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Stat3 enhances transactivation of steroid hormone receptors

Fernando de Miguel[1], Soo Ok Lee[3], Sergio A Onate[2] and Allen C Gao*[3]

Address: [1]Department of Urology, University of Pittsburgh Medical Center, Pittsburgh, PA 15213, USA, [2]Department of Cell Biology and Physiology, University of Pittsburgh Medical Center, Pittsburgh, PA 15213, USA and [3]Department of Medicine and Pharmacology & Therapeutics, Roswell Park Cancer Institute, Buffalo, NY 14263, USA

Email: Fernando de Miguel - demifx@msx.upmc.edu; Soo Ok Lee - soook.cholee@roswellpark.org; Sergio A Onate - onate@pitt.edu; Allen C Gao* - allen.gao@roswellpark.org

* Corresponding author

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Abstract

Background: Steroid hormone receptors (SHRs) are members of the superfamily of ligand-activated transcription factors that regulate many biological processes. Co-regulators act as bridging molecules between the SHR and general transcription factors to enhance transactivation of target genes. Previous studies demonstrated that Stat3 is constitutively activated in prostate cancer and can enhance prostate specific antigen (PSA) expression and promote androgen independent growth. In this study, we investigate whether Stat3 can enhance steroid hormone receptors activation.

Methods: CV-1 cells in which plasmids expressing androgen receptor (AR), glucocorticoid receptor (GR), progesterone receptor (PR) or estrogen receptor (ER) were cotransfected with a constitutively active STAT3 mutant.

Results: Stat3 stimulates the transcriptional activity of all four SHR tested, AR, GR, PR and ER, in a hormone-dependent manner. Stat3 acts in a synergistic fashion with other coactivators such as SRC-1, pCAF, CBP, and TIF-2 on the transcriptional activity of these SHR. In addition, Stat3 significantly enhanced the sensitivity of androgen receptor in response to androgen. STAT3 did not affect the specificity of AR for other steroid hormones other than androgen or binding of AR to other hormone responsive elements.

Conclusions: These findings suggest that Stat3 can enhance the transactivation of AR, GR, PR and ER, and activated Stat3 could have a role in the development or progression of a hypersensitive AR.

Introduction

Steroid hormone receptors (SHRs) are members of a family of ligand-activated transcription factors that regulate many biological processes, including metabolism, reproduction, and development. In the absence of ligand such as androgen, glucocorticoid, progestin, and mineralocorticoid, the SHR maintains a cytosolic inactive state by association with heat shock proteins and/or other proteins such as corepressors. Upon ligand binding, the SHR undergoes conformational changes that involve releas e from the repressor proteins, and translocation to the nucleus where it can bind to specific hormone responsive sequences in the DNA of genes regulated by steroid hormones [1,2]. The mechanism by which SHRs affect the rate of RNA polymerase II-directed transcription likely involves the interaction of receptors with components of the transcription preinitialization complex. This interaction may be direct, or it may occur indirectly through the

action of coactivators, which act as bridging factors. To date, numerous coactivator molecules have been isolated and characterized, encompassing several different families [3,4]. Most of these cofactors are expressed in a wide variety of cell types and can interact with more than one type of nuclear receptor. The recent findings that members of the several different families of coactivators possess intrinsic histone acetyltransferase activity suggests that activated SHRs, and nuclear receptors in general, may also recruit these cofactors to remodel chromatin structure for better accessibility of the transcriptional machinery to DNA [5,6].

The JAK/STAT signaling pathway is involved in many cytokines, hormones, and growth factors mediated signaling pathways to regulate a variety of biological responses, including development, cell differentiation, proliferation and survival [7,8]. Once STAT proteins are activated by tyrosine-phosphorylation, form homo or heterodimers that are translocated to the nucleus, where they can bind to specific sequences in the DNA, thereby stimulating gene transcription. Similarly to nuclear receptors and to other transcription factors, STAT proteins can interact with coactivators to modulate their transcriptional activity [9–12]. Other reports have shown direct interactions between several members of the JAK/STAT signaling pathway with SHRs [13–16]. Stat3 is one of the seven members of the STAT family of proteins that has been shown to modulate the expression of several genes related to control cell cycle, proliferation and apoptosis, such as Cyclin D1, c-myc, and Bcl-xL, respectively [17]. Accordingly, alterations in the activity of STAT3 have been associated with cell transformation and cancer progression [18–20].

Prostate cancer is the most commonly diagnosed cancer, and the second leading cause of death from cancer in North American men. Prostate cells are dependant on androgen to keep their normal functions. Activation of androgen receptor (AR) in prostate cells is a key step in developing and progression of prostate cancer. Most patients respond initially to androgen deprivation or antiandrogen regiments, but eventually the tumor relapses in an androgen-independent stage with a poor outcome. Several possible mechanisms have been suggested to explain this activation of AR, including mutations in the gene encoding AR that alter the specificity for androgens, overexpression of the AR protein itself, crosstalk to other signal transduction pathways, and altered levels or activity of coactivators [21–23]. Several groups including ours have demonstrated a role of activated Stat3 in the proliferation and survival of prostate cancer cells by a mechanism that involves the AR [24–28].

In this study, we examined how Stat3 activates the SHRs including AR. We studied whether Stat3, a transcription factor itself, could act as a coactivator for AR and for other SHRs. We report here that a constitutively active form of Stat3 stimulates transcriptional activity of SHRs in a hormone-dependent manner. We also report that active Stat3 can act synergistically with other coactivators to stimulate SHRs transcriptional activity. Moreover, while Stat3 did not affect specificity of AR to other steroid hormones rather than androgen, or binding of AR to other HREs, Stat3 significantly increased the sensitivity of AR to androgen. These results can add more light to the mechanism by which SHRs, and AR in particular, regulate gene expression.

Results

Effect of Stat3 on AR-mediated transcriptional activity

Stat3 has been shown to enhance AR-mediated PSA expression and AR transcriptional activity in prostate cancer cells [16,25,28]. We used two well-characterized androgen-responsive luciferase reporter plasmids to study the effect of Stat3 on AR-mediated transcriptional activity. Both reporter plasmids contain several AREs that are required for androgen induction. As comparison, we used a saturating dose, 10 nM, of a synthetic androgen, R1881. CV-1 cells do not express endogenous AR, therefore, all experiments were performed in the presence of ectopic wild-type human AR. CV-1 cells showed a strong response to R1881 compared to vehicle-treated cells with both androgen-responsive reporters used (Fig. 1). Cotransfection of a plasmid expressing a constitutively active mutant Stat3, Stat3C, that forms homodimers and translocates to the nucleus without tyrosine phosphorylation, affected AR transcriptional activity in a dose-response manner (Fig. 1A) but only in the presence of hormone (Fig. 1B and 1C). Constitutively activated Stat3 also activates another androgen-responsive reporter contained the fragment -286/+28 of the rat Probasin (PB) promoter (Fig. 1C). Wild-type Stat3, that is in a latent cytosolic state without tyrosine phosphorylation nor dimerization, showed no additional stimulatory effect over the androgen-treated cells (Fig. 1)

Effect of STAT3 on transcriptional activity of other SHRs

Having demonstrated that Stat3 enhance AR-mediated gene transcription, we next investigated the role of Stat3 on transcriptional activation with other SHRs. For GR and PR, we used the same reporter plasmid as for AR, ARE-TATA-luciferase, since it shows promiscuous response to all three SHRs [30]. For ER, we used ERE-TATA-luciferase reporter that is specific for estrogen. CV-1 cells were also cotransfected with plasmids expressing ectopically GR, PR or ER. Like the AR, Stat3 enhances all three other SHRs tested in the presence of the corresponding hormone (Dex for GR, R5020 for PR, or E_2 for ER) (Fig. 2). Taken together, Stat3 enhances four different SHRs transcriptional activity in the presence of their

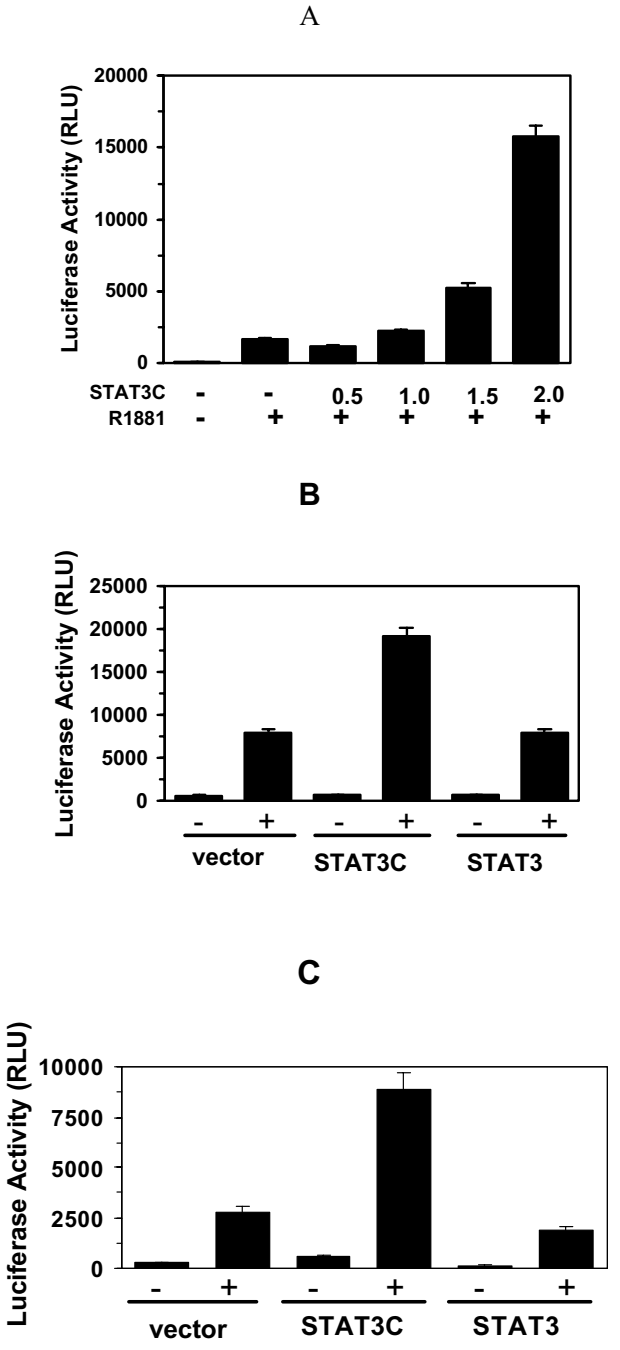

Figure 1
Active Stat3 stimulates androgen-induced AR transcriptional activity. CV-1 cells were cotransfected with AR responsive reporter plasmids, ARE-TATA-luciferase (**A, B**) or PB(-286/+28)-luciferase (**C**), AR, and Stat3C, S tat3, or empty vector. After transfection, cells were treated for 36–40 h with 10 nM R1881 or 0.1% ethanol as vehicle in 5% charcoal stripped serum in phenol red-free DMEM. Luciferase activity was determined in cell extracts and corrected per μg of protein. Data are expressed as mean ± S.E. of three independent experiments.

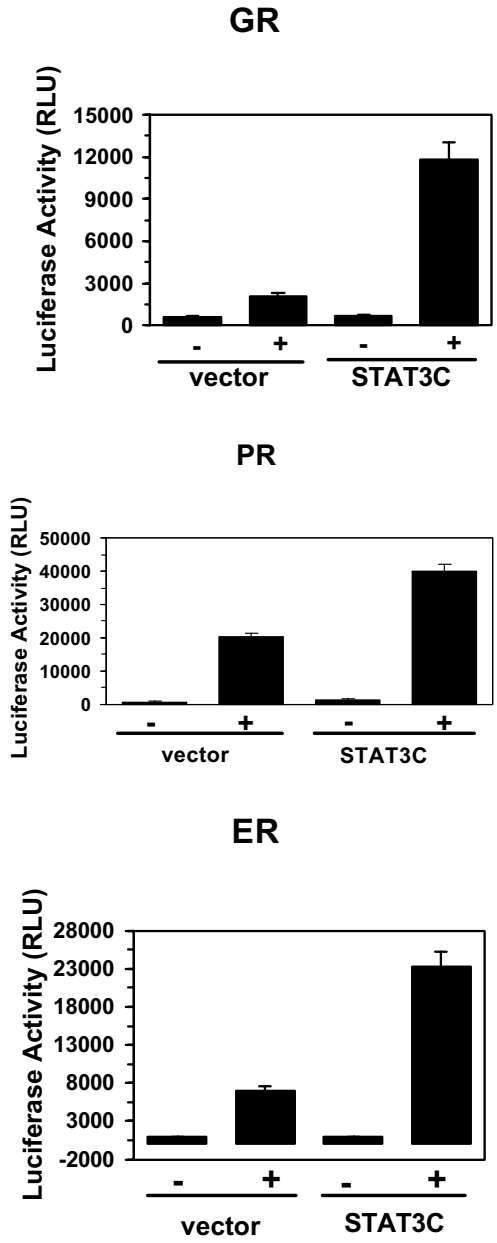

Figure 2
Active Stat3 stimulates hormone-induced transcriptional activity of other SHRs. CV-1 cells were cotransfected with specific luciferase reporter plasmids (ARE-TATA-luciferase for GR and PR, and ERE-TATA-luciferase for ER), their corresponding SHR expressing plasmid (GR, top; PR, middle; and ER, bottom), and Stat3C plasmid or empty vector. After transfection, cells were treated for 36–40 h with 10 nM of the corresponding hormone (Dex for GR; R5020 for PR; and E_2 for ER) or 0.1% ethanol as vehicle in 5% charcoal stripped serum in phenol red-free DMEM. Luciferase activity was determined in cell extracts and corrected per μg of protein. Data are expressed as mean ± S.E. of three independent experiments.

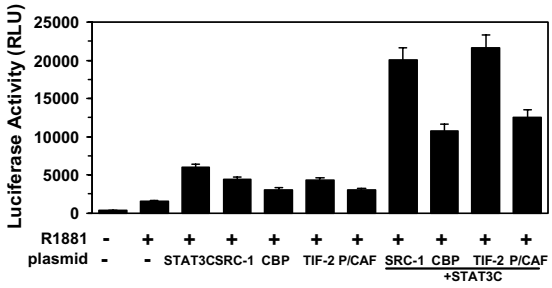

Figure 3
Active Stat3 stimulates AR transcriptional activity similarly as and synergistically with other coactivators. CV-1 cells were cotransfected with ARE-TATA-luciferase, AR, and Stat3C or different coactivators (SRC-1, CBP/p300, TIF2 or P/CAF), individually or in combination. All wells contained the same total amount of DNA. After transfection, cells were treated for 36–40 h with 10 nM R1881 or 0.1% ethanol as vehicle in 5% charcoal stripped serum in phenol red-free DMEM. Luciferase activity was determined in cell extracts and corrected per μg of protein. Data are expressed as mean ± S.E. of three independent experiments.

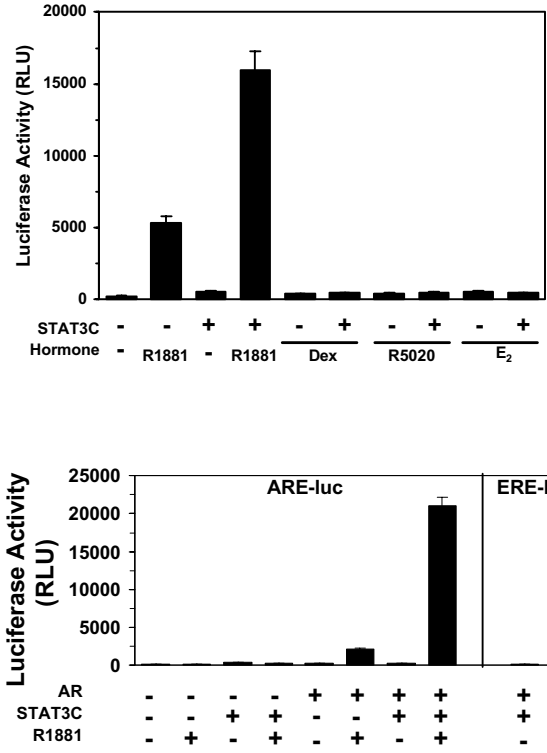

Figure 4
Stat3 does not affect specificity of AR for non-androgen steroid hormones or non-androgen responsive elements. (Top) CV-1 cells were cotransfected with ARE-TATA-luciferase, AR, and Stat3C or empty vector, and treated for 36–40 h with 10 nM each different steroid hormones (R1881, Dex, R5020, or E_2) or 0.1% ethanol as vehicle in 5% charcoal stripped serum in phenol red-free DMEM. (Bottom) CV-1 cells were cotransfected with ARE-TATA-luciferase (left) or ERE-TATA-luciferase (right), AR, and Stat3C or empty vector, and treated for 36–40 h with 10 nM R1881 or 0.1% ethanol as vehicle in 5% charcoal stripped serum in phenol red-free DMEM. Luciferase activity was determined in cell extracts and corrected per μg of protein. Data are expressed as mean ± S.E. of three independent experiments.

corresponding ligand, suggesting that active Stat3 acts as a coactivator for nuclear receptors.

We next compared the coactivator activity of Stat3 to that of other known coactivators for AR. CV-1 cells were transfected with the same amount (2 μg) of plasmid expressing Stat3C, SRC-1, CBP/p300, TIF-2 or P/CAF, respectively. Cells transfected with either coactivator or Stat3C showed comparable and higher luciferase activity in response to R1881 than cells treated with hormone alone in the presence of empty vector (Fig. 3). None of the coactivators tested showed any luciferase activity in the absence of hormone (data not shown). Stat3 is comparable to other coactivators on AR transcriptional activity: SRC-1, CBP/p300, TIF2, P/CAF.

It has been reported that coactivators in combination can enhance transcriptional activity of SHRs compared to when they are delivered to cells individually [29,30]. Recently, Giraud et al [12] reported a direct interaction of Stat3 with SRC-1 and CBP/p300. We determined whether Stat3 could act in a synergistic/additive way when administered to cells simultaneously to other coactivators. As Fig. 3 shows, coexpression of active Stat3C simultaneously to SRC-1, CBP/p300, TIF-2 or P/CAF, resulted in a more efficient enhancement of the reporter transcription activity driven by AR as compared with any of the coactivators expressed separately. A similar enhancement in luciferase activity with simultaneous delivery of coactivators was observed with PR and ER (data not shown).

Stat3 did not affect the specificity of AR for non-androgen steroid hormones or non-AREs

Altered responses of AR include activation by non-androgen ligands or recognition of other hormone responsive elements different from AREs [22,23]. We studied if active Stat3 could be responsible for this behavior of AR. CV-1

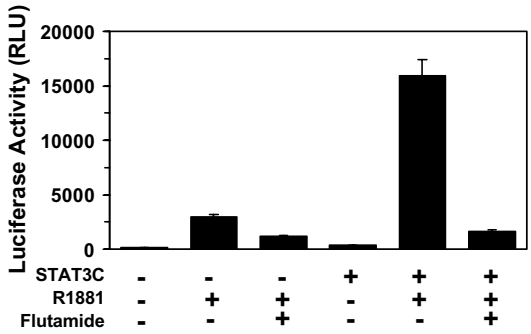

Figure 5
Inhibition of AR transcriptional activity by flutamide is not blocked by active Stat3. CV-1 cells were cotransfected with ARE-TATA-luciferase, AR, and Stat3C or empty vector, and treated for 36–40 h with 10 nM R1881 or 0.1% ethanol as vehicle in the presence or absence of 10 μM flutamide, in 5% charcoal stripped serum in phenol red-free DMEM. Luciferase activity was determined in cell extracts and corrected per μg of protein. Data are representative of three independent experiments. Data are expressed as mean ± S.E. of three independent experiments.

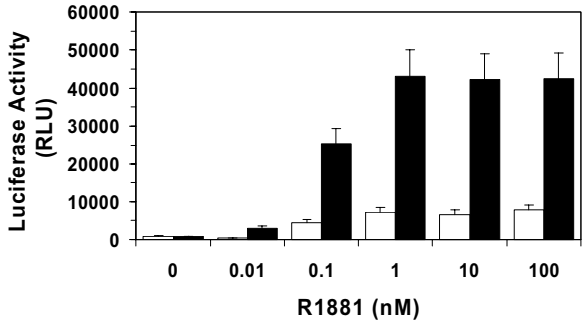

Figure 6
Active Stat3 modifies the sensitivity of AR for androgen. CV-1 cells were cotransfected with ARE-TATA-luciferase, AR, and Stat3C (solid bars) or empty vector (empty bars), and treated for 36–40 h with different doses of R1881 or 0.1% ethanol as vehicle, in 5% charcoal stripped serum in phenol red-free DMEM. Luciferase activity was determined in cell extracts and corrected per μg of protein. Data are expressed as mean ± S.E. of three independent experiments.

cells were cotransfected with a reporter plasmid responsive to androgen (ARE-TATA-luciferase) in the presence or absence of AR and constitutively active Stat3C (Fig. 4 top) and cells were treated with different steroid hormones. Stat3C did not alter the specificity of AR for other steroid hormones, since it showed transcriptional activity only in response to androgen but not to Dex, R5020 or E_2 (Fig 4, top).

To test whether Stat3 could alter the specificity of AR to other HREs, CV-1 cells were cotransfected with reporter plasmids responsive to androgen (ARE-TATA-luciferase) or to estrogen (ERE-TATA-luciferase), in the presence or absence of AR and constitutively active Stat3C (Fig. 4 bottom). With ARE-TATA-luciferase reporter, luciferase activity was dependent on the presence of AR and androgen, without any effect of Stat3C on the basal levels of transcription (Fig 4, bottom left). Stat3C did not modify the sensitivity of AR for other hormone responsive elements, since there was no response on the AR transcriptional activity when we used an estrogen-specific responsive reporter plasmid, ERE-TATA-luciferase (Fig 4, bottom right).

Stat3 does not prevent inhibition of AR by the antiandrogen flutamide

Another altered response of AR is manifested in the so-called "flutamide withdrawal syndrome" or more recently broadened to "antiandrogen withdrawal syndrome". In this condition, it is shown a decrease in serum levels of PSA after removal of flutamide or other antiandrogens from the treatment regiment [33]. We studied if active Stat3 could be involved in this paradoxical effect of antiandrogens. CV-1 cells cotransfected with an androgen responsive reporter plasmid (ARE-TATA-luciferase), AR, in the presence or absence of constitutively active Stat3C, were treated with androgen and flutamide (Fig. 5). As expected, flutamide blocked substantially the induction of ARE-TATA-luciferase activity by R1881 in the absence of active Stat3C. Similarly, the inhibitory effect of the antiandrogen on AR was not prevented by the presence of constitutively active Stat3C.

Stat3 affects sensitivity of AR for androgen

Other mechanism by which AR could show an altered response results from an increase in its sensitivity to very low levels of androgen [22,23]. We tested the role of active Stat3 in this possibility cotransfecting CV-1 cells with an androgen responsive reporter (ARE-TATA-luciferase), AR, in the presence or in the absence of constitutively active Stat3C. Cells were then treated with different doses of androgen. Maximal transcriptional activity of AR was at 1 nM R1881 both in the presence and absence of Stat3C (Fig. 6). Stat3C enhanced luci ferase activity driven by AR even at the minimal dose of R1881 tested, 10 pM, which showed no luciferase activity in the absence of Stat3C.

Discussion

Regulation of gene transcription mediated by hormone-activated SHRs involves interaction with components of

the transcription complex. Coactivators are members of the transcription complex, which act as bridging factors to achieve optimal transcription activity. Coactivators are probably present in the nucleus in preassembled multicomplex units ready to associate to activated SHRs. The role of the coactivators could be just as bridging factors, although some of the members of the p160 family and CBP/p300 present histone acetyltransferase activity [34,35]. Stat3 is a member of the JAK/STAT signaling pathway, which is a transcription factor itself that after activation binds to specific sequences in DNA to regulate expression of genes related to proliferation, differentiation and cell survival. Constitutive activation of Stat3 has been associated with cell transformation and cancer progression. Previous data including from our group showed activation of AR in prostate cancer mediated by activation of Stat3 [24–28].

Here we show that constitutively active Stat3 enhances transcriptional activity of SHRs, AR, GR, PR, and ER, in a comparable extent to the stimulation elicit by other coactivators, and even more, in a synergistic manner to these other coactivators. This stimulation is independent of the DNA binding activity of Stat3, since none of the luciferase reporters responsive to SHRs used throughout this report contain the DNA sequence responsive to Stat3 [7]. We cannot rule out, however, that Stat3 is affecting an independent event that in turn activates SHR transcriptional activity. Nevertheless, direct protein-protein interaction has been documented by coimmunoprecipitation experiments between Stat3 and AR [16,25], GR [13,14], PR [36], and ER [37], suggesting that Stat3 might be involved directly in transcriptional activity elicit by SHRs. Moreover, Stat3 has also been directly associated with several coactivators, such as SRC-1 and CBP/p300 [9,12]. Recruitment of coactivators to nuclear receptors takes place via a common motif in the coactivators containing a core consensus sequence LXXLL (L, leucine; X, any aminoacid) [38]. Most of the coactivators contain more than one of these motifs, raising the question regarding specificity of these motifs to specific activating domains in the nuclear receptors. Stat3 also contains in its N-terminus one of this motifs (^{221}LAGLL^{225}) [39]. Moreover, Stat3 also presents a Ser at -2 position of the LXXLL sequence, which in the case of the coactivator TRBP defines selectivity for nuclear receptors [40]. Phosphorylation of Stat3 has been reported to occur only in ^{705}Tyr and in ^{727}Ser, allowing dimerization and full transactivating activity [41]. Whether this ^{219}Ser next to the LXXLL motif is involved in the coactivator activity of Stat3, and the interaction of Stat3 with other coactivators only takes place in the context of Stat3 transcription factor activity or also can be part of the general mechanism of the transcription complex formation requires further studies.

Activation of AR is a driving force in development and progression of prostate cancer. Several mechanisms could be involved in this AR activation [21–23]. Changes in the specificity of AR broadening the responsive spectrum to other steroid hormones different to dihydrotestosterone, can be caused by genetic mutations affecting different regions of AR. We studied if active Stat3 could be sufficient to alter the specificity of a wild-type AR to non-steroid hormones. Our results indicate that native AR, with no mutations, is responsible only to androgens, being Stat3 not sufficient to alter this feature of AR. Activated Stat3 d oes not affect the specificity of AR to bind to other HREs different form ARE. Other altered response of mutated AR is the paradoxical agonistic effect of antiandrogens. Also in this case, Stat3 could not reverse the antagonistic activity of flutamide on a wild-type AR. These results point to mutations in AR as a necessary step in some of the altered responses of AR seen in advanced prostate cancer.

Another possible mechanism by which a prostate cancer circumvents the low levels of androgens resulting from androgen ablation therapy is by increasing the sensitivity of AR to very low levels of androgens [42]. Stat3 increased the sensitivity of AR to a dose of androgen that showed no activity in the absence of Stat3, in the absence of a mutated AR. The constitutive activation of Stat3 found in prostate cancer [27,43] could be an initial step in the clonal selection of malignant cells affecting not only Stat3-regulated genes but also AR, as part of the transcription complex recruited in response to activated AR. Recently, overexpression of SRC-1 and TIF-2 has been found in prostate cancer recurrence after androgen deprivation therapy [44], providing a molecular basis for AR activation that could be similar to the one displayed by Stat3 in its role as coactivator.

In conclusions, we report here that: 1) a constitutively active form of Stat3 stimulates transcriptional activity of SHRs in a hormone-dependent manner; 2) active Stat3 can act synergistically with other c oactivators to stimulate SHRs transcriptional activity; 3) Stat3 did not affect specificity of AR to other steroid hormones different than androgen, or binding of AR to other HREs; 4) Stat3 increases the sensitivity of AR to androgen. These results can help to elucidate the mechanism by which SHRs in general, and AR in particular, regulate gene expression.

Methods

Plasmids

Luciferase reporter plasmids containing specific responsive elements for AR, GR, and PR (GRE-TATA-luciferase) and ER (ERE-TATA-luciferase) have been described elsewhere [29,30]; another androgen-responsive reporter contained the fragment -286/+28 of the rat Probasin (PB)

promoter, and was obtained from Dr. R. Matusik, Vanderbilt University, TN [31]. An expression plasmid for wild-type hAR was kindly provided by Dr. C. Chang, University of Rochester, NY. Expression plasmids for hGR, hPR α and ER have been also described [29,30]. Wild-type inactive form of Stat3 (pCAGGS-HA-Stat3) was from Dr. T. Hirano, Osaka University, Japan [32]. The plasmid expressing a constitutively active form of Stat3 (Stat3C-Flag) was obtained from Dr. J. Darnell, jr. [18]. This constitutively activated Stat3 (a mutant produced by substitution of the cysteine residues within the COOH-terminal loop of the SH2 domain of Stat3) induces cellular transformation and tumor formation in nude mice [18]. The plasmids to express other coactivators (SRC-1, CBP/p300, TIF2 and PCAF) have been described elsewhere [29,30].

Cells and transfections

CV-1 cells were maintained in 10% fetal bovine serum (Bio-Whittaker, Walkersville, MD), 100 u/ml penicillin, 100 μg/ml streptomycin in DMEM. Twenty four hours before transfection, cells were plated in 12 well plates (1.2 × 105 per well) in 5% charcoal stripped serum (Hyclone, Logan, UT), antibiotics, and 2 mM L-glutamine in phenol red-free DMEM. For transfections, we used (per well) 0.2 μg reporter, 0.2 μg receptor, and 2 μg coactivator or empty vector. In experiments studying synergy of coactivators, the total amount of DNA was kept constant at 4.4 μg per well. Transfections were performed with Superfect reagent according to the manufacturer's protocol (Qiagen, Valencia, CA). Three hours after transfection, medium was removed and hormones added in phenol red-free DMEM containing 5% charcoal stripped serum and antibiotics. Dihydrotestosterone (DHT), dexamethasone (Dex), estradiol (E_2), and the synthetic anti-androgen flutamide were from Sigma (Saint Louis, MO). The synthetic analogs of androgen and progesterone, R1881 and R5020, respectively, were from New England Nuclear (Boston, MA).

Luciferase activity

Thirty six hours after incubation with hormones, luciferase activity was determined in cell extracts according to manufacturer's suggestions (Promega, Madison, WI). Luciferase activity was normalized per μg protein, determined by Bradford assay (Coomasie Plus, Pierce, Rockford, IL). Data are presented as a representative experiment, which was independently repeated at least three times.

Authors' Contributions

DeMiguel and Lee carried out the entire experiments. Onate and Gao conceived of the study, and participated in its design and coordination.

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