E, addition of increasing amount of CBP greatly potentiated the synergistic action of GATA-4 and STAT1 on the ANF promoter.

Next, we determined which promoter elements were required for GATA-4 and STAT1 cooperativity. As shown in Figure 7A, deletion of sequences between -695 and -135 bp decreased maximal activation levels but did not impair GATA-4/STAT1 synergy suggesting that STAT1 binding to DNA was dispensable. Consistent with this, mutation of the STAT element in the context of the full length promoter reduced but did not abolish synergy. In contrast, mutation of the GATA sites abrogated both GATA responsiveness and GATA/STAT synergy. A minimal promoter driven by multimerized GATA binding sites was cooperatively activated by GATA-4 and STAT1. Together, these results indicate that GATA elements are necessary and sufficient for GATA/STAT synergy and suggest that GATA factors may be able to recruit STAT proteins to target promoters. To test this, we performed co-immunoprecipitation assays which revealed that GATA-4 was indeed able to physically interact with STAT1 *in vivo* (Fig. 7B). Interestingly, removal of the N-terminal but not the C-terminal domain of STAT1 abolished physical association with GATA-4 (Fig. 7C), a finding that would explain the inability of N-terminal deleted STAT1 (330-713) to synergize with GATA-4 (Fig. 6D). Finally, to confirm the relevance of a GATA-dependent pathway for STAT1 action, ANF-luc constructs containing the -695 wild-type or GATA element mutated ANF promoter, were tested for their ability to respond to STAT1 in cardiomyocytes. As shown in Figure 7A, whereas the wild-type promoter was dose-dependently activated by co-transfected STAT1, mutation of the GATA sites abrogated STAT1 activation. These results – which are consistent with those of Figure 2C - indicate that, *in vivo*, interaction with GATA factors bound to the promoter is required for STAT1 activation of the ANF promoter.

Next, we probed the *in vivo* association of GATA-4 and STAT proteins with the endogenous ANF gene in cardiomyocytes. For this, we performed chromatin immunoprecipitation assays using AT1aR activated neonate cardiomyocytes in primary cultures. As shown in Figure 8, we found specific enrichment of GATA-4 and STAT1/3 over both the distal and proximal domains of the ANF promoter. These results indicate that the ANF gene is a direct transcriptional target for GATA-4 and STAT1/3.

Finally, we tested the effects of GATA-4 and STAT1 on other growth factor inducible promoters. For this, we used the c-fos, Bcl-x_L and VEGF promoters. The c-fos and Bcl-x_L promoters are inducible by various cytokines and/or growth factors that act through the JAK-STAT pathway and they contain well characterized STAT binding sites (68,84). These promoters also contain GATA binding sites and are activated by GATA transcription factors (3,49). The VEGF promoter was recently shown to be activated by STAT3 (51,80), and our own results indicate that it is also a GATA target. As shown in Figure 9A, all three promoters were synergistically activated by GATA-4 and STAT1. Given that, in the case of c-fos and ANF, we previously showed that GATA-4 cooperates with SRF (49) and since mutation of the proximal ANF SRE also affects cooperative interaction between the STAT element and the proximal promoter (Fig. 2B), we tested for possible functional cooperation between STAT, GATA-4 and SRF. We were unable to detect any cooperativity between STAT proteins and SRF on the ANF or c-fos promoters using several DNA concentrations of STAT and SRF expression vectors (data not shown). However, when GATA-4, STAT1 and SRF were simultaneously added, super activation of both ANF (Fig. 9B) and c-fos (Fig. 9C) driven reporters was observed suggesting that GATA-4 is able to recruit STAT1 to a

GATA/SRF ternary complex and that STAT1 may act as an inducible coactivator of this transcriptional complex.

DISCUSSION

Extracellular stimuli-induced regulation of cell fate requires the execution of a complex program of transcriptional events. How specificity of the transcriptional response is achieved remains largely undefined although it is now widely accepted that combinatorial interactions of signaling pathways and transcription factors play a major role in this process [reviewed in (4)]. In the present work, we examined the mechanisms that transduce transcriptional response to a biologically important peptide hormone acting through a G protein-coupled receptor. Our results indicate that nuclear integration of AII signaling involves cooperative interaction between the signal regulated transcription factors STAT and the tissue-specific family of GATA proteins whose activity is also directly modulated by various intracellular signaling cascades. These data provide new insight into the mechanisms underlying signaling specificity of the AT1R. Moreover, the finding of combinatorial interaction between STAT and GATA proteins have broad implication for understanding cytokine and growth factor signaling in health and disease.

Angiotensin II (AII) regulation of ANF transcription. AII is a major cardioregulatory hormone and drugs that inhibit its biosynthesis or its receptor are widely used for the treatment of human cardiovascular conditions. Surprisingly, the mechanisms by which AII regulates transcription in the heart remain undefined. Within the heart, AII targets both myocytes and nonmyocytes (64,74). Except for the c-fos

gene (61), few direct transcription targets of AII in cardiomyocytes have been identified. The data presented show that ANF is a direct transcription target of AII and that upregulation of ANF does not require AII-induced hypertrophy. Thus, studies of AII regulation of the ANF promoter offered the opportunity to identify AII-mediated transcriptional mechanisms without the confounding input of hypertrophy-induced pathways. The results show that AII, via its type 1 receptor (AT1R), activates the ANF promoter through two major signaling cascades JAK-STAT and PKC-GATA-4. Interestingly, MAPK signaling did not appear to play a role in relaying AII signaling at the level of the ANF promoter. This may reflect a more specific role for the MAPK pathway in the growth (hypertrophic) effects of AII that are dependent on the EGF receptor (74). In contrast, ANF is a cardioprotective hormone that antagonizes cardiac hypertrophy (30). ANF is also a well-known antagonist of AII (39). Thus, ANF activation by AII may be part of a negative feedback loop that serves to attenuate AII action. In this case, the signaling pathways linking AT1R to ANF transcription i.e. PKC and JAK-STAT, would be predicted to be cardioprotective. Several studies support this conclusion. First, STAT proteins have been reported to transduce cardioprotective signals (29,37,83). Although the mediators of this beneficial effect are not defined. Interaction of STAT with GATA-4, a cardioprotective transcription factor (3), and activation of ANF and/or VEGF, may explain at least part of STAT cardioprotective effects. Second, although numerous hypertrophic signals were shown to activate different PKC isoforms, the bulk of the evidence argues against an involvement of PKC in initiating and/or maintaining hypertrophy (11,28). In fact, upregulation of PKCβ in the adult heart has been shown to have beneficial effects (75). Importantly, pharmacologic

inhibition of PKC did not block AII-induced hypertrophy (74) but did abrogate AII activation of c-fos transcription (62) and ANF biosynthesis (34). In the future, it will be interesting to map the specific domains of the AT1R that are required for ANF transcription and determine whether they can be dissociated from those involved in mediating AII hypertrophic response. Such studies could ultimately lead to the development of novel therapeutic drugs that selectively target specific AII-activated signaling pathways.

GATA-4, an integrator of cell signaling in the heart. Cooperative interactions between cell-specific transcription factors and signal activated regulators represent one of the most effective means to achieve cell and target gene specificity by signaling pathways. The experiments presented in this paper reveal that GATA-4 plays an essential role for ANF promoter activation in response to AII. In this context, GATA-4 acts as an integrator of two signaling pathways: PKC, which targets its C-terminal domain resulting in enhanced DNA binding activity, and the JAK-STAT pathway which potentiates its activity through protein:protein interaction with STATs. GATA-4, a member of the GATA family of tissue-specific zinc finger proteins, is a critical regulator of cardiogenesis (26,58). In postnatal cardiomyocytes, GATA-4 is essential for maintaining the cardiac genetic program (14) and for the adaptive response of the heart to numerous stimuli including hormones and work overload (15,42,57). GATA-4 actions involve combinatorial interactions with other cell-restricted or inducible transcription factors including Nkx2.5, Mef2, SRF, NFAT, SMAD and c-fos [reviewed in (73)]. GATA-4 activity is also directly modulated by signaling cascades; ERK and p38 MAPK phosphorylate and enhance GATA-4 transcriptional activation domains (15,41) while

glycogen synthase kinase (GSK3β) phosphorylates the GATA-4 DNA binding domain and inhibits nuclear GATA-4 accumulation (50). Through its cooperative interaction with other transcriptional regulators, GATA-4 serves as a nuclear integrator of several signaling pathways, most notably, calcineurin, MAPK, PI3 kinase and receptor serine-threonine kinases. Our findings that PKC and JAK-STAT also converge on GATA-4 expand the role of GATA-4 as transcriptional mediator of epigenetic signals and will be important in understanding the mechanisms of action of numerous other cardioregulators. For example, opioid receptor agonists were found to enhance ES cell differentiation into cardiomyocyte via PKC-dependent activation of GATA-4 (78). On the other hand, some cytokines acting through a gp130-coupled receptor have been shown to require GATA elements for transcriptional regulation of target promoters (47).

Our findings that GATA binding sites were sufficient to support GATA/STAT synergy and that GATA-4 was able to physically associate with STAT1 in living cells is especially noteworthy as they raise the possibility that STAT proteins can activate target promoters via GATA binding sites. Synergistic action of the GATA/STAT complex does not appear to be due to an effect of STAT on GATA-4 binding to its site (data not shown). The structure-function analysis indicates that at least one of the transcriptional activation domains of GATA-4 is required; in the case of STAT1, the presence of the N-terminal domain appears to contact GATA-4 and the C-terminal activation domain which is known to interact with CBP/p300 (31), is required for maximal synergy (Fig. 6). GATA-4 as well as several other GATA factors also associate with CBP/p300 through the DNA-binding domain leading to enhanced transcriptional activity (6,7,33). Together,

with ability of CBP to further enhance synergy, these data suggest that the GATA/STAT functional likely involves co-recruitment of CBP/p300.

GATA/STAT interaction and cell specificity of cytokine action. The involvement of GATA and STAT proteins in cytokine signaling is well documented in the hematopoietic system. For example, both STAT5 and GATA-1 are essential for the survival effects of erythropoietin on erythroid progenitors (68,81) and dominant negative GATA proteins block IL13 action; these proteins also mediate the effects of thrombopoietic cytokines in erythroid and megakaryotic differentiation (32,36). Similarly, in the lymphoid system, both GATA-3 and STAT5/6 mediate cytokine-induced development of CD4+ cells and the differentiation of T helper type 2 (TH2) cells (54). Interestingly, cooperation between GATA and STAT has been suggested by the finding that the TH2 locus control region require both GATA-3 and STAT6 for generating/maintaining an open chromatin configuration (70). More recently, GATA-1 was found to mediate interferon 8 induction of the human major histocompatibility complex class 1b gene; in this case, GATA-1 binds to a weak site adjacent to the interferon response element and the presence of both sites results in superactivation (5). Thus, the GATA/STAT synergy described in this work is likely to represent a general mechanism that could account for cell specific effects of cytokine/growth factors acting through tyrosine kinase coupled receptors. This may be particularly relevant to understanding cytokine action in hematopoietic cells. In this respect, we demonstrated that, at least, two targets of GATA and STAT action in hematopoietic cells, namely the Bcl-x and c-fos promoters, are synergistically activated by GATA and STAT proteins

(Fig. 9). Whether this paradigm extends to other target genes in hematopoietic and other cell types deserves to be tested.

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FIGURE LEGENDS

Figure 1. ANF is induced by AII in cardiomyocytes. A) ANF transcript levels were quantified in the ventricles of AT1R transgenic mice (TG) and non-transgenic (Wt) littermates by QPCR. The results shown represent the mean ± SEM of 17 Wt and 8 TG mouse ventricles. **B)** and **C)** AII increases the activity of the ANF promoter in cardiomyocytes and in C2C12 cells in an AT1R dependent fashion. **B)** The -695ANF promoter-driven luciferase reporter construct was transfected into cardiomyocytes (Left) that were treated for 12 h with 100 nM AII in presence or absence of 100 nM Losartan (Los), an AII antagonist. The right panel is a Northern blot showing changes in endogenous ANF mRNA levels in ventricular cardiomyocyte cultures in response to 24 h treatment with 100 nM AII. **C)** The -695ANF promoter luciferase construct was cotransfected with a rat AT1a, a dominant negative AT1a mutant (N74D) or an AT2 receptor expression vector in C2C12 cells. The cells were treated or not with 100 nM AII plus or minus 100 nM Los for 12 h. The results shown in **B** and **C** are the mean ± SEM of at least six independent determinations and represent the ratio of promoter activity + AII over promoter activity + vehicle.

Figure 2. Mapping AII response elements on the ANF promoter. A) and **B)** C2C12 cells were cotransfected with various ANF promoter constructs and the AT1aR expression vector and then treated with or without 100 nM AII for 12 h. In **A)** reporter constructs were driven by the wild-type -695 or -135 ANF promoter and deletion or point mutants thereof. In **B)** C2C12 cells were cotransfected with reporter constructs driven by the minimal -57 or -135ANF promoters plus or minus the distal STAT element and point mutants thereof. Note how the distal STAT containing domain cooperates with the

proximal GATA/SRE. The data shown represent the mean ± SEM of at least six independent determinations. **C)** ANF promoter elements required for AII response in cardiomyocytes. Cells were treated with 100 nM AII for 24 h. Wt = -695ANF-luc, mut GATA = -695ANF-luc with mutated GATA sites, Del STAT = -640ANF-luc. The data are from one representative experiment carried out in duplicate (out of 3).

Figure 3. **A)** The distal ANF promoter harbours a low affinity STAT binding site. Electrophoretic mobility shift assays (EMSA) were performed using nuclear extracts of C2C12 cells cotransfected with STAT1α and AT1aR expression vectors and treated with AII. Binding to the ^{32}P-labelled Ly-6E STAT element was competed with 25X of cold probe (self) or with 25X and 50X unlabelled ANF oligonucleotide containing the putative STAT site (ANF). **B)** EMSAs were performed using the STAT containing ANF probe (ANF STAT) and nuclear extracts prepared from STAT1 expressing C2C12 cells (left panel) or from pooled hearts of wild-type (Wt) or AT1R transgenic (TG) mice (middle and right panels). Specific binding was blocked by an anti-STAT1 antibody (left panel). Binding on the ANF STAT probe was competed with 25X excess of cold probe (self) or with 25X and 50X labelled probes containing STAT binding sites from the indicated promoters (middle panel). The right panel shows increased STAT binding in AT1R transgenic hearts (TG) compared to wild-type littermates (Wt). Note unchanged OCT-1 binding in the same extracts. **C)** Western blots were carried out on whole cell extracts prepared from the hearts of wild-type (Wt) and AT1R transgenic mice (TG) using the indicated antibodies. In each case, the first lane is a positive control containing extracts from cells overexpressing the relevant transcription factor. **D)**

Sequences of the various STAT elements used. The core sequences are indicated in bold.

Figure 4. Involvement of JAK, PKC and PLC but not PI3K or MAPK in AT1a-dependent ANF transcriptional activation. A) C2C12 cells were cotransfected with the -695ANF-luc construct and the AT1aR expression vector and treated or not with AII in the presence of inhibitors for various pathways: p38 MAPK (SB 203580, 10 μM), ERK (PD 98059, 50 μM), PI3K (LY 294002, 50 μM), phospholipase C (U-73122, 5 μM), PKC (GF 109203X, 5 μM) and JAK2 tyrosine kinase (AG490, 25 μM). Inhibitors were applied 30 min before the AII treatment. **B)** Maximal AII-induced ANF transactivation requires JAK binding to AT1aR. C2C12 cells were cotransfected with wild-type AT1aR (Wt) or YIPP motif mutant (IIGG) and -695ANF-luc constructs containing the wild-type (Wt) or a STAT mutant (STAT mut). The inset shows total and phospho-STAT1 levels in AII-treated C2C12 cells transiently transfected with intact AT1aR or the IIGG mutant. The first lane is a positive control from AII-treated C2C12 cells overexpressing STAT1α. Note decreased pSTAT1 levels in IIGG expressing cells relative to cells expressing wild-type receptor. **C)** PKCβ enhances AII-induced ANF transactivation. C2C12 cells were cotransfected with the -695ANF-luc construct and the AT1aR expression vector plus or minus PKC (the catalytic subunit), ERK1 or ERK2 expression vectors. The expression vectors are described in Material and Methods. **D)** AII-induced PKC targets GATA elements. C2C12 cells were cotransfected with AT1aR and the indicated ANF-luc constructs and stimulated with AII in presence or absence of the PKC inhibitor (GF). In all cases, AII treatment was for 12 h. Data shown are mean ± SEM of six independent determinations.

Figure 5. **A)** GATA-4 contains a conserved PKC phosphorylation site within the C-terminal activation domain. The putative PKC phosphorylation site is shown in bold. **B)** *In vitro* PKC phosphorylation of GST GATA-4 fusion proteins containing either the N- (1-207) or C-terminal (329-443) transactivation domain. Ponceau staining was used to show protein loading. The right panel shows the effect of serine to alanine mutation within the conserved PKC motif (S419A, S420A) on PKC phosphorylation. **C)** Effect of mutating the PKC phosphorylation residues on GATA-4 transcriptional activity. In the left panel, C2C12 cells were cotransfected with a 3xGATA-luc reporter and increasing concentrations of wild-type (Wt) HA-GATA-4 (1-425) or mutated (Mut) HA-GATA-4 (S419A, S420A). The right panel shows the effect of PKC inhibition with 5 μM GF 109203X on the -135ANF promoter activation with GATA-4. The data shown represent the mean ± SEM of at least four independent determinations. **D)** Effect of the same mutation on GATA-4 nuclear levels (top panel) and DNA binding activity (lower panel). Nuclear extracts were prepared from C2C12 cells transfected with the wild-type (Wt) or mutant (PKCmut) GATA-4 (described above). The amount of GATA-4 was determined by Western blot using anti-HA antibody (upper panel). The binding activity was determined by EMSA using increasing concentration of nuclear extracts and the -120ANF GATA site as probe. **E)** Effect of AII on endogenous GATA-4 levels and DNA-binding activity. Nuclear extracts (NE) were prepared from cardiomyocytes infected with Ad-AT1aR and treated or not with 100 nM AII for 20 min. DNA binding (upper panel) was performed as above. The specificity of the GATA-4 binding was determined by supershift with the anti-GATA-4 (αG4) antibody or by competition with the cold ANF GATA oligonucleotide (self). GATA binding is indicated by the arrow head and the

supershift by the asterisk. The amount of GATA-4 in the nuclear extracts was determined by Western blot using anti-GATA-4 antibody. **F)** The AII-GATA-4 pathway in cardiomyocytes. Cardiomyocytes were cotransfected with the -695ANF-luc construct and wild-type (Wt) or two dominant negative forms of GATA-4, one with the ability to bind DNA but lacking transcriptional activation domains (DBD) and one lacking DNA binding activity but retaining the ability to interact with cofactors (ZN mut). The data are the mean ± SEM of one representative experiment out of four that were qualitatively identical.

Figure 6. A) AII potentiates STAT1α-induced transactivation of ANF. NIH 3T3 were cotransfected with the -695ANF-luc construct and the STAT1α expression vector and treated with 100 nM AII (AII) or vehicle (Ctl) for 12 h. **B)** Synergistic activation of ANF transcription by GATA-4 and STAT1α. Cells were cotransfected with the -695ANF-luc and GATA-4 vectors, in presence or absence of various STAT expression vectors. The results shown are from C2C12 cells but similar results were also obtained in NIH 3T3. **C)** and **D)** Structure-function analysis of GATA-4 and STAT1α using the -695ANF-luc reporter. The various GATA-4 or STAT1α expression vectors are shown schematically. **E)** Effect of the CBP coactivator on GATA-4/STAT1 synergy. The experiments were done as described in C) and D) above. The data shown represent the mean ± SEM of n=6 for A and B; n=4 for C, D and E.

Figure 7. A) Mapping of the DNA elements required for GATA-4/STAT1α synergy. NIH 3T3 cells were cotransfected with the indicated ANF-luc constructs or a minimal BNP promoter driven by multimerized GATA binding sites (3XGATA) and with GATA-4, STAT1α or both expression vectors. The data shown represent the mean ± SEM of at

least six independent determinations. **B and C)** GATA-4 interacts physically with STAT1α i*n vivo*. In **B)**, nuclear extracts from 293T cells transfected with STAT1α and/or HA-GATA4 were immunoprecipitated using an anti-HA antibody, separated on 8% SDS-PAGE, transferred to PVDF membranes, and subjected to immunoblotting using either anti-STAT1 or anti-HA antibodies (lower two panels). The top panels are Western blots carried out on the nuclear extracts used for the immunoprecipitation. In **C)**, similar experiments were done using Flag-GATA-4 and HA-tagged full length or N- and C-deleted STAT1α. Immunoprecipitation was carried out with the anti-Flag antibody. The position of the different STAT1 proteins as revealed with the anti-HA antibody is indicated by asterisk. **D)** GATA elements are essential for ANF transactivation by STAT1α in cardiomyocytes. Increasing amounts of the STAT1α expression vector were cotransfected in cardiomyocytes with the -695ANF-luc construct (Wt) or a similar construct containing point mutations in the two high affinity GATA sites (GATA mut). The data shown represent the mean ± SEM of four independent determinations.

Figure 8. *In vivo* association of GATA-4 and STAT proteins with the ANF promoter. **A)** Schematic representation of the various STAT and GATA elements on the ANF promoter. Putative elements correspond to *in silico* identified consensus sequences. The location of the sets of primers used in the QPCR is depicted below. **B)** Enrichment of GATA-4 and STAT1/3 proteins on the endogenous ANF promoter as revealed from chromatin immunoprecipitation (ChIP) experiments. The results shown are from one representative experiment out of two carried out in duplicate on primary cardiomyocytes stimulated for 48 h with 100 nM AII. The data are expressed as the ratio of ChIP with

the respective antibodies over that obtained with IgG. For details, see Material and Methods.

Figure 9. A) Functional cooperation between GATA-4 and STAT1α is not restricted to the ANF promoter. NIH 3T3 cells were cotransfected with the -1176 VEGF, -360 c-fos or -757 Bcl-x promoter-driven luciferase reporters and GATA-4, STAT1α or both expression vectors. **B)** and **C)** Functional cooperation between STAT1α, GATA-4 and SRF. Cells were cotransfected with -137ANF (B) or -360 c-fos (C) promoter-driven luciferase reporter constructs with various combinations of SRF, GATA-4 and STAT1α. The data shown represent the mean ± SEM of n = 4-6 in C2C12 cells.

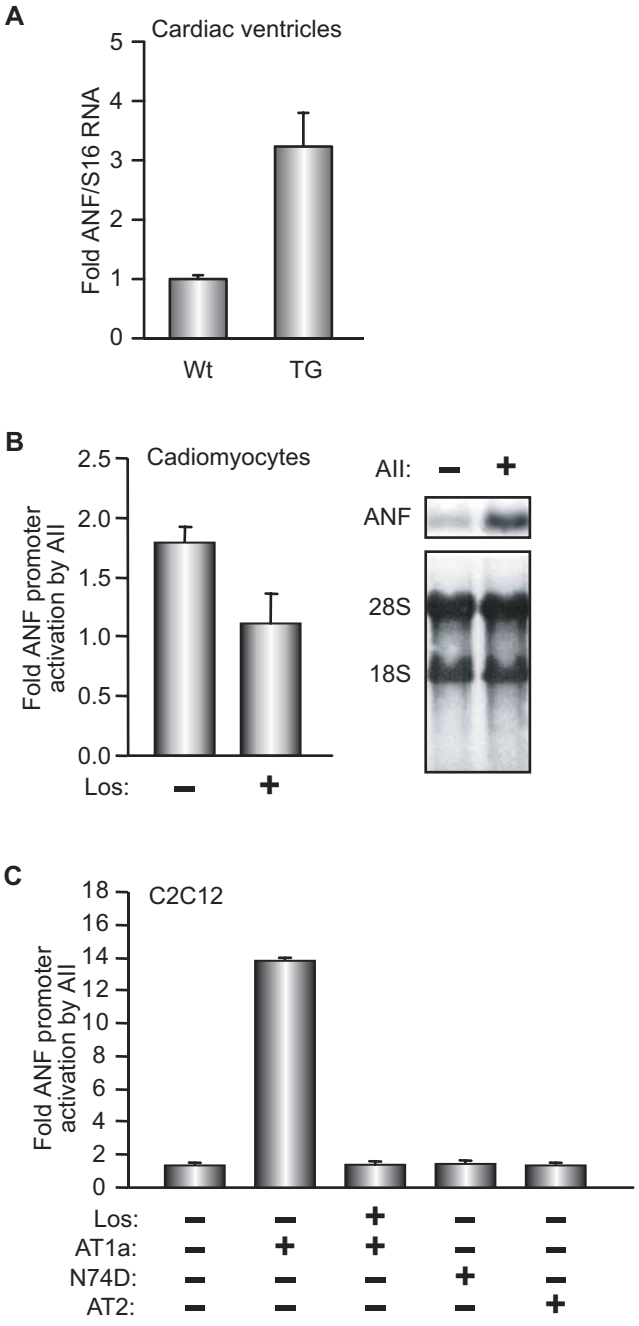


Figure 1, Wang et al.

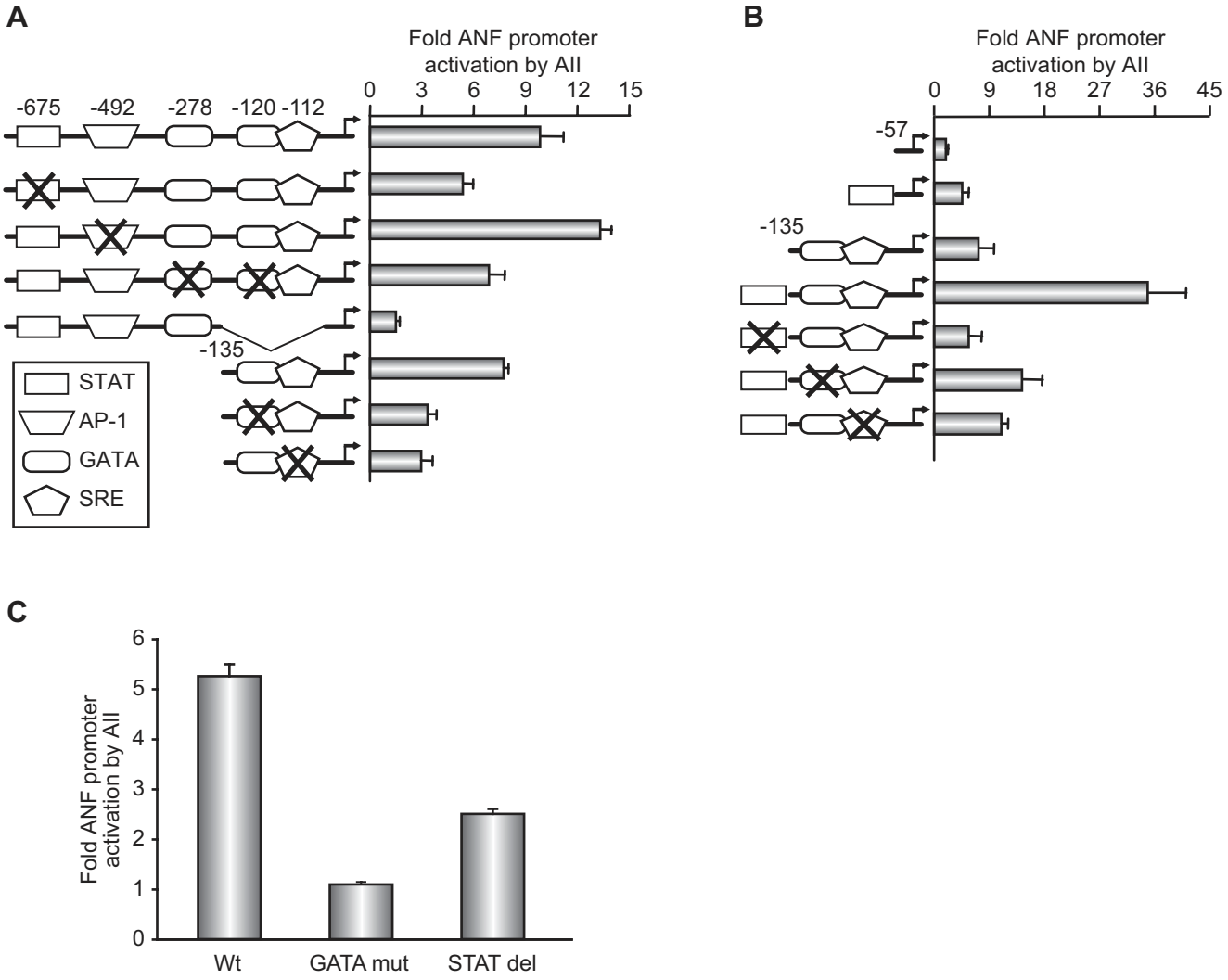
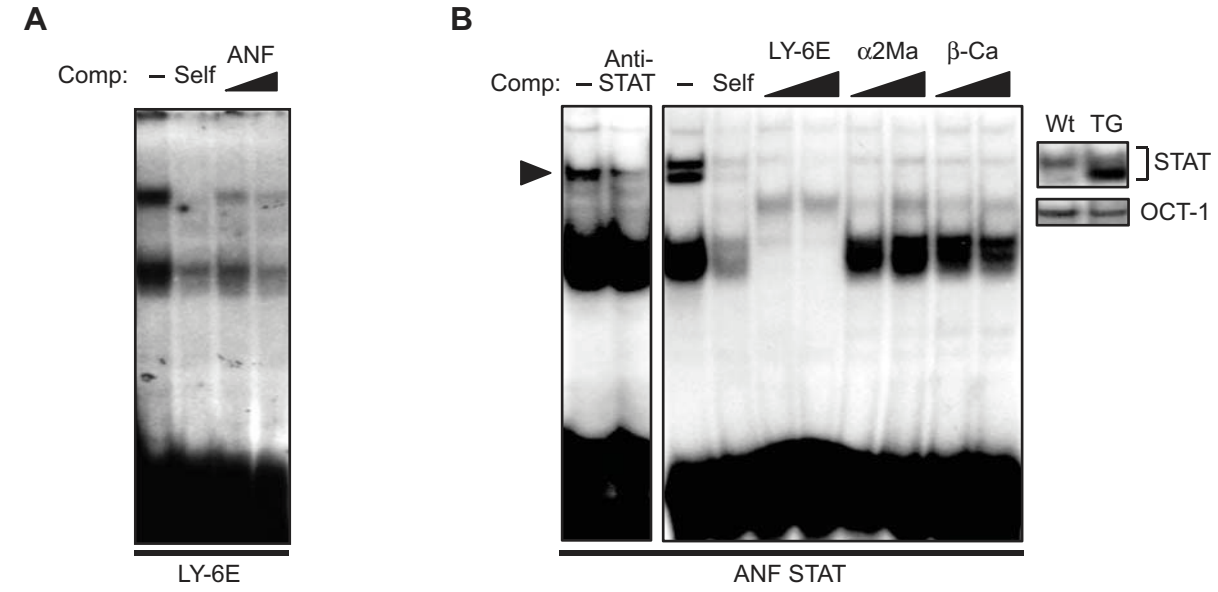
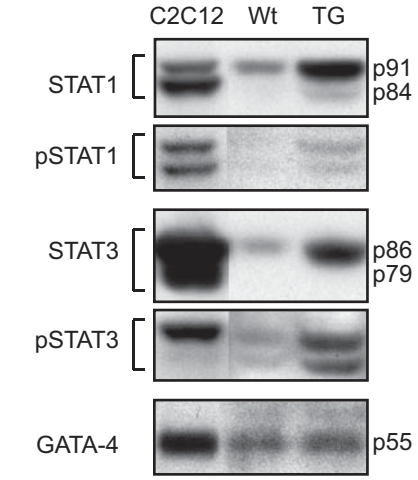


Figure 2, Wang et al.





D

GC**TTTAGAGGAAA**CG	Rat ANF
AT**ATTCCTGTAAG**TG	Murine LY-6E
TCC**TTCTGGGAATT**CC	RAT α2-macroglobulin
GA**TTTCTAGGAATT**CA	Goat β-casein

Figure 3, Wang et al.

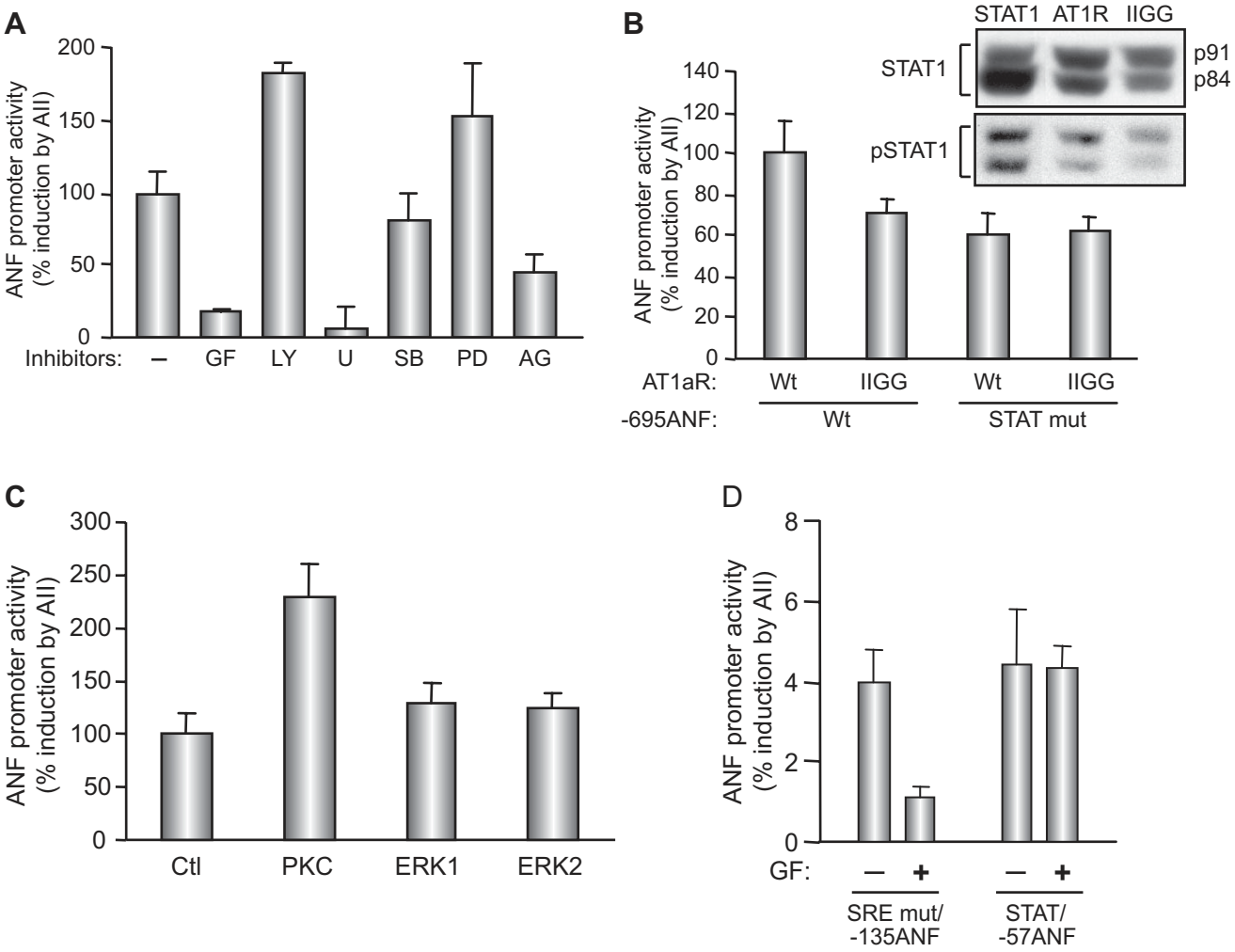


Figure 4, Wang et al.

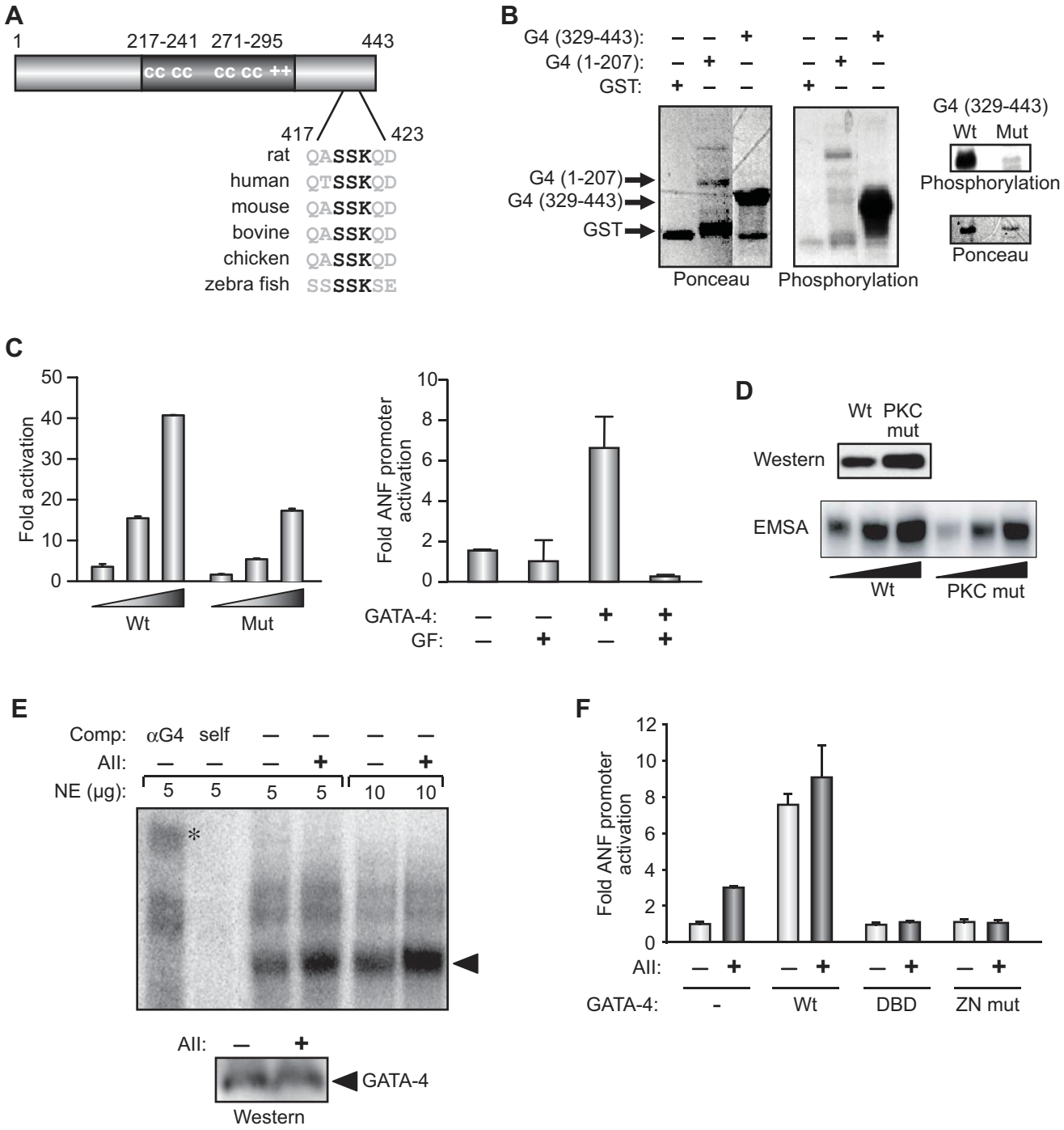


Figure 5, Wang et al

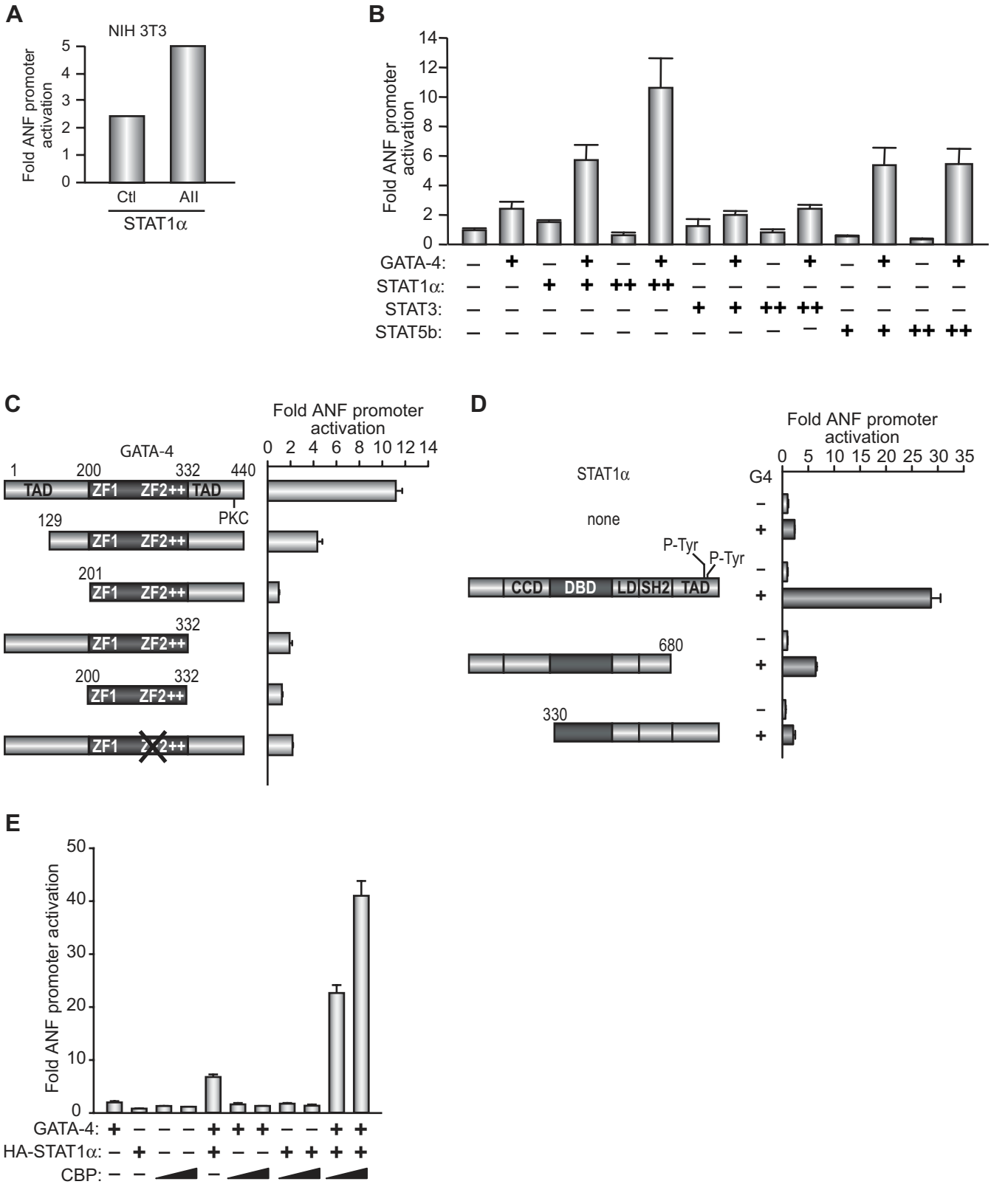


Figure 6, Wang et al.

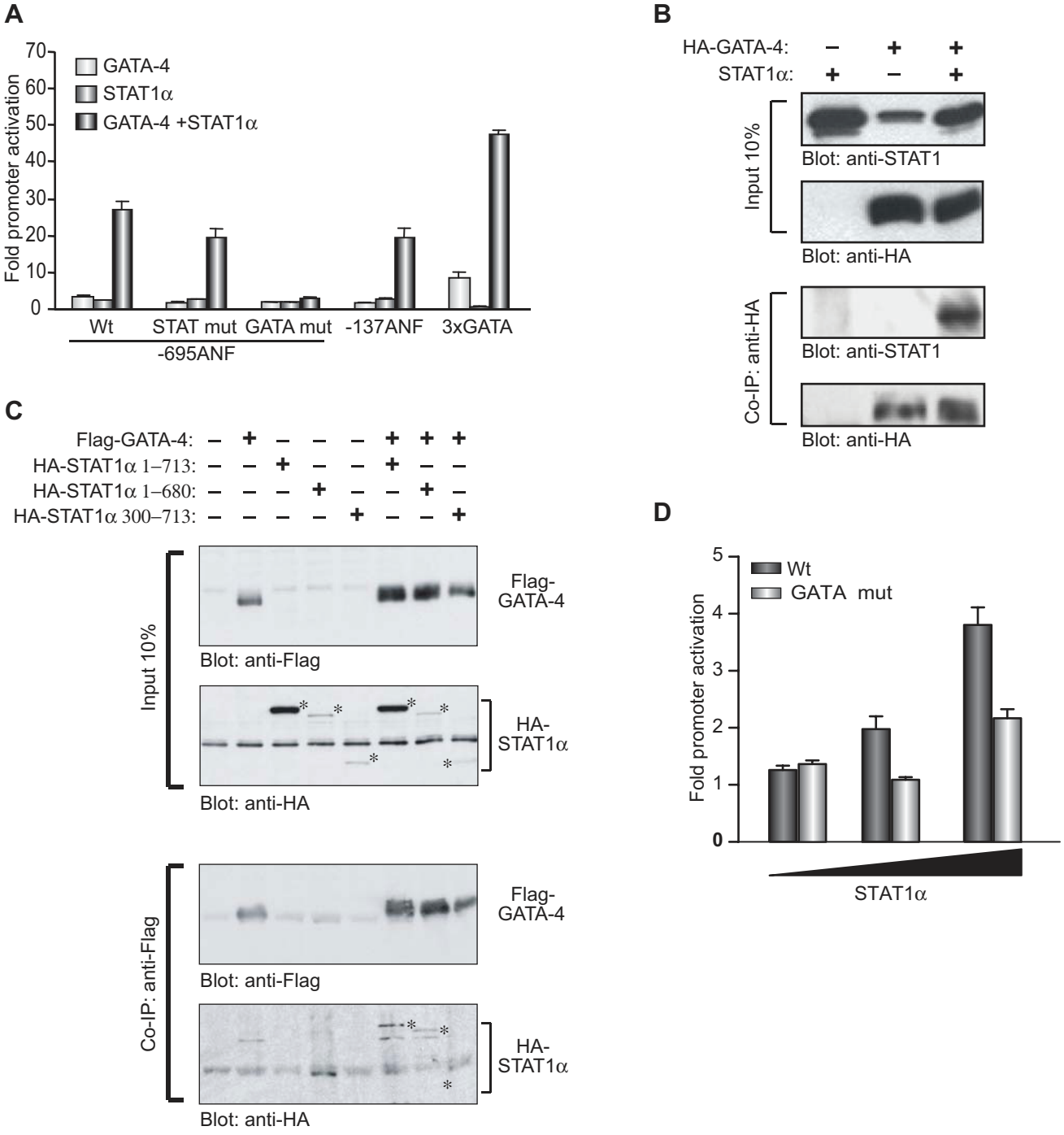


Figure 7, Wang et al

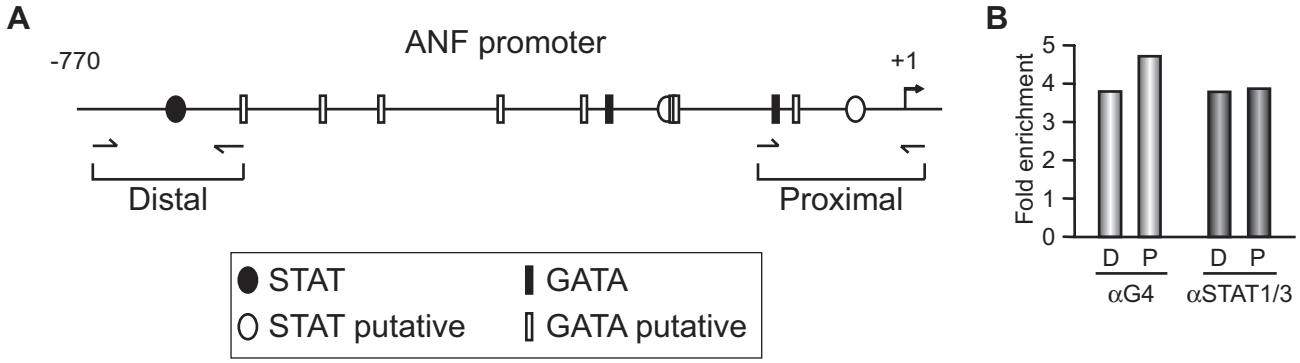


Figure 8, Wang et al.

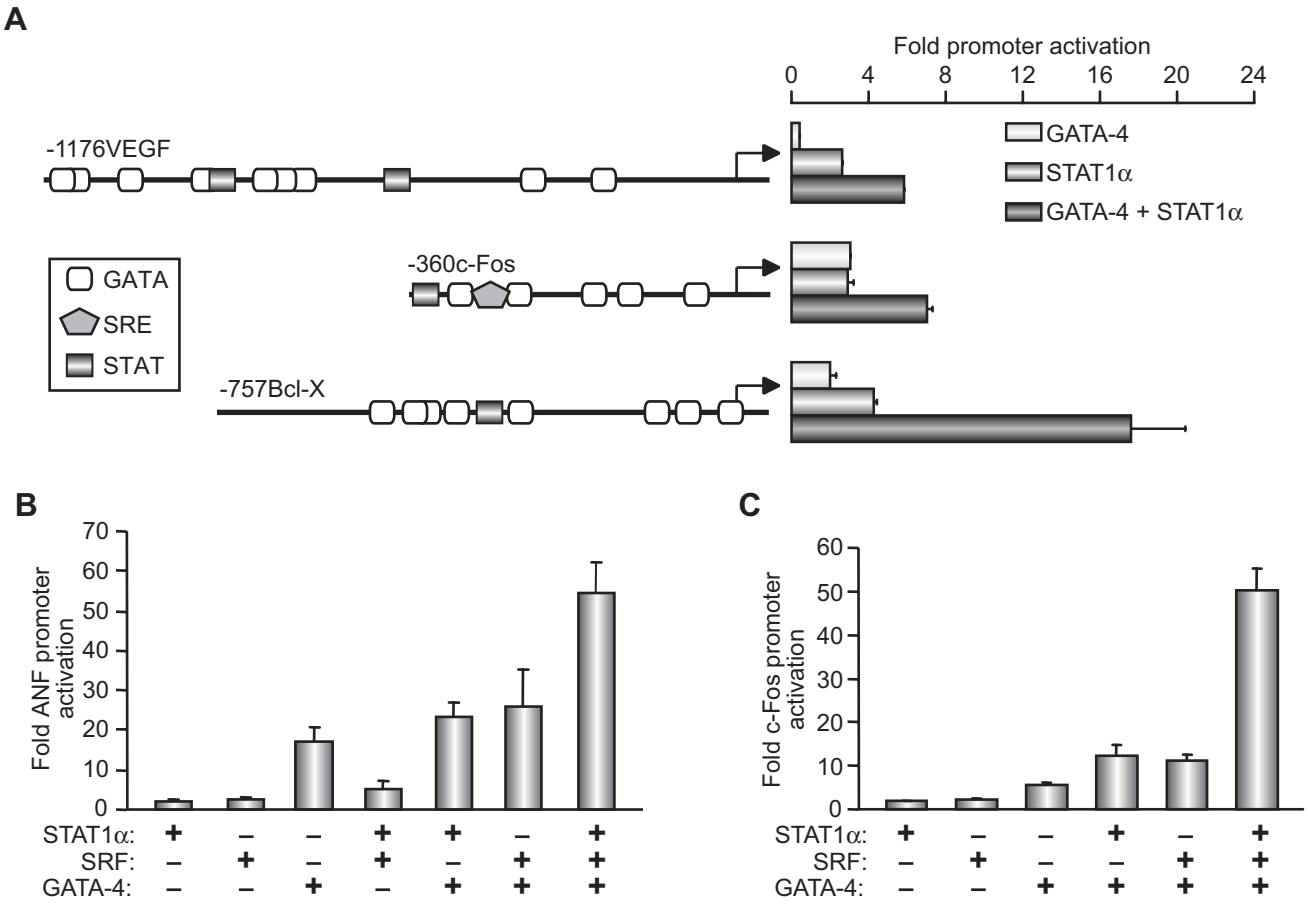


Figure 9, Wang et al