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Genistein-Mediated Attenuation of Tamoxifen-Induced Antagonism from Estrogen Receptor-Regulated Genes

Janice A. Schwartz,*,†,[1] Gouzhen Liu,‡ and Sam C. Brooks*,§

**Karmanos Cancer Institute, ‡Graduate Program in Cancer Biology, †Department of Physiology, and §Department of Biochemistry and Molecular Biology, School of Medicine, Wayne State University, Detroit, Michigan 48201*

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In this study we demonstrate that physiologic concentrations of genistein are sufficient to mediate agonism and to reverse the repressive effects of 4-hydroxytamoxifen on estrogen receptor (ERα)-responsive reporter genes. We also show that overexpression of the steroid receptor coactivator (SRC-1) potentiates transactivation by genistein-activated ERα and that coexpression of CBP (the cAMP response element binding protein coactivator) synergistically increases this signal. Exogenous expression of a nuclear receptor corepressor (NCoR) was, however, unable to alter genistein-mediated transactivation. In *in vitro* binding assays, we show that genistein, but not 4-hydroxytamoxifen, induces a direct interaction between radiolabeled ERα and a GST–SRC-1 fusion protein. More importantly, coincubation with genistein and 4-hydroxytamoxifen or genistein treatment following preincubation of the ER with 4-hydroxytamoxifen also resulted in a strong physical interaction with SRC-1. These findings imply that genistein-induced shifts in the coregulator status of ERα may be involved in transcriptional regulation and suggest that tamoxifen-mediated antagonism at ER-dependent genes is sensitive to attenuation by low levels of genistein.

Key Words: **genistein; 4-hydroxtamoxifen; estradiol; estrogen receptor α; steroid receptor coactivator-1; CBP; nuclear receptor corepressor.**

Genistein, a major constituent of soybeans, has been attributed with diverse biochemical activities which suggest that it acts as a chemopreventative (1–4). Epidemiologic studies show that the incidence of mammary and other cancers is inversely related to the dietary intake of soy (5, 6). Estradiol (E2), on the other hand, has been shown to stimulate mammary tumor growth (7), and is considered a risk factor in the development and progression of breast cancer. Tamoxifen, a partial antiestrogen with antagonistic actions in mammary tissue, has become the endocrine treatment of choice for breast cancer patients (8). Results from recent tamoxifen trials in women who are at higher than normal risk for the development of breast cancer have been very promising. Genistein has also been shown to exert antagonistic and antiproliferative actions in mammary and other tissues as well as in cultured cell lines (9–11). These effects generally occur, however, at concentrations ranging from ~10 to 100 μM, where multiple cellular targets are likely to be involved (2, 3, and refs. within, 12). At lower concentrations, genistein can act as an agonist in estrogen-dependent target cells. And, indeed, recent reports have shown that at concentrations of between 0.01 to 1.0 μM, genistein can induce pS2 mRNA expression and promote ER-positive breast cancer cell proliferation (11, 13–17). These concentrations of genistein are comparable to those found in Asians consuming a soy-rich diet (18, 19) and are, therefore, likely to be physiologically relevant. Consistent with these findings, we show that at concentrations of less than 1 μM, genistein acts as a potent agonist of ER-dependent activities in transiently transfected HeLa cells and that in the presence of 4-hydroxytamoxifen (4OHT), genistein induces transactivation to levels which are similar to those of E2. These findings point to the possibility that, at concentrations which are readily obtainable from the diet, genistein may function to diminish the antagonistic properties of tamoxifen. We also show that these genistein-mediated effects are sensitive to further modulation by exogenous coregulator overexpression.

MATERIALS AND METHODS

Cell maintenance, transfection, and CAT assays. HeLa cells were maintained in Dulbecco's modified Eagle's medium F12 (DMEM F12) supplemented with 10% fetal calf serum (Gibco BRL) plus 0.5% gentamicin (Sigma), and subcultured one time per week. For transfections, the cells were passaged into growth media containing heat-

[1] To whom correspondence should be addressed at Department of Physiology, Wayne State University School of Medicine, 540 E. Canfield, Detroit, MI 48201. Fax: 313 577 4112. E-mail: jschwart@med.wayne.edu.

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inactivated, dextran-coated charcoal-stripped serum. Transient transfections were performed using the Superfect reagent (Qiagen) according to the manufacturer's instructions. HeLa cells were cotransfected overnight with 0.5 μg HEGO-pSG5 (hERα) and 2.0 μg pEREBLCAT (estrogen responsive reporter) alone or along with 1.5 μg of either of the corresponding empty expression vectors (pBK-CMV, pCR3.1, or pCEP4) or expression vectors for hSRC-1 (pBKCMV-SRC-1), hSRC-1a (pCR3.1-SRC-1a), mCBP (pRc/RSV-CBP), or mNCoR (pCEP4-NCoR), or the indicated combinations, using enough DNA so that the total DNA concentration in each sample was maintained at 2.5 μg for hERα and pEREBLCAT alone, or 4.0μg for the hERα plus pEREBLCAT in the presence of coregulator proteins. The transfected cells were then incubated in the absence (NH, no hormone) or in the presence of either estradiol, genistein, 4-hydroxytamoxifen (4OHT), or ICI164,384, or combinations (at the indicated concentrations) for 24 h prior to harvesting. Lysates were normalized for protein concentration and assayed for CAT activity. AMBIS phosphoimaging was used for quantification. The percent conversion for each sample was expressed as either: the fold change in CAT activity compared to those of basal levels, the percent maximum (relative to induction by maximally stimulating concentrations of E2, set at 100%), or as the percent of control (arbitrarily set at 100%). The value of controls were determined from cells which had been transfected exactly as the ligand-treated cells but which had been incubated with media deprived of any hormone/ligand supplementation. These experiments were performed 2 or more times and are represents as the averages (which had <20% variation) or as the mean ± SEM. Genistein and estradiol were obtained from Sigma (St. Louis, MO), 4OHT was purchased from RBI (Natick, MA), and ICI164,384 was a gift from Dr. Wakeling (Zeneca Pharmaceuticals).

In vitro translation and GST pull-down assays. [^{35}S]Methionine labeled hERα was made by coupled transcription/translation (TNT Quick Coupled Transcription/Translation Promega, Madison, WI) using the pSG5-hERα (HEGO) plasmid as a template. The GST–SRC-1 (amino acids 1–1062) and GST–SRC-1a (amino acids 1–1442) fusion proteins were made by cloning the corresponding cDNAs into the pGEX-6p-x vector (Pharmacia Biotech, Piscataway, NJ), according to suggested protocols. Briefly, *Escherichia coli* bacterial cells BL-21(DE3)pLysE (Novagen, Madison WI), carrying the fusion protein plasmids, were induced for 1.5 h by 0.2 mM IPTG, lysed and analyzed for GST activity. Two micrograms of the Sepharose 4B-GSH conjugated fusion protein was allowed to incubate with 5 μl of the *in vitro* translated protein in 500 μl of Hepes buffer (50 mM KCl, 20 mM Hepes, 2 mM EDTA, 0.1% NP-40, 5% glycerol, 0.5% nonfat dry milk, 5 mM DTT, pH 7.9) at 4°C overnight or at 37°C for 1 h, with 10^{-6} M of each ligand. Unbound proteins were washed away by 4 stringent washes with 500 μl of the Hepes buffer. Bound proteins were eluted by boiling in 30 μl 1× SDS loading buffer and then resolved by SDS–PAGE. The gels were fixed for 30 min in the protein fixing solution, soaked in Amplify fluorographic reagent (Amersham Life Science Inc, Arlington Heights, IL) for 20 min, dried and then visualized by autoradiography. These experiments were performed two or more times.

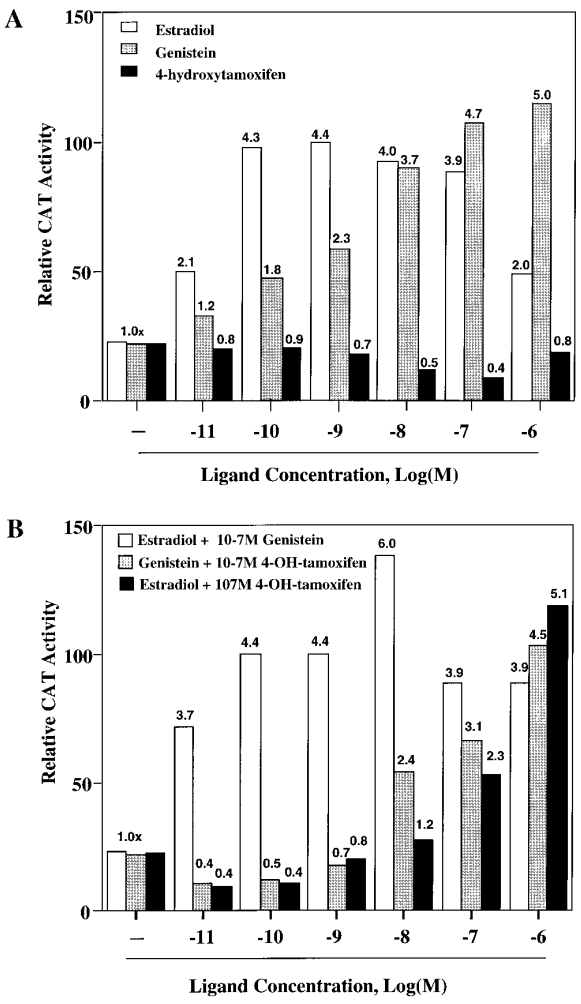

FIG. 1. Influence of genistein, relative to E2 or 4OHT, on ERα-dependent reporter expression. ER-deficient HeLa cells were transiently cotransfected with 0.5 μg of an expression vector for hERa, pSG5-HEGO, and 2.0 μg of the estrogen responsive reporter, pEREBLCAT, using Superfect (Qiagen). After an overnight incubation, the cells were washed and incubated an additional 24 h in the absence of ligand or with ethanol vehicle alone (NH, controls), or with media containing either E2, genistein, or 4OHT alone (A) or in combination (B), at the indicated concentrations, in the same experiment. CAT activities were determined from lysates which had been normalized for protein concentration and are represented here as the percent of activity obtained in response to maximally stimulating concentrations of E2, arbitrarily set at 100%. Fold change, relative to controls, are shown above each bar. The data shown indicate the average of duplicate experiments, with <20% variation.

RESULTS AND DISCUSSION

To test the genistein inducibility and transactivation potential of the human ERα (hERα), transient transfection assays were conducted using ER-deficient HeLa cells (Fig. 1). These cells were cotransfected with an estrogen responsive reporter containing one ER binding site, pEREBLCAT (20) and an expression vector for the hERα, pSG5-HEGO (21). Incubation with 10^{-9} M E2 resulted in 4.4-fold activation of this promoter, relative to untreated controls (Fig. 1A). Treatment with genistein resulted in even higher levels of ERα-driven reporter activity, reaching 5.0-fold (over control levels) at a concentration of 10^{-6} M (Fig. 1A). Notably, at concentrations as low as 10^{-8} M, genistein-stimulated transactivation was 84% of that induced by maximally stimulating concentrations of E2 (10^{-9} M), 107% at 10^{-7} M, and 113% at 10^{-6} M genistein. By contrast, 4-OHT (the active form of tamoxifen) was antagonistic in this system. Treatment with 4OHT repressed basal

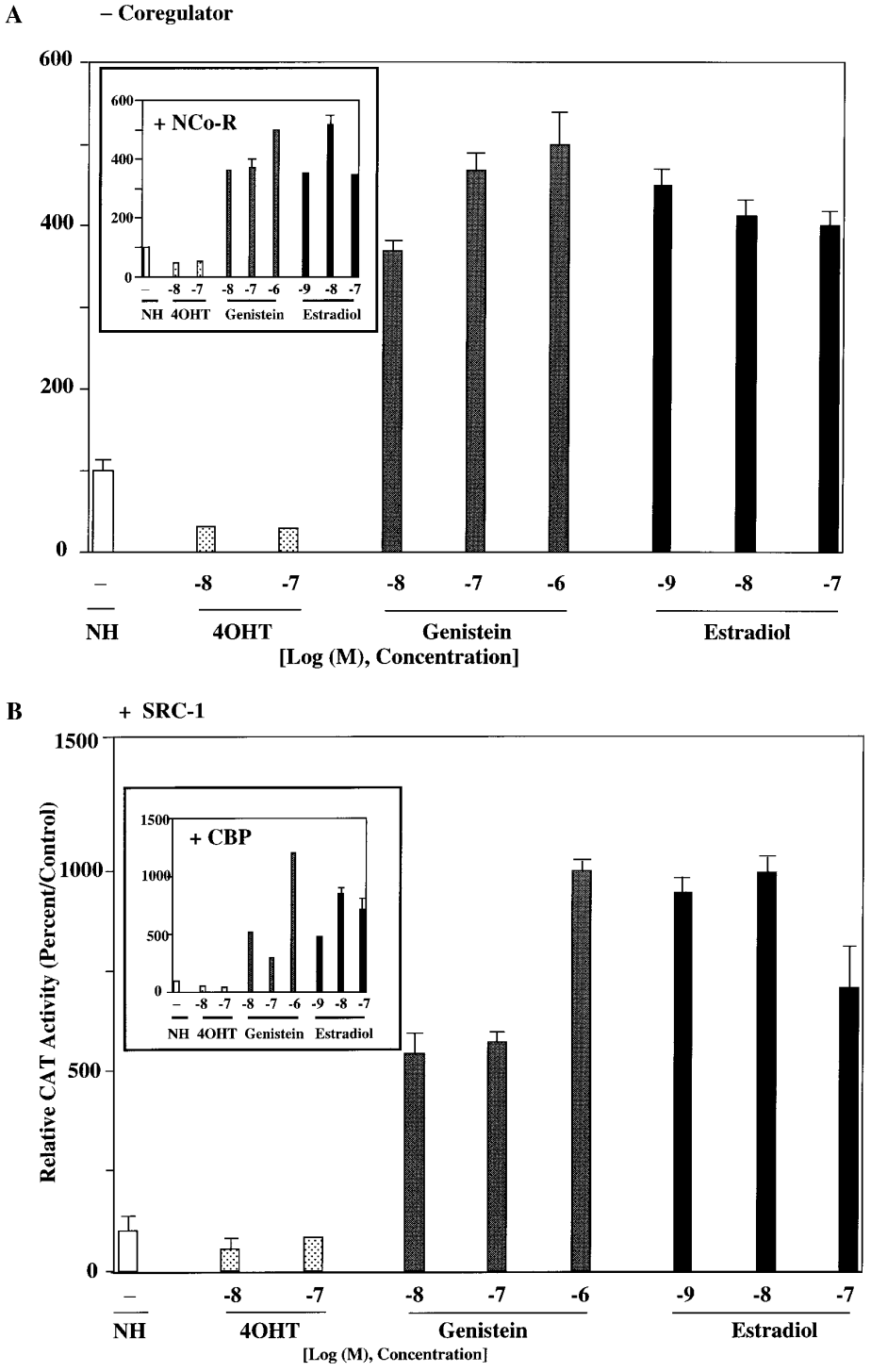

FIG. 2. Influence of coregulator coexpression on genistein-driven activation of pEREBLCAT by ERα. Hela cells were cotransfected with 2.0 μg of pEREBLCAT, 0.5 μg of pSG5-HEGO (hERα), and 1.5 μg of pCEP4 (the empty expression vector) or 1.5 μg of expression vectors for NCo-R (inset, A), SRC-1 (B), or CBP (inset B); or with 0.75 μg of SRC-1 plus 0.75 μg of CBP together (inset C), so that the total DNA concentration (4.0 μg) was constant for each sample. The transfected cells were incubated for 24 h in the absence of hormone (NH, controls) or in the presence of 4OHT, genistein, or E2 at the indicated concentrations (A & B), or with 10^{-8} to 10^{-6} M genistein in the presence of 10^{-7} M 4OHT (C). CAT activities were determined as in Fig. 1, only here the results are represented as the percent of control, which is arbitrarily set at 100%. Shown here are the mean values ± SEM. calculated from two or more independent experiments. Note that the slightly lower values obtained for genistein plus 4OHT in the absence of coregulator (− coregulator) in this group of experiments (C) compared to those shown in Fig. 1B, may have been due to the coexpression of the empty pCEP4 expression vector.

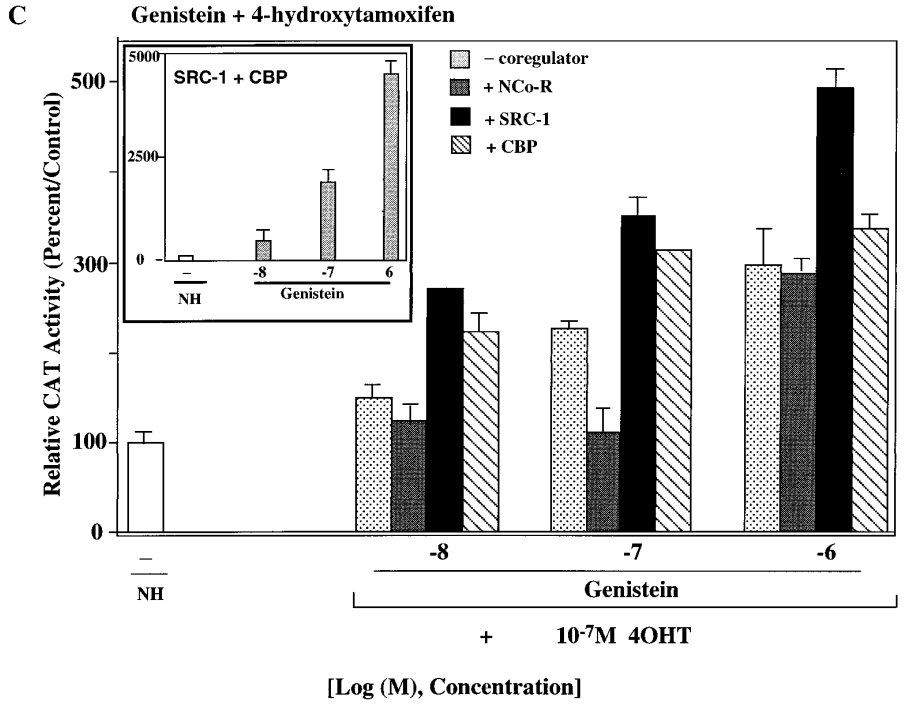

FIG. 2—*Continued*

levels of activity over the entire concentration range used, achieving maximum reductions of greater than 50% at a concentration of 10^{-7} M (Fig. 1A). These findings indicate that at low concentrations, genistein is a potent inducer of ERα-driven gene expression in transiently transfected cells.

To test for synergism between E2 and genistein and to assess the influence of genistein (in comparison to that of E2) on 4OHT-mediated repression of ERα-driven reporter expression, transiently cotransfected HeLa cells were incubated with combinations of E2, 4OHT, and/or genistein. No synergism was observed in response to cotreatment of transfected HeLa cells with E2 over a range of concentrations in the presence of 10^{-7} M genistein (Fig. 1B). Instead, non-additive increases in the levels of reporter activity were observed, with the exception of transfectants treated with very low levels of E2 (10^{-11} M) in the presence of 10^{-7} M genistein, which exhibited transactivation levels which were slightly greater than additive. Next we examined the ability of maximally repressive concentrations of 4OHT (10^{-7} M) to antagonize E2- or genistein-mediated agonism over a range of concentrations (Fig. 1B). Here we find that 4OHT exerts efficient repression of ERα-driven transcriptional activity in the presence of 10^{-11} to 10^{-8} M concentrations of E2. At higher concentrations of E2, agonism is observed even in the presence of 10^{-7} M 4OHT. Interestingly, 4OHT-mediated antagonism can also be reversed by the simultaneous administration of genistein, in a concentration-dependent manner. In the presence of 10^{-7} M 4OHT, 2.4- to 4.5-fold increases in reporter activity are observed in response to cotreatment with 10^{-8} to 10^{-6} M genistein, respectively. The results presented here suggest that low physiologic levels of genistein, such as those which may be obtained from dietary sources (3, 18, 19), may be able to attenuate and even reverse the repressive effects of 4OHT, on ERα-regulated genes. Furthermore, these findings are consistent with the recent studies of Wang and Kurzer which have shown that tamoxifen is unable to block DNA synthesis induced by low levels of genistein in breast cancer cell lines (22).

To investigate whether or not coregulatory proteins are involved in the transcriptional activities exerted by genistein-activated ERα, HeLa cells were cotransfected with expression vectors containing cDNAs for the hERα, and either CBP (23), SRC-1 (24), N-CoR (25), or an empty expression vector (pCEP4, designated "– coregulator"), along with the estrogen responsive reporter construct, pEREBLCAT. The transfected cells were treated with E2, genistein, and/or 4OHT, at the indicated concentrations, or left untreated (controls, designated "NH") and the CAT activities were determined. In Fig. 2, values for the relative CAT activities are represented as the percent of control, arbitrarily set at 100%. Figure 2A shows that, like E2, genistein acts as a potent inducer of ER-dependent transcriptional activity in the absence of coregulator and that these levels are not significantly affected by the overexpression of the nuclear corepressor, NCo-R (inset).

Coexpression of either SRC-1 or CBP (Fig. 2B and inset, respectively) further augment genistein- and E2-stimulated agonism, leaving the low transactivation levels mediated by 4OHT unaffected. When both SRC-1 and CBP are simultaneously coexpressed, they synergize to enhance genistein- (inset in Fig. 2C) as well as E2- (not shown) mediated agonism from this reporter gene. Finally, we show that in the presence of NCoR (Fig. 2C), 10^{-6} M genistein is required to overcome the repressive effects of 4OHT; in the presence of SRC-1, 10^{-8} M genistein is sufficient to overcome 4OHT-induced antagonism. Similar findings were obtained for SRC-1a, the full-length form of SRC-1 using this promoter construct, as well as others (data not shown). Collectively, these results show that the concentration of genistein required to overcome tamoxifen-mediated antagonism can be influenced by the level and composition of coregulators in the cell and imply that the consequences of genistein intake may become more significant in certain pathologic conditions where coactivator is overexpressed (26) or where changes in the interactions with corepressors predominate (27).

Next we wanted to know whether the functional activities of genistein-stimulated hERα observed *in vivo* resulted from direct physical interactions between coregulator and the genistein-activated ERα. For this we used *in vitro* protein-protein binding assays (GST pull-downs) and assessed the interactions between the ligand-activated ERα and the GST–SRC-1 (or GST–SRC-1a) fusion proteins. This strategy was employed in order to rule out potential contributions of bridging or intermediary proteins, such as those which may be present in cellular coimmunoprecipitation or 2-hybrid binding assays. Here, we examined the influence of different ligands on the ability of radiolabeled, full-length hERα (synthesized *in vitro* by quick coupled transcription/translation) to physically bind to the SRC-1 or SRC-1a coactivators. SRC-1 and -1a coactivators were synthesized in bacteria as GST fusions and then immobilized individually on an agarose matrix. Non-recombinant GST was employed in each experiment as a negative control. SDS–polyacrylamide gel electrophoresis was used to resolve the radiolabeled proteins which remained bound to the GST derivatives after washing and elution, and autoradiography was used for visualization.

Figure 3 shows that the interaction between [^{35}S]-labeled, full-length hERα and the bacterially produced GST–SRC-1 recombinant increases in response to incubation with either estradiol (lane 2) or genistein (lane 5), and decreases in response to treatment with either of the antiestrogens: 4-hydroxytamoxifen or ICI164,384 (lanes 3 and 4 respectively), compared to the levels observed in the absence of ligand (lane 1). Next, we examined the ability of genistein to induce ER-coactivator interactions in the presence of 4-hydroxytamoxifen. Figure 3 shows that although there is little or no interaction between radiolabeled hER and GST–SRC-1 in the presence of 4-hydroxytamoxifen alone (lane 3), coincubation with equimolar concentrations of genistein plus 4-hydroxytamoxifen (lane 6) or 4-hydroxytamoxifen preincubation (overnight, 4°C) followed by genistein treatment (lane 7), result in dramatic increases in the binding of these two proteins. As expected, coincubation with equimolar concentrations of estradiol plus 4-hydroxytamoxifen also induced a strong interaction between [^{35}S]-hERα and GST–SRC-1 (lane 8). Finally, coincubation of genistein with ICI164,384 did not promote any significant interaction between [^{35}S]-labeled hERα and GST–SRC-1 (lane 9).

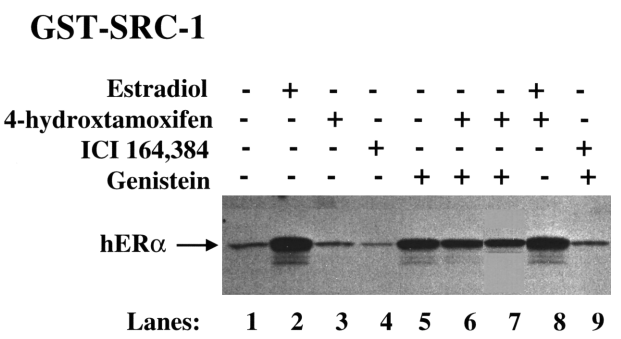

FIG. 3. *In vitro* binding interaction between hERα and SRC-1. [^{35}S]-labeled hERα was incubated with GST alone (not shown) or GST–SRC-1 in the absence of ligand (lane 1), in the presence of 10^{-6} M: E2 (lane 2), 4OHT (lane 3), ICI164,384 (lane 4), or genistein (lane 5). Also shown is the *in vitro* binding induced by: coincubation with equimolar concentrations of 4OHT plus genistein (lane 6), 4OHT plus E2 (lane 8), or genistein plus ICI164,384 (lane 9); or preincubation with 4OHT (overnight, 4°C) followed by an additional incubation (1 h, 37°C) with genistein (lane 7), each at 10^{-6} M. These experiments were done two or more times, shown is a representative experiment.

We also tested interactions between the [^{35}S]-labeled, *in vitro* translated, full-length hERα and bacterially produced GST–SRC-1a fusion protein, representing the full-length version of SRC-1 (28), in the presence and absence of these ligands (Fig. 4). Here, we show that although there is no interaction between [^{35}S]-labeled ERα and GST alone (lane 1), basal interactions (in the absence of any ligand) with GST–SRC-1a (lane 2, Fig. 4) occurred to a greater extent than those which were obtained for the GST–SRC-1-[^{35}S]-hERα interaction (lane 1, Fig. 3). Nevertheless, incubation with E2 or genistein increased the interaction between [^{35}S]-hERα and GST–SRC-1a (lanes 3 and 6, Fig. 4), while treatment with 4-hydroxytamoxifen or ICI164,384 decreased it (lanes 4 and 5, Fig. 4). These latter findings are comparable to those which were observed for the interaction between [^{35}S]-labeled hERα and GST–SRC-1 in response to incubation with the same ligands (Fig. 3).

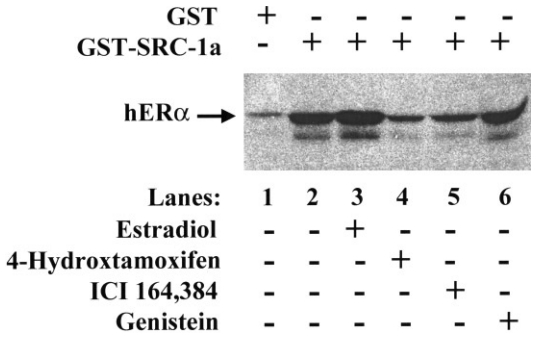

FIG. 4. *In vitro* binding interaction between hERα and SRC-1a. GST interaction assays were performed exactly as described in the legend to Fig. 3, only here the *in vitro* translated/transcribed [^{35}S]-labeled hERα was incubated with either GST alone (lane 1) or the GST–SRC-1a recombinant (lanes 2–6) in the absence or presence of the indicated ligands, each at 10^{-6} M. Shown is a single representative experiment.

In sum, our results indicate that at concentrations ranging from 10 nM to 1.0 μM, genistein functions as a pure agonist in stimulating ERα-dependent transactivation of reporter genes and that these effects are sufficiently potent to mediate dose-dependent attenuation of antagonism by 4OHT. Additional influences were observed in the presence of coregulatory protein overexpression.

SRC-1, for example, functioned to lower the concentration of genistein required to mediate agonism from the pEREBLCAT reporter in the presence of 4OHT, while NCoR seemed to stabilize the antiestrogenic effects of 4OHT despite the presence of genistein. Furthermore, our *in vitro* results show that genistein promotes direct binding of the hERα to SRC-1 and that it does so in the absence as well as in the presence of 4OHT. The genistein-mediated agonism which was observed in cell culture, therefore, may rely upon direct physical interactions between the genistein-activated ER and relevant coregulator proteins. Currently, these investigations are being expanded to include the hERβ form of the receptor.

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