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Novel role of signal transducer and activator of transcription 3 as a progesterone receptor coactivator in breast cancer

Cecilia J. Proietti*, Wendy Béguelin, María Celeste Díaz Flaqué, Florencia Cayrol, Martín A. Rivas, Mercedes Tkach, Eduardo H. Charreau, Roxana Schillaci, Patricia V. Elizalde**

Instituto de Biología y Medicina Experimental (IBYME), CONICET, Vuelta de Obligado 2490, Buenos Aires C1428ADN, Argentina

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ABSTRACT

Interactions between progesterone receptor (PR) and signal transducer and activator of transcription 3 (Stat3)-mediated signaling pathways have already been described. In the present study, we explored the capacity of Stat3 to functionally interact with progesterone receptor (PR) and modulate PR transcriptional activation in breast cancer cells. We found that the synthetic progestin medroxyprogesterone acetate (MPA) induced the association of a PR/Stat3 complex in which Stat3 acts as a coactivator of PR. We demonstrated that Stat3 activation is required for MPA modulation of the endogenous genes bcl-X and $p21^{CIP1}$ which are involved in MPA-induced cell cycle regulation. Stat3 activity as a coactivator of PR was observed in both the classical and nonclassical ligand activated-PR transcriptional mechanisms, since the effects described were identified in the bcl-X promoter which contains a progesterone responsive element and in the $p21^{CIP1}$ promoter which carries Sp1 binding sites where PR is recruited via the transcription factor Sp1. The data herein presented identifies a potential therapeutic intervention for PR-positive breast tumors consisting of targeting Stat3 function or PR/Stat3 interaction which will result in the inhibition of PR function.

1. Introduction

Progesterone receptor (PR) and the signal transducer and activator of transcription 3 (Stat3) are major players in the breast cancer scenario. In its classical mechanism of action, PR acts as a ligand-activated transcription factor on promoters containing progesterone response elements (PREs) [1]. Alternatively, PR may alter gene expression non-classically, where the receptor tethers to other transcription factors bound to DNA [2]. In both cases, the process results in the recruitment of coregulators, chromatin remodeling complexes, and the general transcriptional machinery [3]. The identity of cofactors and transcriptional complexes recruited by activated PR in the classical and nonclassical transcriptional mechanisms is still being studied [4]. In addition to its direct transcriptional effects, PR activates signal transduction pathways in breast cancer cells through a rapid or nongenomic mechanism [5,6].

Experimental and clinical evidence points to a direct role for PR in breast cancer. Treatment of Brca-1-deficient mice with the anti-progestin RU486 prevented mammary tumorigenesis [7]. In addition, the recent extensive, randomized, and controlled Women's Health Initiative trial revealed that postmenopausal women who undergo a combined estrogen and progestins hormone replacement therapy suffer a higher incidence of breast cancer than women who take estrogen alone [8,9]. Finally, we have long demonstrated that progestins exert sustained proliferative response *in vivo* in the previously described estrogen receptor (ER) and PR-positive C4HD model of mammary carcinogenesis induced by the synthetic progestin medroxyprogesterone acetate (MPA) in female BALB/c mice [10,11]. All these findings strongly support the idea that through PR, progestins play an important role in the etiology and/or progression of breast cancer.

On the other hand, Stat3 is a member of the Stat family of proteins and has dual functions: as a signaling molecule in the cytoplasm and as a transcription factor following nuclear translocation [12,13]. Accumulated evidence including our own findings indicates that Stat3 plays a key role in breast tumorigenesis [11,13–16]. Constitutive Stat3 tyrosine phosphorylation and DNA-binding activity have been found in breast cancer cell lines and in breast tumor samples [14,15,17–19]. This unique family of proteins was found to be involved in crosstalks with several members of the steroid hormone receptor superfamily signaling pathways. These

* Corresponding author at: Laboratory of Molecular Mechanisms of Carcinogenesis, IBYME, Vuelta de Obligado 2490, Buenos Aires C1428ADN, Argentina. Tel.: +54 11 4783 2869; fax: +54 11 4786 2564.
** Corresponding author.
E-mail addresses: cproietti@ibyme.conicet.uba.ar (C.J. Proietti), patriciaelizalde@ibyme.conicet.uba.ar (P.V. Elizalde).

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crosstalks are of a bidirectional nature, where steroid hormone receptors regulate Stat-dependent transcription and, conversely, where Stats are able to modulate steroid hormone-mediated transcription [20–22]. Indeed, we have shown that progestins induced transcriptional activation of Stat3 in breast cancer cells, which is an absolute requirement for progestin-mediated *in vitro* and *in vivo* breast cancer growth [16].

In the present study, we explored the significance of the interaction between PR and Stat3 in gene expression. We chose to study two endogenous genes involved in cell cycle regulation and modulated by progesterone: bcl-X [23,24] and $p21^{CIP1}$ [2,25,26]. Bcl-X_L is a member of the Bcl-2 family of antiapoptotic regulatory proteins, which plays a critical role in the control of programmed cell death. The 5′upstream region of the murine bcl-X gene contains five different promoters (promoters P1–P5) which exhibit a tissue-specific pattern of promoter usage [27] and six different Bcl-X isoforms can be generated by alternative splicing. It was previously demonstrated that the fourth promoter (P4) contains two PREs located at positions −317 and −291 upstream the transcription start site [28]. PR and glucocorticoid receptor (GR) act on the same common hormone responsive element (HRE) on DNA, designated PRE or GRE depending on the receptor involved [29–31]. In this latter study, the authors showed that the synthetic glucocorticoid dexamethasone induced GR and RNApolII recruitment to these HREs evidencing that hormone treatment transcriptionally activates this region. It has also been demonstrated that the aforementioned HREs confer progestin responsiveness to the promoter when assessed in gene reporter assays [28] but PR interaction with this promoter sequence remains to be investigated. Human bcl-X promoter was not reported to harbor PREs in the proximal region. In addition, accumulated findings, including ours, indicate that Stat3 activation contributes to Bcl-X_L upregulation [16,32–34]. The precise sequences involved in the transcriptional regulation of bcl-X promoter by Stat3 have not been identified [34] but no functional Stat3 binding sites were reported in the bcl-X proximal promoter. $p21^{CIP1}$ was originally described as a cyclin-dependent kinase inhibitor involved in the blockage of cell cycle progression. The role of $p21^{CIP1}$ in breast cancer is complex and its upregulation is associated with resistance to apoptosis induced by chemotherapeutic agents [35]. This effect is due to the fact that $p21^{CIP1}$ expression prevents cells from entering M phase of the cell cycle, where they would become susceptible to the cytotoxicity exerted by the chemotherapeutic agents [36]. Accumulating evidence indicates that both proteins are upregulated by progestins in breast cancer cells [16,23,28,37,38]. Our present findings identify progestin-activated Stat3 as a coactivator of PR in its classical and nonclassical transcriptional mechanism of action in breast cancer cells.

2. Experimental

2.1. Animals and tumors

Experiments were carried out with virgin female BALB/c mice raised at the Institute of Biology and Experimental Medicine of Buenos Aires. All animal studies were conducted as described previously [11] and in accordance with the highest standards of animal care as outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institute of Biology and Experimental Medicine Animal Research Committee. The C4HD tumor line is of ductal origin, expresses PR and ER, and lacks glucocorticoid receptor expression [39].

2.2. Antibodies

The following antibodies were used for Western blots: phospho-Stat3 (Tyr705) (B-7), total Stat3 (C-20), Bcl-X_L (S-18), p21 (C-19), and Sp1 (PEP2) all from Santa Cruz Biotechnology (Santa Cruz, CA); PR (clone hPRa7), and actin (clone ACTN05), from Neomarkers (Freemont, CA), phospho-PR (Ser294) from Affinity BioReagents (Rockford, IL) and HRP-conjugated secondary antibody from Vector Laboratories (Burlingame, CA). The antibodies used for immunoprecipitation experiments, chromatin immunoprecipitation (ChIP), and sequential ChIP assays were the mouse monoclonal anti-PR (clone hPRa7), the rabbit polyclonal anti-Stat3 (C-20) and the anti-Sp1 (PEP2) antibodies. IgG (Sigma–Aldrich) was used as negative control.

2.3. Cell cultures and treatments

Primary cultures of epithelial cells from C4HD tumors were performed as described [39]. T47D cells were obtained from American Type Culture Collection. T47D-Cat0 cells were kindly provided by Dr. Nancy Weigel (Baylor College of Medicine, TX, USA) [40]. Medroxyprogesterone acetate (MPA) and RU486 used for the treatments of C4HD and T47D cells were purchased from Sigma–Aldrich (Saint Louis, MI).

To evaluate the effects of RU486 on MPA-induced Bcl-X_L and $p21^{CIP1}$ expression, PR transcriptional activation and Sp1, PR and Stat3 interaction, cells were preincubated for 90 min with RU486, before addition of MPA.

2.4. Western blots and immunoprecipitations

Lysates were prepared from cells subjected to the different treatments and proteins were subjected to SDS-PAGE as previously described [11]. Membranes were immunoblotted with the antibodies detailed in each experiment. When phospho(p)-protein antibodies were used, filters were reprobed with total protein antibodies. Signal intensities of pStat3 and pPR bands were analyzed by densitometry and normalized to total protein bands. Similarly, signal intensities of PR, Stat3, Bcl-X_L and $p21^{CIP1}$ bands were normalized to actin bands. Data analysis showed a significant increase in Bcl-X_L and $p21^{CIP1}$ levels by MPA treatment as compared to non-treated cells, and a significant inhibition of MPA-induced proteins expression when Stat3 siRNA was used ($P < 0.001$). The Nuclear and Cytoplasmic Extraction Reagents technique (Pierce Biotechnology) was performed as per manufacturer's instructions. Nuclear association between PR and Stat3 was studied by performing coimmunoprecipitation experiments using 200 μg of nuclear protein lysates as described [11]. The association among Sp1, PR, and Stat3 was studied by performing coimmunoprecipitation experiments. Total cell lysates (1 mg protein) were immunoprecipitated using a Sp1 (PEP2) antibody both from Santa Cruz Biotechnology, Santa Cruz, CA). As a control for the specificity of the protein interaction, lysates were also immunoprecipitated with normal rabbit IgG.

2.5. Plasmids and transient transfections

The luciferase reporter plasmid downstream the murine bcl-XP4 promoter which contains two PREs at sites −317 and −291 relative to the initiation of transcription (bcl-XP4-luc), and construct containing a deletion of 95 bp including both PREs were kindly provided by Dr. Adalí Pecci (Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires) [28]. The luciferase reporter vector controlled by the human $p21^{CIP1}$ promoter region which contains two progestin responsive Sp1 binding sites at positions −83 and −65 relative to the initiation of transcription (p21P93-S-Luc) was kindly provided by Dr. Xiao-Fan Wang (Duke University Medical Center, NC, USA). The *Renilla* luciferase expression plasmid RLCMV was obtained from Promega (Madison, WI). Dominant negative Stat3 expression vector, Stat3Y705-F, which carries a tyrosine to phenylalanine substitution at codon 705 that