RACK1 and CIS Mediate the Degradation of BimEL in Cancer Cells

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RACK1 is a 7-WD motif-containing protein with numerous downstream effectors regulating various cellular functions. Using a yeast two-hybrid screen, we identified dynein light chain I as a novel interacting partner of RACK1. Additionally, we demonstrated that RACK1 formed a complex with DLC1 and Bim, specifically BimEL, in the presence of apoptotic agents. Upon paclitaxel treatment, RACK1, DLC1, and CIS mediated the degradation of BimEL through the ElonginB/C-Cullin2-CIS ubiquitin-protein isopeptide ligase complex. We further showed that RACK1 conferred paclitaxel resistance to breast cancer cells in vitro and in vivo. Finally, we observed an inverse correlation between CIS and BimEL levels in both ovarian and breast cancer cell lines and specimens. Our study suggests a role of RACK1 in protecting cancer cells from apoptosis by regulating the degradation of BimEL, which together with CIS could play an important role of drug resistance in chemotherapy.

Bim (Bcl-2-interacting mediator of cell death), a BH3 domain-containing protein, is expressed predominantly in hematopoietic, neuronal, and epithelial cells (1). Bim has at least 18 different splicing variants, among which BimEL (extra long), BimL (long), and BimS (short) are the three major isoforms (2). BimL and BimEL expression are tightly regulated at both the transcriptional and posttranslational levels. Transcriptionally, growth factors can down-regulate Bim mRNA expression through the Akt-FOXO3 pathway (3). Posttranslationally, under normal conditions, Bim is regulated either by binding to microtubules via DLC1 (dynein light chain 1) (4) or by directly binding to 14-3-3 (5). Upon apoptotic stimulation, Bim is released from the microtubules and translocates to the mitochondrial outer membrane. There, Bim promotes apoptosis (4) either through inhibition of anti-apoptotic Bcl-2 proteins, such as Bcl-2, Bcl-xL, and Mcl1 (6), or through activation of pro-apoptotic proteins, such as Bax (7). It has been shown that Bim functions as a tumor suppressor and is also a determinant in paclitaxel sensitivity in both renal carcinoma (8) and non-small cell lung carcinoma (9). Bim-null mice are generally healthy and fertile, although the number of Bim−/− offspring is only about 50% of the normal counterparts. The major defect of these mice is the dysregulation of leukocyte homeostasis, leading to systemic lupus erythematosus-like autoimmune disease in aged mice (10).

RACK1, a 7-WD motif-containing protein, regulates numerous cellular properties through its multiple downstream effectors. RACK1 is an important component of the ribosome and has been shown to regulate protein synthesis (11, 12). Through different signaling pathways, RACK1 is involved in controlling apoptosis and contributes to tumor growth in vivo. First, RACK1 has been shown to mediate c-Jun N-terminal kinase activation via protein kinase C and promote melanoma growth in nude mice (13). Second, RACK1 forms a complex with insulin and insulin-like growth factor 1 receptors to regulate STAT3 activation and mediates insulin receptor/insulin-like growth factor 1 receptor-induced protection against apoptosis of ovarian cancer cells (14). Additionally, RACK1 interacts with p73 and prevents p73-mediated cell death (15). Finally, RACK1 interacts with E1A and rescues E1A-induced yeast growth inhibition and mammalian cell apoptosis (16). The expression level of RACK1 is elevated during angiogenesis (17) and in colon carcinoma, non-small cell lung carcinoma (17), and melanomas (13). These observations suggest a positive role of RACK1 in protecting cells from apoptosis and in promoting tumorigenesis. RACK1 has been reported as an E3 ligase component to mediate the degradation of ΔNp63α, a member of the p53 family (18), and to interact with ElonginC and mediate the degradation of HIF-1α (19), suggesting a role of RACK1 in proteasome-mediated protein degradation.

The specificity of proteasome-mediated protein degradation is determined by distinct E3 ligase complexes. The SOCS (suppressor of cytokine signaling)-box-containing E3 ligase family members are composed of ElonginB/C and Cullin2 or Cullin5 and SOCS-1 or SOCS-3. Together, they form the ECS (ElonginB/C-Cullin-ŠSOCS-box)-Roc complex (20). Besides SOCS-1 and

4 The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; SH, Src homology; HA, hemagglutinin; HIF, hypoxia-inducible factor; GST, glutathione S-transferase; siRNA, small interfering RNA; FACS, fluorescence-activated cell sorter; CMV, cytomegalovirus.
SOCS-3, CIS (cytokine-inducible Src homology 2 domain-containing protein) also has the SOCS-box domain and could potentially be part of the E3 complex (21). Overexpression of CIS has been shown to suppress BCR/ABL-mediated cell transformation, whereas proteasome inhibitors relieved the suppressive phenotype, hinting at the involvement of CIS in proteasome-mediated protein degradation (22). CIS has been shown to be overexpressed in breast cancer lines and in breast carcinomas (23). In the CIS transgenic model, the mice displayed aberrantly increased CD4+ T cell population, prolonged survival of T cells after T cell receptor activation (24), and shifted Th1 versus Th2 T cell balance (25). This implies an independent role of CIS other than that in inhibiting the well-known cytokine signaling, such as that mediated by interleukin-2, interleukin-3, and growth hormone. CIS transgenic mice resemble the phenotype of Bim-null mice in terms of T cell proliferation and survival (10), pointing to a functional relationship between CIS and Bim.

To identify novel interacting partners of RACK1, yeast two-hybrid screening was performed. We found that DLC1 interacted with RACK1. Subsequently, we observed that DLC1 interacted with RACK1 upon apoptotic stimulation in mammalian cells. Furthermore, we demonstrated that RACK1 formed a complex with DLC1 and BimEL in the presence of paclitaxel. Moreover, RACK1 promoted proteasome-mediated BimEL degradation via a Cullin/CIS E3 ligase complex. Our study suggests that RACK1 plays an important role in protecting stress or apoptotic agent-treated cancer cells from apoptosis through degradation of BimEL, which may contribute to tumor formation in vivo and drug resistance during cancer therapy.

**EXPERIMENTAL PROCEDURES**

**Cells and DNA Transfection**—Human embryonic kidney (HEK) 293T cells, ovarian cancer line SKOV3, colon carcinoma-derived ovarian metastasis line SW626, and breast cancer stable line MDA-MB-468, MCF7, and MCF7-I4 were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (Sigma). Briefly, MCF7-I4 cells were derived from MCF7 by consecutive cycles of selection of invasive MCF7 cells through a Matrigel-coated Boyden chamber (26). SKOV3, SW626, and MCF7 were transfected with Lipofectamine 2000 according to the manufacturer’s (Invitrogen) instructions, HEK293T cells were transfected by calcium phosphate coprecipitation methods, and MB-MDA-468 cells were transfected by FuGENE reagent (Roche).

**Plasmids and Their Construction**—phEF-RACK1-HA has been described previously (27). pEF-BimEL (mouse) was kindly provided by Dr. David C. Huang (University of California, Berkeley). Expression plasmids containing CIS were kindly provided by Drs. Tracy Willson and Douglas Hilton. cDNA of human DLC1 was purchased from the American Type Culture Collection (ATCC 927281) and cloned into the phEF-Neo vector. pcDNA-HA-ubiquitin and HA-Cullin2 constructs were generous gifts from Dr. Zhen-Qiang Pan at Mount Sinai School of Medicine.

**Antibodies**—Anti-RACK1, goat anti-rabbit horseradish peroxidase, and goat anti-mouse horseradish peroxidase were purchased from BD Biosciences. Anti-GST antibody was from Amersham Biosciences. Anti-CIS antibody was from Santa Cruz Biotechnology. Anti-Bim antibody was purchased from Chemicon. Anti-FLAG, anti-tubulin, rabbit anti-mouse secondary antibody, and anti-rat horseradish peroxidase antibodies were purchased from Sigma. Anti-glyceraldehyde-3-phosphate dehydrogenase and anti-lamin B1 antibodies were purchased from Biodesign Co. Anti-HA antibody was purchased from the Hybridoma Center at Mount Sinai School of Medicine.

**Preparation of Cell Lysates, Immunoprecipitation, SDS-PAGE, and Immunoblotting**—For direct immunoblot analysis, cell lysates were prepared with ice-cold radioimmune precipitation assay buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.4 mM phenylarsine oxide, and 25 mM NaF). For coimmunoprecipitation, cell lysates were prepared with Nonidet P-40 lysis buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM EDTA, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, and 1% aprotinin). Cell lysates or immunoprecipitates were separated by SDS-PAGE and immunoblotted with appropriate antibodies as described (14, 27).

**RNA Interference**—Double-stranded siRNA construction and synthesis as well as pRS-shRNA of RACK1 for preparation of MCF7-I4 stable clones have been described previously (13, 14). The construction and synthesis of double-stranded siRNA of CIS and DLC1 were according to the manufacturer’s instruction (Ambion). The target sequence for the CIS siRNA template is 5’-AATGTACGCATTGAGTATGCC-3’; the target sequences for DLC1 are 5’-AACATAGAGAAGGACATTGC-3’ and 5’-AACCTCGGTATGTGACA-3’.

**Annexin V Staining Assay**—MCF7-I4 cells that stably overexpress RACK1 and control phEF clones were seeded at 1 × 10^6 cells and cultured at 37 °C overnight. 200 nM paclitaxel was added into the medium to treat the cells for 72 h. Cells were collected and double-stained with propidium iodide and annexin V-fluorescein isothiocyanate according to the manufacturer’s instructions (BD Biosciences). FACS analysis was performed to count the apoptotic cells that were stained with annexin V but not with propidium iodide.

**Colony Formation Assay and Anoikis Assay**—MCF7-I4 cells that stably overexpress RACK1 or MCF7 cells transiently transfected with RACK1 siRNA or CIS siRNA were trypsinized, counted, and resuspended. 5 × 10^4 cells were used for a colony formation assay (27) with or without addition of paclitaxel (10 nM), and 10^6 cells were used for anoikis assay as described (28). For quantifying the colony formation assay, the total area of colonies was quantified by ImageTool software; for anoikis assay, cells were stained with trypan blue, and dead cells were counted with a hemocytometer.

**In Vivo Tumor Growth**—BALB/c nude mice were injected with 1 × 10^6 MCF7-I4-phEF or MCF7-I4-RACK1–3 cells (five mice in each group) in the flank subcutaneously. Tumor growth was monitored by measuring three dimensions and calculated as follows: tumor volume (mm^3) = length × width × height/0.52. 18 days after injection, mice were injected intraperitoneally with either blank vehicle solution or 5 mg/kg paclitaxel (stock solution is formulated with 50% ethanol and 50% Cremophor EL (10 mg/ml), diluted with 0.9% sodium chloride to 0.5 mg/ml for injec-
tion). At the end point, mice were sacrificed, and tumors were dissected for tumor weight measuring and protein extraction for biochemical analysis. In all experiments, a two-tailed t test assuming equal variances for non-paired samples was used with \( p < 0.05 \); the exact \( p \) values were indicated in individual figures.

The animal experiments were performed under the protocol approved by the Institutional Animal Care and Use Committee of Mount Sinai School of Medicine. The collection of anonymous tumor samples was according to the institutional review board-approved waived human subject protocol at the University of Southern Florida.

**RESULTS**

RACK1 Associated with DLC1 and BimEL upon Apoptotic Stimulation—To identify novel interacting partners for RACK1, we performed a yeast two-hybrid assay and found that DLC1 interacted with RACK1. Coincubation assay was carried out in HEK293T cells transiently transfected with FLAG-DLC1 to confirm the yeast two-hybrid result. The interaction between RACK1 and DLC1 was detected upon treatment of the cells with UV irradiation, paclitaxel, or staurosporine (Fig. 1A). Based on this observation and a previous report of the Bim-DLC1 complex (4), we hypothesized that RACK1 may be involved in the regulation of Bim-DLC1-related apoptosis. To test our hypothesis, we first examined whether RACK1 also interacted with Bim. In MCF7 breast cancer cells, endogenous RACK1 was able to be coimmunoprecipitated with anti-Bim antibody, which was greatly enhanced upon paclitaxel or staurosporine treatment (Fig. 1B). As previously reported by Puthalakath et al. (4), endogenous DLC1 associated with BimEL constitutively in HEK293T cells (Fig. 1C). Interaction between endogenous DLC1 and RACK1 was significantly
enhanced by paclitaxel treatment (Fig. 1C). To examine whether RACK1 can interact with DLC1 and BimEL directly, in vitro binding assays were carried out with GST-DLC1, His-RACK1 or its C-terminal deletion mutant His-WD1–4, the N-terminal deletion mutant His-WD5–7, and His-BimEL. These constructs were expressed and purified from Escherichia coli. An equal amount of GST-DLC1 was incubated with GST (negative protein control), RACK1, or its deletion mutants. His-RACK1 and His-WD1–4 directly interacted with GST-DLC1 (Fig. 1D, left panel). His-WD5–7 failed to pull down DLC1 (Fig. 1D, left panel). Because His-WD1–4 is able to bind to GST-DLC1, we have focused our initial effort within this sequence. According to a predicted model of RACK1 (29), we synthesized a peptide, \textsuperscript{138}CKYTVQDES\textsuperscript{146}, including the entire outskirt \(\beta\) sheet of blade 3 and named pWD4. We found that pWD4 was able to inhibit the RACK1-DLC1 interaction in a dose-dependent manner (Fig. 1D, right panel), suggesting that it may overlap with the RACK1 binding sequence for DLC1. By contrast, an unrelated peptide, pSH2, had no effect on the RACK1-DLC1 interaction. RACK1 also interacted with BimEL directly in vitro, which was further enhanced in the presence of DLC1 (Fig. 1E). The interaction between RACK1 and BimEL was also through the N-terminal portion of RACK1, the WD1–4 domain (supplemental Fig. S1). These in vitro binding data support the intracellular association of these proteins described above and suggest that RACK1 interacted directly with both DLC1 and BimEL.

Because Bim is sequestered to microtubules by DLC1 and released to mitochondria upon apoptotic stimulation, we tested whether RACK1 is involved in this process. To this end, velocity sedimentation in a glycerol gradient was performed with cell lysates from MCF7 cells and HEK293T cells overexpressing FLAG-DLC1. Under normal conditions, BimEL distributed along with DLC1 and \(\beta\)-tubulin, confirming that BimEL is sequestered to microtubules through DLC1 (supplemental Fig. S2A); upon paclitaxel treatment, both DLC1 and BimEL shifted from the heavier to the lighter fractions 16–20 (peaked at 18–19 and 18–20 for BimEL and DLC1, respectively) and corresponded to the sedimentation positions of a mitochondrial outer membrane protein, Tom20 (supplemental Fig. S2A). No global distribution change of tubulin, RACK1, BimL, and BimS (data not shown) was observed, suggesting the paclitaxel-induced release of BimEL and DLC1 from microtubules was specific in these two cell lines. To detect the distribution of RACK1-BimEL-DLC1 complexes, a coimmunoprecipitation experiment was done with the glycerol gradient fractions. Little interaction between RACK1 and BimEL (supplemental Fig. S2B) or between RACK1 and DLC1 (supplemental Fig. S2C) was detected in the controlled cells; however, paclitaxel treatment led to the interaction between RACK1 and BimEL in MCF7 cells (supplemental Fig. S2B) and between RACK1 and DLC1 in HEK293T cells (supplemental Fig. S2C). Because of technical restriction, we failed to coimmunoprecipitate all three proteins in the same cells. The observation suggested that the interaction between RACK1 and the BimEL-DLC1 complex might be a regulatory step for BimEL translocation.

**RACK1 Promoted BimEL Degradation in Paclitaxel-treated Cells**—Having established that RACK1 interacts with both DLC1 and BimEL, we proceeded to examine the role of RACK1 in the BimEL level because we found that the proteasome inhibitor MG-132 could significantly enhance the interaction between RACK1 and BimEL in MCF7 cells (Fig. 1F). To further examine whether RACK1 is involved in controlling the BimEL level, different cancer cells were transiently transfected with either RACK1 or RACK1 siRNA. In the RACK1-overexpressing breast cancer lines, MDA-MB-468 and MCF7, BimEL expression decreased dramatically at 48 and 24 h after paclitaxel treatment, respectively (Fig. 2A). A similar observation was made in the ovarian cancer lines, SKOV3 and SW626, respectively (supplemental Fig. S3A). The reduction of BimEL by RACK1 overexpression was proteasome-dependent, because the proteasome inhibitor, MG-132, significantly reversed the BimEL level (Fig. 2A). Reverse transcription-PCR was performed to assess

**FIGURE 2. RACK1 mediated BimEL ubiquitination and degradation in paclitaxel-treated cells.** A, MDA-MB-468 or MCF7 cells were transiently transfected with phEF-Neo or phEF-RACK1. 24 h later, cells were treated with \(100 \text{ nm}\) paclitaxel for 24 h. MDA-MB-468 cells were transiently transfected with two independent siRNAs of RACK1 or scrambled control for siRNA1. 72 h later, cells were treated with \(100 \text{ nm}\) paclitaxel for 24 h. C, MCF7 cells were transiently transfected with \(1 \text{ mg}\) of HA-ubiquitin (Ub) expression plasmid and either phEF-RACK1 or phEF-Neo. 24 h later, cells were treated with \(100 \text{ nm}\) paclitaxel, with or without \(1 \mu\text{g}\) MG132 for 6 h. Cell lysates were collected with Nonidet P-40 lysis buffer, and immunoprecipitation was performed with \(1 \text{ mg}\) of total protein lysates. The result was the representative of three independent experiments. Con, control; Pac, paclitaxel; IP, immunoprecipitation; IB, immunoblotting; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
whether the RACK1-mediated decrease of BimEL was due to reduced transcription. As shown in supplemental Fig. S3B, the BimEL mRNA level did not change in RACK1-overexpressing paclitaxel-treated cells. These observations suggest that RACK1-mediated regulation was not at the level of mRNA but at the level of protein stability.

To confirm that RACK1 regulated the BimEL level in paclitaxel-treated cells, RACK1 siRNAs were transiently transfected into MDA-MB-468 cells. Knockdown of RACK1 resulted in a higher level of BimEL compared with the control in paclitaxel-treated MDA-MB-468 cells (Fig. 2B). Analogous to RACK overexpression results, down-regulation of RACK1 by siRNA in MCF7 cells did not affect the BimEL mRNA level (supplemental Fig. S3B). Similar results were observed in MCF7-14 cells (a subline of MCF7) stably expressing short hairpin RNA of RACK1 (supplemental Fig. S3C). Additionally, we showed that overexpression of RACK1 in HEK293T cells promoted the polyubiquitination of BimEL upon paclitaxel treatment, which was further enhanced by the treatment of MG132 (Fig. 2C). Taken together, RACK1 reduced the BimEL protein level in paclitaxel-treated cells through a proteasome-dependent pathway.

**CIS Regulated BimEL Ubiquitination and Degradation—**RACK1 has been implicated as a component in the E3 ligase complex to enhance the ubiquitination and degradation of ΔNp63α, a member of the p53 family (18). A recent report showed that RACK1 interacted with ElonginC and mediated the degradation of HIF-1α (19). We observed previously that paclitaxel enhanced the interaction between RACK1 and CIS in HEK293T cells (data not shown). CIS belongs to SOCS family and is implicated to be a subunit in the RING domain-based E3 ligase complex, containing ElonginB/C-Cullin2-SOCS family members (21). These observations prompted us to investigate whether RACK1 and CIS were involved in E3 ligase complex-mediated BimEL ubiquitination and degradation.

To determine whether CIS is involved in the degradation of BimEL, we overexpressed CIS in HEK293T cells. The overexpression of CIS led to a decreased BimEL level in a dose-dependent manner (Fig. 3A). Inversely, transient knockdown of CIS resulted in significant accumulation of BimEL in MCF7 cells (Fig. 3A). Similar results were also observed in SKOV3 and SW626 cells (Fig. 3B), supporting that CIS plays a regulatory role in BimEL degradation. Furthermore, we found that CIS overexpression could significantly promote the ubiquitination of endogenous BimEL, which was further enhanced by paclitaxel and MG132 treatment (Fig. 3C). These data point to the role of CIS as a component in the E3 ligase complex to regulate the degradation of BimEL.

**CIS, RACK1, and DLC1 Were Mutually Dependent to Mediate the Degradation of BimEL via the E3 Ligase Complex—**To examine whether RACK1, BimEL, and CIS form a protein complex, a coimmunoprecipitation assay was performed. The paclitaxel treatment caused enhanced interaction between RACK1 and CIS (Fig. 4A, left panel), which confirmed our previous unpublished observation. Reciprocal coimmunoprecipitation revealed that the interaction between BimEL and CIS was enhanced upon paclitaxel treatment (Fig. 4A, right panels). Together with the observations described above, we have shown that 1) RACK1, CIS, and BimEL can interact and potentially form a triple-protein complex and 2) both CIS and RACK1 regulated BimEL protein levels via a proteasome-dependent pathway.

CIS, like other SOCS/CIS family members, contains a short N-terminal domain, a central SH2 domain, and a C-terminal SOCS-box. Because of the structural similarity, CIS can potentially associate, via its SOCS-box, with ElonginB and ElonginC (the Elongin B/C complex) and Cullin2, thereby forming the RING domain-based E3 ligase complex. Additionally, Cullin2 has already been shown to form an E3 ligase complex with other SOCS family members (21). Coimmunoprecipitation revealed that Cullin2 also interacted with CIS (Fig. 4B). We also detected association of Cullin2 with RACK1 and BimEL (Fig. 4C), which was enhanced upon paclitaxel treatment. These results suggest that CIS regulated BimEL degradation through the Cullin2-based E3 ligase-proteasome pathway. Indeed, overexpression of CIS and Cullin2 in HEK293T cells led to the decreased level of

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5 W. Zhang, G. Z. Cheng, J. Gong, U. Hermanto, C. S. Zong, J. Chan, J. Q. Cheng, and L.-H. Wang, unpublished observations.
BimEL (Fig. 4C), supporting our hypothesis that Cullin2 participated in the degradation of BimEL.

To address whether RACK1- and CIS-mediated degradation of BimEL is mutually dependent, we transiently knocked down RACK1 to see whether this would revert the CIS-mediated degradation of BimEL (Fig. 4D). Overexpression of CIS enhanced degradation of BimEL upon 24-h paclitaxel treatment in MCF7 cells. However, knockdown of RACK1 significantly reverted CIS-mediated degradation of BimEL (Fig. 4D), suggesting that CIS-regulated degradation of BimEL is dependent on RACK1.

We also examined whether DLC1 is involved in this complex, as well as its role in the BimEL level. Endogenous DLC1 association with CIS in a paclitaxel-dependent manner (Fig. 1C), in a similar way as its interaction with RACK1 (Fig. 1C). Knockdown of its expression by specific siRNAs of DLC1 resulted in accumulation of BimEL in MCF7 cells (Fig. 4E), consistent with previous observation (30). Paclitaxel-induced interaction between RACK1 and BimEL was significantly decreased in HEK293T cells expressing siRNAs of DLC1 (Fig. 4F), suggesting that DLC1 was involved in the interaction between RACK1 and BimEL, in agreement with in vitro binding experiment (Fig. 1E). More importantly, down-regulation of DLC1 in HEK293T cells inhibited CIS- and RACK1-mediated BimEL degradation in paclitaxel-treated cells (Fig. 4G), implicating DLC1 in the protein complex as controlling degradation of BimEL.

**RACK1 and CIS Confer Paclitaxel Resistance and Anchorage-independent Survival through BimEL Degradation**—To explore the biological role of RACK1-promoted BimEL degradation, we examined the effects of RACK1-mediated BimEL degradation on paclitaxel resistance and on anchorage-independent survival, as well as on growth by the assays for resistance to paclitaxel treatment, anoikis, and for formation of colonies in soft agar. Anchorage-independent survival and growth are important properties of cancer cells. A previous report has shown BimEL-mediated anoikis, a phenomenon of cell apoptosis and death due to deprivation of cells from attachment to the extracellular matrix (31). Because RACK1 and CIS mediated BimEL degradation, we hypothesized that RACK1 and CIS may confer resistance to anoikis and paclitaxel treatment.

BimEL has been shown to be involved in paclitaxel-mediated cell death (8); we examined whether it is similarly implicated in breast and ovarian cancer cells studied here. A panel of normal epithelial or tumor cells were transiently transfected with BimEL. BimEL expression did not significantly lead to apoptosis under normal conditions (data not shown), whereas paclitaxel treatment resulted in massive cell death in cells overexpressing BimEL (supplemental Fig. S4A). Down-regulation of BimEL by siRNA in SW626 and SKOV3 cells (to 40 and 50%, respectively, data not shown) prevented paclitaxel-induced cell death.
RACK1 and CIS in BimEL Degradation

![Image](https://www.jbc.org/content/283/24/16422/data/FIGURE5_C.jpg)

**FIGURE 5.** RACK1 and CIS conferred paclitaxel resistance in vitro. **A**, MCF7-I4 cells stably overexpressing RACK1 were seeded at 1 × 10⁵ cells/ml of 1.3% methylcellulose medium (left panel). MCF7 cells transiently transfected with 15 nm RACK1 siRNA or scrambled control were seeded at 1 × 10⁵ cells/ml of 1.3% methylcellulose medium (white bars, right panel), or 1 × 10⁴ monolayer cells were treated with 100 nm paclitaxel (gray bars, right panel). Cells were recovered 48 h later, followed by trypan blue staining to count the dead cells. **B**, MCF7-I4 cells stably overexpressing RACK1 were subjected to colony assay with or without the addition of 10 nm paclitaxel. **C**, MCF7 cells transiently transfected with siRNA for CIS or control siRNA were subjected to an anokis assay for 48 h (left panel), or 1 × 10⁴ monolayer cells were mock-treated with ethanol (white bars, right panel) or treated with 100 nm paclitaxel (gray bars, right panel). The dead cells were counted by trypan blue staining. **D**, MCF7 cells transiently transfected with siRNA for CIS or control siRNA were subjected to colony assay with or without the addition of 10 nm paclitaxel (left panel). Cell lysates were extracted with radioimmuneprecipitation assay buffer 48 h after siRNA transfection, followed by SDS-PAGE and immunoblotting (right panel). Total colonies were quantified using ImageTool. All the results were from three independent experiments.

As reflected by trypan blue staining, all three RACK1 overexpression clones displayed greatly enhanced colony formation ability (Fig. 5B). This result suggested that RACK1 was able to confer resistance to paclitaxel treatment of the cells, especially under anchorage-independent conditions. In terms of CIS-mediated effects, transient knockdown of CIS via siRNA in MCF7 cells greatly sensitized the cells to anoikis and paclitaxel, as reflected by trypan blue staining of dead cells (Fig. 5C) and by FACS to quantify the cells in the sub-G₁ fractions (supplemental Fig. S5D). Similar to the situation with RACK1, down-regulation of CIS in MCF7 cells showed only an ~25% reduction in colony formation without paclitaxel treatment (Fig. 5D, white bars); however, down-regulation of CIS dramatically sensitized the cells to paclitaxel treatment in soft agar.

Death by 20 and 15%, respectively, at the 48-h time point (supplemental Fig. S4B), suggesting that BimEL is a significant mediator of paclitaxel-induced cell death. The effect of BimEL in paclitaxel-mediated cell death is underestimated, considering the inefficient knockdown of the BimEL level.

We tested the function of RACK1 in mediating resistance to paclitaxel treatment and anokis. Three representative MCF7-I4 stable clones overexpressing RACK1 (the pooled stable cell culture RACK1-1 and two other single cell-derived stable clones, RACK1-2 and RACK1-3 (supplemental Fig. S5A), showed a dramatically decreased BimEL level after paclitaxel treatment, which was reversed by the treatment of MG-132 (supplemental Fig. S5B).

The anokis assay was performed with the MCF7-I4 stable clones overexpressing RACK1. RACK1 overexpression in MCF7-I4 significantly protected the cells from anokis (Fig. 5A, left panel), whereas MCF7 cells transiently transfected with two independent RACK1 siRNAs sensitized the cells to anokis and paclitaxel treatment, as reflected by trypan blue staining to count the dead cells (Fig. 5A, right panel) and by FACS analysis to quantify the cells in sub-G₁ fractions, reflecting apoptosing and dead cells (supplemental Fig. S5C). Another assay to measure the ability of anchorage-independent survival and growth is soft agar colony formation assay. In light of our observation that RACK1 could confer resistance to anokis and paclitaxel, we hypothesized that RACK1 would increase cancer cell colony formation ability, especially in the presence of paclitaxel. No significant change in colony formation ability in soft agar was observed in the RACK1-overexpressing clones without concurrent treatment of paclitaxel (data not shown). By contrast, in the presence of 10 nm paclitaxel, all three RACK1 overexpression clones displayed greatly enhanced colony formation ability (Fig. 5B).

To test whether RACK1 expression in breast cancer cells conferred paclitaxel resistance in vivo, we injected MCF7-I4-phEF cells and MCF7-I4-RACK1–3 cells into nude mice subcutaneously. Significant tumor growth was noticed 12–18 days after injection (Fig. 6B). RACK1 overexpression significantly enhanced tumor growth in vivo (Fig. 6B, left panel), which was...
in agreement with our previous observation in the melanoma system (13). Intraperitoneal injection of paclitaxel led to dramatic inhibition of tumor growth in the MCF7-I4-phEF group but no significant inhibition of tumor growth in the MCF7-I4-RACK1–3 group (Fig. 6B, right panel). The daily average tumor growth rates were significantly different in MCF7-I4-phEF groups with or without paclitaxel treatment; however, the difference in the MCF7-I4-RACK1–3 groups was not statistically significant, suggesting that RACK1 overexpression rendered the cells resistant to paclitaxel (supplemental Fig. S6B).

Biochemical analysis revealed that in the paclitaxel-treated MCF7-I4-RACK1–3 tumors, the BimEL expression level was dramatically decreased when compared with that of the MCF7-I4-phEF group (Fig. 6C). Additionally, cleavage of lamin B1, an apoptotic marker downstream of caspases, was greatly decreased in the I4-RACK1 tumors (Fig. 6C), correlating with the reduced expression level of BimEL. These results strongly suggest that RACK1 plays an important role in paclitaxel resistance of cancer cells by promoting the degradation of BimEL.

In breast carcinomas and breast cancer cell lines, the CIS expression level is highly elevated compared with their corresponding counterparts (23). Moreover, the RACK1 level has been shown to be up-regulated in various tumors and during angiogenesis (13, 17). To explore the clinical significance of our observations, we performed a comprehensive analysis of protein expression levels in human ovarian tumor specimens.
observation of RACK1- and CIS-mediated BimEL degradation, we examined the expression levels of RACK1, CIS, and BimEL in 16 breast cancer lines, 60 breast tumors, and 53 ovarian tumors. RACK1 showed significant expression in 95% of all the samples (Fig. 6D and data not shown). In agreement with a previous study (23), CIS was significantly expressed in more than 50% of the samples (supplemental Fig. S6C). BimEL and CIS expression were further quantified by Image J. We found an inverse correlation between the expression of CIS and BimEL (Fig. 6, D and E, and supplemental Fig. S6C). The inverse correlation was found in 69% of the breast cancer lines, 57% of the breast carcinomas, and 63% of the ovarian carcinomas (Fig. 6E). A chi-square test showed a significant difference between the actual expression pattern and expected expression pattern of CIS and BimEL in ovarian tumor samples and breast cancer cell lines, with p < 0.05 (Fig. 6E and supplemental Fig. S6C). Breast tumors only showed marginal significance, with p = 0.07 (Fig. 6D). RACK1 expression is high in most of those samples, probably because of its critical roles in cells, especially its role in ribosome (11, 12); thus no inverse correlation of RACK1 and BimEL was observed. These observations further support the regulatory roles of CIS in determining BimEL levels and implicate its potential role in the development of drug resistance in tumors.

**DISCUSSION**

We propose a model for RACK1- and CIS-mediated degradation of BimEL in cancer cells (Fig. 7). Apoptotic agents, such as paclitaxel and staurosporine, cause the formation of the RACK1, BimEL, and DLC1 protein complex. Subsequently, RACK1 promotes degradation of BimEL in paclitaxel-treated cells likely by bridging BimEL to the ElonginB/C-Cullin2-CIS E3 ligase complex for polyubiquitination and proteasome-dependent degradation. Ub, ubiquitin.

FIGURE 7. A model for RACK1- and CIS-mediated degradation of BimEL. Apoptotic agents like paclitaxel cause the protein complex formation between RACK1, BimEL, and DLC1. RACK1 promotes degradation of BimEL in paclitaxel-treated cells likely by bridging BimEL to the ElonginB/C-Cullin2-CIS E3 ligase complex for polyubiquitination and proteasome-dependent degradation. Ub, ubiquitin.
observations point to the importance of the homeostatic equilibrium concentration of RACK1 in the normal physiology of cells. The notion that expression of a scaffold protein, depending on its level, could be either promoting or inhibitory in its functions has been demonstrated with KSR1 in mitogen-activated protein kinase signaling (34) and with c-Jun N-terminal kinase interacting protein (JIP) in c-Jun N-terminal kinase signaling (35, 36). Our stable RACK1-expressing MCF7-14 cells displayed an enhanced growth of tumor and resistance to paclitaxel. This is in agreement with the previously reported effect of RACK1 on cancer cell growth (14).

The molecular detail of how RACK1 mediates the formation of the BimEL and CIS-containing E3 ligase complex is still unclear. Although constitutive tyrosine phosphorylation of RACK1 in HEK293T cells has been reported before (27), which may provide a binding site for the SH2 domain of CIS, i.e. it brings BimEL to the CIS-containing E3 complex within the favorable proximity. If a competitive peptide or small molecule could be designed to abolish the specific RACK1-BimEL or RACK1-CIS interaction, it could be a potential choice for combinatorial chemotherapy with paclitaxel.

Although Bim knock-out mice mainly showed defects in hematopoietic cells (10), in vitro and in vivo experiments have established the roles of Bim in initiating apoptosis in neurons (37) and osteoclasts (38), in lumen formation in cultured mammary acini (39), and in paclitaxel-mediated tumor killing (8, 9). Our study suggests that RACK1 and CIS play a critical role in regulating the BimEL level, which in turn confers drug resistance, and promote anchorage-independent survival and growth. This is corroborated by our observation of the negative correlation of the expression level between CIS and BimEL in cancer cells and tumor samples. It will be interesting to know whether those tumors with a high expression level of CIS correlate with paclitaxel resistance in patients. We hypothesize that overexpression of RACK1 in cancer cells would have a similar effect. Subsequent studies with fresh micro-dissected tumor cells and appropriate clinical treatment response data are needed to fully elucidate this condition.

The anti-apoptotic role of RACK1 is not restricted to epithelial cells. RACK1 has been shown to protect dexamethasone-induced apoptosis in T lymphocytes (40). In CD4 promoter-driven CIS transgenic mice, elevated CIS expression promoted T cell proliferation and survival upon T cell receptor activation when compared with the T cells in the control mice (24). Bim-null mice showed perturbed T cell development and elevated mature T cells. Additionally, Bim is required for apoptosis of autoreactive thymocytes (10, 41). Although elevated activation of mitogen-activated protein kinases has been proposed to explain the enhancement of T cell proliferation and survival in CIS transgenic mice (24), BimEL phosphorylation and RACK1/CIS-promoted degradation could be a major alternative mechanism.

Taken together, RACK1 plays an important role in the regulation of the pro-apoptotic protein BimEL in paclitaxel-treated cancer cells by mediating BimEL degradation via the ElonginB/C-Cullin2-CIS E3 ligase complex, leading to paclitaxel resistance. CIS appears to have a regulatory role in the BimEL level in cancer cells. Confirmation of our observation with freshly derived tumor cells will further underscore the clinical significance of RACK1/CIS-mediated regulation of BimEL. Intervention of RACK1-DLC1-BimEL or RACK1-CIS interaction presents a rational design for potential therapeutic regimen in combination with cancer drugs such as paclitaxel.

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