Expression of Nuclear Receptor Interacting Proteins TIF-1, SUG-1, Receptor Interacting Protein 140, and Corepressor SMRT in Tamoxifen-resistant Breast Cancer

Christina M. W. Chan, Anne E. Lykkesfeldt, Malcolm G. Parker, and Mitchell Dowsett

Department of Academic Biochemistry, Royal Marsden Hospital, Fulham Road, London SW3 6JJ, United Kingdom [C. M. W. C., M. D.]; Department of Tumor Endocrinology, Institute of Cancer Biology, Danish Cancer Society, Strandboulevarden 49, DK-2100 Copenhagen, Denmark [A. E. L.]; and Molecular Endocrinology Laboratory, Imperial Cancer Research Fund, WC2A 3PX, London, United Kingdom [M. P.]

ABSTRACT

Regulation of gene transcription as a consequence of steroid receptor-DNA interaction is mediated via nuclear receptor interacting proteins (RIPs), including coactivator or corepressor proteins, which interact with both the receptor and components of the basic transcriptional unit and vary between cell types. The aim of this study was to test the hypothesis that resistance of some breast carcinomas to tamoxifen was associated with inappropriate expression of some of these RIPs. Using Northern analysis, we observed no significant difference between the amount of either TIF-1 or SUG-1 mRNA expressed in parental MCF-7 and MCF-7 tamoxifen-resistant cell lines. However, the expression of RIP140 mRNA was lower in the resistant cell line and in the presence of estradiol, the level of RIP140 mRNA was higher in the resistant cells but not in the parental cells. In a cohort of 19 tamoxifen-resistant breast tumor samples, there was no significant difference in the level of the RIP140 and TIF-1 and corepressor SMRT mRNA compared with tamoxifen-treated tumors (n = 6) or untreated tumors (n = 21). However, SUG-1 mRNA was lower in resistant breast tumors. These data provide no support for increased expression of these RIPs or decreased expression of corepressor SMRT for being a mechanism for resistance of breast tumors to tamoxifen.

INTRODUCTION

Approximately 70% of breast cancers are ER2 positive, but only 60% of these ER-positive tumors respond to tamoxifen therapy. Thus, more than half of all breast carcinomas are de novo or intrinsically resistant to tamoxifen, a problem of great clinical significance. In general, these tumors are resistant to all means of endocrine therapy. In advanced disease, patients responsive to tamoxifen eventually relapse with so-called acquired tamoxifen resistance. In contrast to de novo resistance, tumors with acquired resistance frequently respond to other endocrine therapies, such as aromatase inhibitors or megestrol acetate (1). In most cases, loss of ER cannot explain tumor progression during tamoxifen treatment (2, 3). Indeed, the continued presence of ER would seem to be a prerequisite for the second- and third-line responses. There is now a body of evidence that, at least in some patients, this resistance results from the tumor becoming sensitized to the agonist activity of tamoxifen. For example, the growth of a tamoxifen-resistant MCF-7 xenograft and cells taken from metastatic breast cancer patients with acquired tamoxifen resistance was found to be enhanced by both estradiol and tamoxifen (4–6). In both cases, the growth was suppressed by the pure AE ICI182780. Recent progress in understanding the molecular mechanism of ER signaling has suggested that differential expression of coactivators and/or corepressors might be responsible for this sensitization to tamoxifen agonist activity.

The present model of estrogen action suggests that estradiol binds to the ER, leading to dimerization and to tight binding of ER to its specific DNA target, the estrogen response element. Once the receptor has bound to the estrogen response element, it stimulates transcription from the adjacent promoter. This process is thought to be brought about by the receptor making protein-protein contacts between DNA-bound dimeric ER and other components of the transcriptional complex (e.g., TATA box factor/RNA polymerase II), leading to the formation of an activated transcription complex. Various steroid hormone receptors, including ER, have been shown to bind to TFIIB (7, 8). Additionally, ER has been shown to bind in vitro to hTAF, hTAF30, together with a subset of TFIID complexes (9). hTAF30 interacts with AF-2 in region E of the human ER but not with ER AF-1. TFIID cofactor complexes containing hTAF30 are required for the activity of ER AF-2.

The mechanism(s) by which AEs antagonize the effect of estrogen in target tissues has not been fully elucidated, but it is thought to be mediated by the AE-ER complex. Unlike the estrogen-ER complex, the AE-ER complex is thought to competitively inhibit the estradiol-dependent activation of AF-2 (10). In contrast, its agonistic effect may be attributable to its ability to stabilize the AF-1 binding site on DNA and thus lead to AF-1 activation (11). Various studies have shown that estradiol and tamoxifen induce different conformations of the HBD/AF-2 region (12–15), and this may account for the differential ability of estrogens and AE to activate the AF-2 trans-activation function.
Recent discoveries demonstrate an important amplification role for nuclear RIPs including coactivators and corepressors (16). A number of proteins with molecular weights of $M_1$ 160,000, $M_2$ 140,000, and $M_3$ 80,000 have been reported to interact with the AF-2 region of some nuclear receptors (17–20) and have been shown to function as transcriptional coactivators (21). These include the SRC-1 (22) and related proteins such as p/CIP/ACTR/AIB1 (20), TIF-2/GRIP1 (23, 24), RIP140 (25), SUG-1 (19), and TIF-1 (26, 27). Others including NcoR (28) (21). These include the SRC-1 (22) and related proteins such as transcriptional coactivators and corepressors. Ornate et al. (22) reported that SRC-1 enhances the transcriptional activity of multiple steroid receptors in a ligand-dependent manner. In SRC-1 knockout mice, the loss of the SRC-1 gene resulted in partial resistance to steroid hormone (30). Anzick et al. (31) also reported that coactivator AIB1 was amplified in about 10% of 105 unselected primary breast tumors analyzed and was expressed at high levels in 64%. Importantly for the present studies, Smith et al. (32) have shown that the relative expression of coactivators and corepressors can modulate 4-hydroxytamoxifen regulation of ER transcriptional activity. In parallel, Berns et al. (33) in a preliminary small clinical study found that high levels of SRC-1 in breast tumors indicate a good response to tamoxifen in recurrent breast cancer. Thus, the interaction between receptors, basal transcription factors, coactivators, and/or corepressors may play an important role in mediating hormonal regulation and/or gene expression.

Studies have also shown that these RIPs function in different manner. Although SRC-1, AIB1, and TIF-2 stimulate hormone-dependent transactivation by nuclear receptors in transient transfection assays (22, 23, 31), RIP140 and TIF-1 function weakly or not at all under these conditions (25, 27). In addition, Henntu et al. (34) showed that the replacement of lysine-366 to alanine in the COOH terminus of helix 3 in ER reduced the ability of the receptor to bind to SRC-1. However, it has no effect on RIP140, and overexpression of RIP140 increased the transcriptional activity of the mutant receptor dramatically (34). Because different RIPs may function by distinct mechanisms, depending on the nature of the nuclear receptor, it is possible that recruitment of different RIPs to a target gene would mediate nuclear receptor transcription activity via multiple pathways.

On the basis of above studies, we hypothesized that the overexpression of and/or underexpression of some of the RIPs might result in the acquisition of tamoxifen resistance in breast cancer. To test whether an increase or decrease in RIP expression might result in tamoxifen resistance, we compared the expression of TIF-1, SUG-1, RIP140, and corepressor SMRT in: (a) a tamoxifen-resistant MCF-7 cell line versus wild-type cells; and (b) a cohort of tamoxifen-resistant breast tumors versus two sets of control tumors.

**MATERIALS AND METHODS**

**Tumors.** Untreated (control) tumors were excised from 4 premenopausal and 17 postmenopausal women with primary untreated breast cancer. Short-term tamoxifen-treated tumors ($n = 6$) were obtained from postmenopausal women whose tumors had been treated with tamoxifen for 14–21 days. A series of 19 tamoxifen-resistant tumors were obtained from postmenopausal women with primary breast cancer who were treated with tamoxifen (20 mg daily) and categorized as follows: (a) AQ tumors were obtained from postmenopausal women at relapse after initially responding, according to International Union Against Cancer guidelines, after a median duration of 24 months ($n = 12$); (b) de novo tumors were from postmenopausal women who had failed to respond to tamoxifen treatment [median duration of tamoxifen treatment was 4 months ($n = 7$)]. The tumors were snap-frozen immediately after dissection by the histopathologist and stored at $-80^\circ$C thereafter. The ER content of the breast tumors was analyzed as described previously (35).

**Cell Lines.** The MCF-7 cell line was originally obtained from The Breast Cancer Task Force Cell Culture Bank, Manson Research Institute (Worcester, MA). The MCF-7 cell line was gradually adapted to grow in medium with a low serum concentration (36) and afterward maintained in phenol red-free DME/F12 medium containing 1% FCS, 2.5 mM Glutamax (Life Technologies, Roskilde, Denmark), and 6 ng/ml insulin (NovoNordic, Copenhagen, Denmark). The MCF-7/TAMR-1 cell line was established by two series of treatments with $10^{-6}$ M tamoxifen, as described previously (37). Stock cultures of MCF-7/TAMR-1 cells were propagated with $10^{-6}$ M tamoxifen in growth medium as above. One week before the experiment, the MCF-7/TAMR-1 cells were propagated in the absence of tamoxifen. For the experiment, both MCF-7 and MCF-7/TAMR-1 were grown in growth medium containing 5% charcoal-treated FCS for 6 days. Estradiol ($10^{-7}$ M), $10^{-6}$ M tamoxifen, or vehicle (0.1% ethanol) was then added to the medium, and the cells were grown for an additional 3 days. Cells were then harvested using 1 mM EDTA in calcium and magnesium-free PBS, and the pellet was stored at $-80^\circ$C until use.

**RNA Extraction.** Frozen tumor samples were pulverized in a Polytron (Kinematica GmbH, Littau, Switzerland). Total RNA from the tumor samples and cells was extracted using TRIzol (Life Technologies, Inc., Scotland, United Kingdom) according to the manufacturer’s instructions, by quantification using spectrophotometry.

**Northern Blot Analysis.** Total RNA (10 $\mu$g) was denatured and electrophoresed on a 1.0% w/v agarose gel containing 2.2 M formaldehyde, 40 mM 4-morpholinopropanesulfonic acid (pH 7.0), 10 mM sodium acetate, and 1 mM EDTA (pH 8.0). After electrophoresis, gels were then soaked in 0.05 M NaOH for 20 min, rinsed three times with distilled water, and finally soaked in 20× SSPE [3.6 M NaCl, 200 mM sodium phosphate (pH 7.7), and 20 mM Na2EDTA (pH 8.0)] for 45 min. The RNA was transferred onto Hybond-N nylon membranes (Amersham Pharmacia Biotech, St. Albans, United Kingdom), prehybridized in 5× SSPE, 50% w/v deionized formamide, 5× Denhardt’s solution (0.1% w/v PVP, 0.1% w/v BSA, and 0.1% w/v Ficoll), 0.1 mg/ml denatured sonicated salmon sperm DNA (Sigma), 6% w/v polyethylene glycol, and 1% w/v SDS at 42°C for at least 2 h. Fifty ng of TIF-1, SUG-1 (both kindly provided by Professor Pierre Chambon, Centre National de la Recherche Scientifique, INSERM, Strasbourg, France), SMRT (obtained from Dr. R. M. Evans, Howard Hughes Medical Institute, The Salk Institute, La Jolla, CA), RIP140, or GAPDH cDNA were radiolabeled using [$\alpha$-32P]dCTP (DuPont NEN, Stevenage,
United Kingdom) and an oligolabeling kit (Amersham Pharmacia Biotech, St. Alban, United Kingdom) according to the manufacturer’s instructions. Radiolabeled probe DNA (10^6 cpmp/μg of DNA) was then heat-denatured and transferred to the hybridization buffer. After hybridization at 42°C for 16–20 h, membranes were washed twice in 2× SSPE/0.1% w/v SDS at 65°C and prehybridized as above. All data were corrected for variability in loading by expressing the coactivator mRNA levels relative to GAPDH.

**RESULTS**

The amount of mRNA for TIF-1, SUG-1, RIP140 and GAPDH in the MCF-7 and MCF-7/TAM²-1 cells containing E2 and tamoxifen, and vehicle treatment is shown in Figs. 1 and 2. The data show the mean result from three individual experiments. No significant differences were observed in the amount of TIF-1 and SUG-1 mRNA expressed between the cell lines, and estradiol (E2) or tamoxifen had no significant effect on their expression. However, expression of RIP140 mRNA was lower in the resistant cell line in control conditions (P = 0.003). In the presence of E2 and tamoxifen, the level of RIP140 mRNA was increased in the resistant cells but not in the parental cells, but these changes were of small magnitude and marginal statistical significance (P = 0.11 and 0.04, respectively). Expression of the corepressor SMRT appeared to be similar in both cell lines (data not shown).

The relative mRNA levels for RIP140, SUG-1, TIF-1, and SMRT for individual breast carcinomas are shown in Figs. 3 and 4. In each case, values varied widely, even between tumors in the same group. For RIP140 mRNA levels, there were no significant differences between the control (median, 0.18), short-term tamoxifen-treated (median, 1.18), and resistant tumors (median, 0.43) (P = 0.34; Fig. 4A). Similarly, no significant differences were observed in the level of corepressor SMRT (the median values for the control, short-term tamoxifen and resistant groups were 0.22, 0.91, and 0.41, respectively; Fig. 4D). TIF-1 mRNA levels were significantly lower in short-term tamoxifen tumors (median, 3.46; P = 0.014) and approached a significantly lower level in the resistant tumors (median, 3.9) when compared with control (median, 4.66; P = 0.06; Fig. 4C).

In contrast, SUG-1 mRNA levels were significantly lower in the resistant tumors (median, 0.44) as compared with control tumors (median, 0.72, P = 0.014; Fig. 4B) and significantly higher in short-term tamoxifen-treated tumors (median, 2.06) when compared with either control (P = 0.016) or resistant tumors (P = 0.0005).

Since SUG-1 mRNA levels were significantly lower in resistant tumors, the data were also analyzed in separate groups according to the type of tamoxifen resistance: (a) AQ tumors; and (b) DN tumors. No significant differences were observed between the control and AQ groups (median, 0.57, P = 0.089), but levels were significantly lower in the DN group (median, 0.35; P = 0.016) than the control group (median, 0.44; Fig. 5).

Since de novo resistance is frequently associated with ER negativity, the breast tumors were then grouped with respect to their ER status. SUG-1 mRNA was significantly lower in the ER-negative resistant tumor group (median, 0.36) than in the ER-negative control tumor group (median, 1.70, P = 0.028; Fig. 6B). No significant differences were observed between the ER-positive control group and resistant tumor group (Fig. 6A). There were no significant differences in TIF-1, RIP140, and SMRT mRNA in either ER-positive groups or ER-negative groups (data not shown). The tumors were also subdivided according to their ER status in both control and resistant groups; only minor differences were observed (data not shown).

**DISCUSSION**

The majority of breast cancer patients will receive tamoxifen at some time during their treatment. It is now known that tamoxifen can enhance survival in patients with early breast cancer, but many of these will relapse at a later stage...
with disease, which has become resistant to this compound. An understanding of the mechanism of tamoxifen resistance both intrinsic (or de novo) and acquired will help to advance our development of better agents for breast cancer treatment. Absence of ER is accepted as the cause of most but not all de novo resistance. Several mechanisms have been hypothesized as to how acquired resistance could arise. These include loss or mutation of ER; postreceptor alterations, such as changes in signaling pathways; changes in growth factor production/sensitivity or paracrine cell-cell interactions; and altered uptake, retention, or metabolism of the AE (38). However, although some of these may contribute to resistance, none have been shown to have a substantial role in the tamoxifen resistance phenotype of human breast carcinomas.

The discovery of RIPs, including coactivators and corepressors that interact with the AF2 region of steroid receptors in a ligand-dependant manner, provides an additional potential mechanism (16, 22). It has been shown that they can enhance or repress, respectively, receptor-mediated transcription. In the absence of ligand or when bound to an antagonist, nuclear recep-

Fig. 2 Summary of mRNA expression data in the cell lines. The histograms show the expression of TIF-1, SUG-1, and RIP140 in MCF-7 and MCF-7/TAM-R-1 (TAM-R) cells treated with 0.1% ethanol (con), $10^{-9}$ M estradiol (E2), or $10^{-9}$ M tamoxifen (Tam). The mRNA levels are expressed relative to the control mRNA from MCF-7 cells. Bars, SE. *, $P = 0.003$.

Fig. 3 Representative Northern blot analysis of breast tumor RNA samples. The Northern blots were performed as described in Fig. 1. Lanes 1–4, control tumors; Lanes 5–8, tamoxifen-resistant tumors; Lanes 9–14, short-term, tamoxifen-treated tumors.
tors may recruit corepressors to repress transcription; in the presence of an agonist, nuclear receptors associate with coactivators and transcription is initiated.

It has been suggested that imbalance of expression of corepressors or coactivators may play a role in the acquisition of tamoxifen resistance (39). Smith et al. (32) showed that by transfecting coactivator SRC-1 to HepG2 cells, the transfection enhanced the agonistic effect of 4-hydroxyltamoxifen to an extent equivalent to that of estrogen. Jackson et al. (39) have identified a hinge domain-binding coactivator, L7/SPA, which increased the partial agonist activity of antagonists in tamoxifen-occupied ER. Thus, the ratio of coactivators to corepressors that can bind to the transcription complex determine whether the outcome is inhibitory or stimulatory. In hormone-resistant breast cancer in which the agonist activity of the tamoxifen predominates, the ER may be preferentially bound to coactivator. Hence, we and others have hypothesized that the acquisition of tamoxifen-resistant breast cancer might be attributable to the inappropriate expression of RIPs at the transcriptional level. Evidence supporting this has been provided by Lavinsky et al. (40), who showed that corepres-

![Fig. 4](image1.png)

**Fig. 4** Comparison of the relative mRNA levels of the coactivator and corepressor in individual control, short-term tamoxifen-treated, and tamoxifen-resistant breast tumors. Northern blot were performed as described in “Materials and Methods.” Quantification was by PhosphorImager (Molecular Dynamics). Statistical analysis was performed using the Kruskal-Wallis and Mann-Whitney tests. A, RIP140 mRNA; B, SUG-1 mRNA; C, TIF-1 mRNA; D, SMRT mRNA. *p* are from comparison with control level. Bar, median value.

![Fig. 5](image2.png)

**Fig. 5** Comparison of the relative SUG-1 mRNA according to their tamoxifen responsiveness. Resistant tumors were subdivided into AQ and DN. *p* are from comparison with control level. Bar, median value.
sor SMRT and NCoR protein levels are lowered in the whole-cell extract of tamoxifen-resistant MCF-7 human breast cancer cells than in tamoxifen-sensitive MCF-7 cells in a mouse model. Using semiquantitative RT-PCR, Berns et al. (33) observed that the levels of coactivator SRC-1 were lower in patients that did not respond to tamoxifen.

Because MCF-7 breast cancer cells are widely used as a model, we first determined whether tamoxifen or estradiol treatment would affect the expression of RIP140, TIF-1, SUG-1, and SMRT in parental MCF-7 and MCF-7/TAMR -1 cells and whether values differed between the cell lines. The expression of SRC-1 and NcoR-1 levels in these cell lines were also investigated; however, their levels were too low to be accurately quantified, and they were omitted in this series. No changes in the level of TIF-1, SUG-1, or the corepressor SMRT were observed. The levels of RIP140 were different between the two cell lines; but contrary to our hypotheses, the levels were lower in the resistant cells. Not surprisingly, this relationship was not confirmed in tamoxifen-resistant breast carcinomas because MCF-7 cells represent only one phenotype and thus cannot represent the variable phenotype in breast carcinomas. The median of RIP140 levels were similar in all three tumor groups examined. There were also no differences in SMRT and TIF-1 mRNA levels between the tumor groups. SUG-1 mRNA levels were significantly different between the resistant tumors as compared with the control, but as with RIP140 in the cell lines, the values were lower in the resistant group. The greater part of the differences in SUG-1 levels appeared to be attributable to differences in the ER-negative groups, which are almost certain to be DN. It is unclear why this might occur in tumors, which would normally be considered to be refractory to the effects of tamoxifen. One explanation might be that tamoxifen has systemic effects independent of ER status (e.g., suppression of IGF-1 levels; Ref. 41), which then impact on the SUG-1 levels, primarily in ER-negative tumors. However, to our knowledge, there are no data to support this.

If acquisition of the tamoxifen-resistant phenotype was attributable to the absence or low expression of corepressor or high expression of coactivators, one would expect to observe lower SMRT mRNA levels in resistant tumors and/or higher levels of one or more of the coactivators. However, in each case where differences were observed, these were in the opposite direction to those hypothesized.

This was a relatively small study, and a larger one would be required to confirm the absence of any difference. However, it is clear from this study that any differences between the groups must be relatively minor compared with the wide variability within the groups. There are two caveats to our negative results: (a) it remains possible that the change in the levels of these coactivators/corepressors may be at the level of translation (i.e., protein product); it is at this level that Lavinsky et al. (40) reported differences, rather than at the level of transcription; and (b) there are other coactivators and corepressors that may be more important in determining the agonist/antagonist balance of tamoxifen in breast cancer. Additional studies on the level of translation on a more complete set of modifiers on the same set of breast tumor samples will enable us to determine whether the coactivators or corepressors play a part in the acquisition of tamoxifen resistance, but for the moment, the evidence that we have gathered is negative.

ACKNOWLEDGMENTS

We thank Professor P. Chambon (Centre National de la Recherche Scientifique, INSERM, Strasbourg, France) in providing us with the TIF-1 and SUG-1 cDNA probe. We also thank Dr. R. M. Evans (Howard Hughes Medical Institute, The Salk Institute, La Jolla, CA) for the SMRT cDNA probe. We are grateful to our surgical and pathological colleagues and to Dr. Stephen R. D. Johnston for the collection of the tumor specimens analyzed in this study.

REFERENCES


Expression of Nuclear Receptor Interacting Proteins TIF-1, SUG-1, Receptor Interacting Protein 140, and Corepressor SMRT in Tamoxifen-resistant Breast Cancer

Christina M. W. Chan, Anne E. Lykkesfeldt, Malcolm G. Parker, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/5/11/3460

Cited articles
This article cites 39 articles, 16 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/5/11/3460.full#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/5/11/3460.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link:
http://clincancerres.aacrjournals.org/content/5/11/3460.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.