Targeting β-Transducin Repeat–Containing Protein E3 Ubiquitin Ligase Augments the Effects of Antitumor Drugs on Breast Cancer Cells

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Abstract

β-Transducin repeat–containing proteins (β-TrCP) serve as substrate recognition component of E3 ubiquitin ligases that control stability of important regulators of cell cycle and signal transduction. β-TrCP function is essential for the induction of nuclear factor-κB transcriptional activities, which play a key role in proliferation and survival of cancer cells and are often constitutively up-regulated in human breast cancers. Here we show that inhibition of β-TrCP either by RNAi approach or by forced expression of a dominant-negative β-TrCP mutant suppresses growth and survival of human breast cancer cells. In addition, inhibition of β-TrCP augments the antiproliferative effects of anticancer drugs such as doxorubicin, tamoxifen, and paclitaxel on human mammary tumor cells. These data provide the proof of principle that targeting β-TrCP might be beneficial for anticancer therapies. (Cancer Res 2005; 65(5): 1904-8)

Introduction

Conjugation of proteins with ubiquitin (ubiquitination) and ubiquitin-like proteins have emerged as an important mechanism in regulating neoplastic cell growth and survival (1). It has been long suggested that aberrant ubiquitination of regulatory proteins contributes to cell transformation and tumor progression and, therefore, represents potential target for anticancer therapy (2, 3). Inhibition of protein sumoylation in cells was shown to sensitize neoplastic cells to anticancer drugs (4). Suppression of proteasomal degradation by Velcade (bortezomib) is effective against multiple myeloma; the therapeutic benefits of this drug for patients with other hematologic malignancies as well as with solid tumors are currently under clinical investigation (5). A major mechanism of anticancer effects of proteasomal inhibitors is thought to be the suppression of prosurvival nuclear transcription factor κB (NFκB) due to stabilization of its inhibitors (IκB; ref. 6). Proteasomal degradation of IκB requires phosphorylation-dependent ubiquitination of IκB, which is mediated by β-transducing repeat–containing proteins (β-TrCP; refs. 7, 8). Ensuing NFκB activation contributes to many aspects of tumor development including accelerated cell cycle progression, cell proliferation, tumor initiation and promotion, angiogenesis, metastasis, etc. As a major antiapoptotic factor, it plays a pivotal role in the resistance of tumors to chemotherapy and radiation. Constitutive activation of NFκB is a hallmark of many human malignancies including breast cancer (reviewed in ref. 9). Closely related β-TrCP1 and β-TrCP2 proteins seem to play a redundant role in ubiquitination and degradation of IκB (7). Expression of β-TrCP2 (also termed HOS) is induced in human breast cancer cell lines and primary tumor samples (10). Mammary glands of the β-TrCP1 knockout mice are hypoplastic; conversely, transgenic mice expressing human β-TrCP1 under control of the mouse mammary tumor virus long terminal repeat promoter exhibit hyperproliferation of mammary epithelium concurrent with nuclear localization of NFκB p65/RelA and development of mammary carcinomas (11). These data indicate that β-TrCP may play an important role in regulating growth and survival of mammary cells and development of breast cancer. This provides justification for targeting β-TrCP to limit proliferation and survival of mammary tumor cells. However, the proof of principle for this approach has not yet been established. Here we show that inhibition of β-TrCP by short inhibitory RNA (siRNA) or by expression of a dominant-negative mutant are effective in suppressing growth and survival of human breast cancer cells alone or in combination with various chemotherapeutic agents.

Materials and Methods

Cell Culture and Drug Treatment. Human breast cancer cell lines MDA-MB-468, T47D, and MCF-7 were purchased from American Type Culture Collection (Manassas, VA). The cells were grown in DMEM/F12 (1:1) medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin mixture at 37°C and 5% CO2. Human embryonic kidney cell line 293T purchased from American Type Culture Collection were grown in DMEM with 10% fetal bovine serum, 1% penicillin-streptomycin at 37°C and 5% CO2. T47D cells stably expressing pETH tet-off regulator and hygromycin resistance marker were grown in DMEM with 10% fetal bovine serum, 1% penicillin-streptomycin at 37°C and 5% CO2. T47D cells stably expressing pETH tet-off regulator and hygromycin resistance marker were grown in the presence of hygromycin B (150 μg/mL) and G418 (800 μg/mL). Anticancer drugs doxorubicin, tamoxifen, and paclitaxel were purchased from Sigma (St. Louis, MO), dissolved in DMSO, and added to cell medium. NFκB activity was measured in cells cotransfected with κB-luciferase reporter and Renilla luciferase construct using Dual Luciferase assay (Promega, Madison, WI).

DNA Constructs, Transfection, and Retroviral Transduction. The siRNA against β-TrCP2 (siBTR2) cloned in pSilencer1.0-U6 vector (Ambion, Austin, TX) as well as control siRNA that differs from siBTR2 by two base pair substitution (siCON) were previously described (12). siRNA against β-TrCP1 (siBTR1) were generated in the same vector using 5′-TTCTCAGAGAGAGAAGACTG-3′ as a targeting sequence, pBl-G-HA-β-TrCPΔF and pBabe-puro-HA-β-TrCPΔF were constructed by cloning β-TrCP2ΔF (hemagglutinin (HA)-tagged β-TrCP2 lacking the F-box) into the pBl-G vector (for tet-dependent expression of β-galactosidase and the

Note: W. Tang and Y. Li contributed equally to this work.

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gene of interest, Clontech, Palo Alto, CA) or pBabe-puro vector, respectively. pMIGR1-β-TrCP<sup>F</sup> was constructed by ligating β-TrCP<sup>F</sup> into a bicistronic green fluorescent protein retroviral vector pMIGR1 (13), a gift from Dr. W. Pear (Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA). Tet-off regulator plasmid pETH was kindly provided by Dr. Stuart A. Aaronson (Ruttenberg Cancer Center, Mount Sinai School of Medicine, New York, NY). Cells were transduced using LipofectAMINE Plus (Invitrogen, San Diego, CA) or standard calcium phosphate method. Retrovirus-containing supernatants from 293T cells transfected with pMIGR1- or pBabe-puro constructs, as well as with vesicular stomatitis virus-G and GAG-pol plasmids, were prepared and used for transduction of breast cancer cells in the presence of polybrene (6 μg/ml) for 2 days and puromycin-resistant cells were plated for colony formation and cell accumulation WST-1 assays. Expression of endogenous β-TrCP proteins or expressed HA-tagged β-TrCP<sup>F</sup> protein was analyzed by immunoblotting using anti-β-TrCP HOS-C antibody (14) or anti-HA tag antibody (Roche, Indianapolis, IN) as described elsewhere (12, 15).

Survival of β-Galactosidase–Positive Cells. T47D tet-off cells transfected with either pB1-G empty vector or pB1-G-β-TrCP<sup>F</sup> were seeded in a 96-well plate and incubated in the presence or absence of tetracycline and in the presence of vehicle (DMSO) or anticancer drugs for 48 hours.

β-Galactosidase–positive cells were revealed by staining with 5-bromo-4-chloro-3-indolyl β-D-galactoside (Sigma) and enumerated under light microscope.

Colonies Accumulation Assay. A predetermined number of cells that yields ~100 colonies for each of the cell lines used was seeded into 6-well plates in the medium containing puromycin. Anticancer drugs or DMSO was added to the cells 24 hours later. Cells were grown for 21 days, fixed with 70% cold methanol, stained with Giemsa stain (Sigma), and the colonies of 20 or more cells were counted.

Cell Accumulation WST-1 Assay. Puromycin-resistant breast cancer cells or nontumorigenic human mammary MCF10a cells were plated in 96-well plates in the presence of puromycin. After overnight incubation, the cells were treated with anticancer drugs or DMSO and incubated for additional 72 hours. WST-1 reagents (Roche) was added to the cells and the number of live cells was estimated by measuring the absorbance at 450 nm with a microplate reader.

Apoptosis Assay. Breast cancer cells transduced with pMIGR1 retroviruses that coexpress green fluorescent protein were plated onto glass coverslips placed in 35-mm dishes. Following treatment with drugs or DMSO, the medium was removed and cells were fixed and stained with 4',6-diamidino-2-phenylindole as described previously (15). Cells (400-500) were examined in five to seven randomly selected fields and apoptotic cells exhibiting condensed and fragmented nuclei were scored.

Results and Discussion

We sought to investigate whether β-TrCP levels are important for growth and survival of human breast cancer cells using the RNAi approach that proved efficient in delineating the function of these proteins (12, 16). Whereas siRNA specific against β-TrCP1 or β-TrCP2 efficiently down-regulated exogenously expressed or endogenous β-TrCP species, knockdown of β-TrCP2 led to a more dramatic inhibition of NF-κB activity (Fig. 1A and B). Cotransfection of siRNA and pBabe-puro constructs followed by colony formation assay in the presence of puromycin revealed that knockdown of β-TrCP2 led to a statistically significant inhibition of growth in T47D cells (Fig. 1C). Combination of siBTR2 with anticancer drugs further decreased the growth of these cells. Less efficient growth suppression was observed in cells transfected with siRNA against β-TrCP1 (siBTR1; Fig. 1C). These data indicate
that β-TrCP in general and β-TrCP2 in particular are essential for the maintenance of growth and survival of human breast cancer cells.

To corroborate these results we used a tetracycline inducible system to express the dominant-negative β-TrCP<sup>D</sup>F mutant, which lacks the F-box and, hence, the ability to recruit E3 ubiquitin ligase activity. This mutant has been previously shown to target both forms of β-TrCP and to inhibit NFκB (17). Expression of such a mutant induced by tetracycline withdrawal led to a significant growth inhibition effect in all treatment groups, including DMSO, Figure 3.

**Figure 3.** Retrovirus-mediated expression of the dominant-negative β-TrCP<sup>D</sup>F mutant inhibits growth of breast cancer cells. Growth and survival of T47D cells (A), MDA-MB468 cells (B), and MCF7 cells (C) transduced with an empty pBabe-puro retrovirus (white columns) or pBabe-puro-β-TrCP<sup>D</sup>F cells (black columns) that were cultured in the presence of puromycin and with or without drugs was analyzed by colony formation assay. Average from three independent experiments (each in triplicate). *, P < 0.05, compared with empty virus (Student’s t test). A, inset, expression of HA-tagged β-TrCP<sup>D</sup>F analyzed by immunoblotting with HA antibody.

**Figure 4.** Retrovirus-mediated expression of the dominant-negative β-TrCP<sup>D</sup>F mutant promotes apoptosis in breast cancer cells. The rate of apoptosis in T47D cells (A), MDA-MB468 cells (B), and MCF7 cells (C) transduced with an empty pMIGR1 retrovirus (white columns) or pMIGR1-β-TrCP<sup>D</sup>F cells (black columns) that were cultured with or without drugs (at the indicated concentrations) for 24 hours, fixed, and stained with 4′,6-diamidino-2-phenylindole (DAPI). Apoptotic cells with condensed/fragmented nuclei were scored among green fluorescent protein (GFP)-positive cells. An example of such cell (white arrow) is shown in (B). Average from three independent experiments (each in triplicate). *, P < 0.05, compared with empty virus (Student’s t test).
with the maximum efficiency in cells treated with tamoxifen or paclitaxel (Fig. 2). These results support our findings obtained with siRNA and suggest that inhibition of β-TrCP may augment the antiproliferative effects of anticancer agents in T47D human breast cancer cells.

To investigate whether these findings could be expanded to other breast cancer cell lines we subcloned the dominant-negative β-TrCPΔF mutant in pBabe-puro retroviral vector and used it for transduction of human breast cancer cells. Attenuation of β-TrCP function by retroviral-mediated expression of β-TrCPΔF mutant led to a dramatic decrease of colony formation by estrogen-dependent MCF7 and T47D cells. Combination of β-TrCP inhibition with anticancer drugs resulted in further decrease in cell growth and survival (Fig. 3A and C). WST-1 cell accumulation assay revealed similar results (Table 1). Interestingly, inhibition of β-TrCP also decreased growth of nontumorigenic human mammary MCF10a cells and sensitized these cells to the effects of doxorubicin and paclitaxel but not tamoxifen (Table 1). Conversely, whereas tamoxifen had no effect on hormone-resistant MDA-MB-468 cells, paclitaxel but not tamoxifen (Table 1). Conversely, whereas tamoxifen had no effect on hormone-resistant MDA-MB-468 cells, paclitaxel but not tamoxifen (Table 1). Conversely, whereas tamoxifen had no effect on hormone-resistant MDA-MB-468 cells, paclitaxel but not tamoxifen (Table 1). Conversely, whereas tamoxifen had no effect on hormone-resistant MDA-MB-468 cells, paclitaxel but not tamoxifen (Table 1). Conversely, whereas tamoxifen had no effect on hormone-resistant MDA-MB-468 cells, paclitaxel but not tamoxifen (Table 1). Conversely, whereas tamoxifen had no effect on hormone-resistant MDA-MB-468 cells, paclitaxel but not tamoxifen (Table 1). Conversely, whereas tamoxifen had no effect on hormone-resistant MDA-MB-468 cells, paclitaxel but not tamoxifen (Table 1). Conversely, whereas tamoxifen had no effect on hormone-resistant MDA-MB-468 cells, paclitaxel but not tamoxifen (Table 1).

Expression of β-TrCPΔF leads to an inhibition of ubiquitination and degradation of IκB, which, in turn, results in a decreased activity of a major antiapoptotic factor, NFκB (17). Given that the decrease in growth of human breast cancer cells following inhibition of β-TrCP may result from an accelerated cell death as much as from inhibition of cell proliferation, we sought to investigate whether expression of β-TrCPΔF affects the rate of apoptosis in these cells. Indeed, retrovirus-mediated expression of β-TrCPΔF significantly increased the rate of apoptosis in T47D and MDA-MB-468 breast cancer cells even in the absence of anticancer drugs (Fig. 4A and B). Synergistic effect of β-TrCPΔF mutant with doxorubicin, paclitaxel, or tamoxifen was observed in T47D and MDA-MB-468 cells. The evidence suggests that inhibition of β-TrCP sensitizes human breast cancer cells to apoptosis. A similar effect has been previously observed in melanoma cells treated with ionizing radiation or cisplatin (15). MCF7 cells, which lack caspase 3 (18) and are known to resist inhibition of NFκB (19), were also somewhat less sensitive to apoptosis induced by the β-TrCPΔF mutant (Fig. 4C). Nevertheless, combination of β-TrCP inhibition with anticancer drugs induced an augmented apoptotic response in these cells.

The important role of the ubiquitin pathway in regulating some key regulatory events in mammary tumorigenesis prompted us to explore the inhibition of specific E3 ubiquitin ligases as a potential therapy of breast cancers (20). Our findings presented here collectively indicate that targeting β-TrCP expression and function is detrimental for growth and survival of human breast cancer cells. Furthermore, inhibition of β-TrCP amplifies the effects of anticancer drugs on these cells. Mechanisms of such sensitization are likely to include the conflict of signals from stabilized antigrowth/survival β-TrCP substrates [including IκB, IFNAR1 (21), mitotic inhibitor Emi1 (16, 22), etc.] and those β-TrCP substrates that promote growth and survival (e.g., β-catenin, prolactin receptor (12)]. Future studies will be aimed at identification of suitable inhibitors of β-TrCP function that could improve the therapeutic benefits of anticancer drugs against human breast cancer.

**Table 1. Effect of β-TrCPΔF on growth of human breast cancer cells measured by the WST-1 assay**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>T47D Transduction</th>
<th>MCF7 Transduction</th>
<th>MDA-MB468 Transduction</th>
<th>MCF10A Transduction</th>
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<td>β-TrCPΔF</td>
<td>Vector</td>
<td>β-TrCPΔF</td>
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<td>1248 ± 76</td>
<td>516 ± 19*</td>
<td>780 ± 49</td>
<td>559 ± 39†</td>
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<td>Doxorubicin</td>
<td>1 nmol/L</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
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<td></td>
<td>5 μmol/L</td>
<td>46 ± 6</td>
<td>17 ± 3*</td>
<td>217 ± 38</td>
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<tr>
<td>Tamoxifen</td>
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<td>456 ± 99</td>
<td>137 ± 19*</td>
<td>81 ± 18</td>
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<td>15 μmol/L</td>
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<td>NT</td>
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</tr>
<tr>
<td>Paclitaxel</td>
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<td></td>
<td>250 nmol/L</td>
<td>532 ± 54</td>
<td>217 ± 27*</td>
<td>363 ± 64†</td>
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</table>

NOTE: Cell proliferation is measured by cell/WST1 reagent–generated absorbance at 450 nm minus the background (an absorbance of a tissue culture well containing medium without cells). Average data from three independent experiments (each in triplicate) ± SDs are shown.

Abbreviation: NT, not tested.

*P < 0.01, compared with cells transduced with empty vector (Student’s t test).

†P < 0.05, compared with cells transduced with empty vector (Student’s t test).

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