PLK1 Is Transcriptionally Activated by NF-κB during Cell Detachment and Enhances Anoikis Resistance through Inhibiting β-Catenin Degradation in Esophageal Squamous Cell Carcinoma

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Abstract

Purpose: To investigate the molecular mechanisms through which polo-like kinase-1 (PLK1) takes part in anoikis resistance of esophageal squamous cell carcinoma (ESCC) cells.

Experimental Design: The role of PLK1 in cell anoikis resistance was examined by ectopic gene expression and siRNA-mediated knockdown. Glutathione S-transferase pull-down and co-immunoprecipitation assays were utilized to investigate PLK1-interacting proteins. Electrophoretic mobility shift assay, chromatin immunoprecipitation, and reporter gene assays were carried out to identify the transcription factors responsible for PLK1 expression during anoikis resistance.

Results: We found that detachment of ESCC cells triggers the upregulation of PLK1. Elevated PLK1 expression contributes to protection against anoikis in cancer cells through the regulation of β-catenin expression. Moreover, we showed that, through direct binding to the PLK1 promoter, the NF-κB subunit RelA transcriptionally activates PLK1, which inhibits the ubiquitination and degradation of β-catenin. Inhibition of the NF-κB pathway restores the sensitivity of cancer cells to anoikis by downregulating PLK1/β-catenin expression. In addition, RelA gene amplification and protein overexpression was significantly correlated with PLK1 expression in ESCC tissues.

Conclusions: Our findings suggest that upregulation of PLK1 triggered by cell detachment is regulated by RelA at the transcriptional level. PLK1 protects esophageal carcinoma cells from anoikis through modulation of β-catenin protein levels by inhibiting their degradation. Taken together, this study reveals critical mechanisms involved in the role of RelA/PLK1/β-catenin in anoikis resistance of ESCC cells. Clin Cancer Res; 17(13); 4285–95. ©2011 AACR.

Introduction

Esophageal squamous cell carcinoma (ESCC) is a devastating disease because it metastasizes early and is highly resistant to conventional chemotherapy and radiation therapy. Characterization of the genetic alterations and the downstream effectors that contribute to ESCC metastasis will facilitate the development of effective therapeutic strategies for combating the disease.

The extracellular matrix (ECM) provides adhesive support to tissues and controls numerous signals that regulate diverse cellular processes, such as survival, growth, differentiation (1). Detachment of normal epithelial cells from the ECM typically results in anoikis, which is essential for several morphogenetic and homeostatic processes, such as embryo cavitation (2), postweaning mammary gland regression (3), and elimination of epithelial cells shed into the intestinal lumen (4). In contrast, malignant cells are resistant to anoikis, which leads to enhanced survival after detachment from the supporting matrix and facilitates metastasis (5). Thus, elucidating the molecular mechanisms involved in cancer-associated anoikis resistance is critical for the development of therapies designed to restore the sensitivity of malignant cells to anoikis.

Human polo-like kinase 1 (PLK1) is a highly conserved serine (Ser)/threonine (Thr) kinase that regulates a multitude of mitotic processes. PLK1 has been reported to be upregulated in several solid tumors, such as esophageal, breast, ovarian, prostate, and colon cancer (6–9). Inhibition of PLK1 with antisense oligonucleotides, siRNA, or dominant-negative mutations leads to mitotic catastrophe, apoptosis, and tumor inhibition (10–12). However, the molecular mechanisms that underlie PLK1 overexpression and its anti-apoptotic function in cancer cells are largely unknown.
Translational Relevance

Our work revealed that the RelA–PLK1–β-catenin pathway plays an important role in anoikis resistance in esophageal squamous cell carcinoma (ESCC) cells. Furthermore, we found RelA gene amplification and co-overexpression of RelA and PLK1 proteins in esophageal cancer cell samples. These results suggested that the RelA–PLK1–β-catenin pathway might be a potential target of therapies designed to restore the sensitivity of ESCC cells to anoikis.

In this study, we showed that upregulation of PLK1 triggered by cell detachment enhances anoikis resistance of esophageal cancer cells by inhibiting β-catenin degradation. Most importantly, we show that PLK1 is a direct target of RelA. RelA binds to the PLK1 promoter and regulates PLK1 transcription, which consequently stabilizes the β-catenin protein by disrupting its interaction with GSK-3β and β-TrCP. Altogether, our study reveals critical mechanisms involved in the dysregulation of PLK1 in human tumors and the role of PLK1 in anoikis resistance.

Materials and Methods

Patients and tissue specimens

Tissue specimens from 157 pathologically confirmed ESCCs and adjacent histologically normal tissues were obtained from patients who had undergone single-stage curative esophagectomy at the Cancer Hospital, Chinese Academy of Medical Sciences, Beijing, China. The samples were obtained following written informed consent from patients and with the approval of the Institutional Review Board. Of the 157 samples, 125 and 53 samples were used for immunohistochemistry and for genomic real-time PCR, respectively. Twenty-one samples were used for immunohistochemistry and for genomic real-time PCR, respectively. Twenty-one samples were used for immunohistochemistry and for genomic real-time PCR, respectively. Twenty-one samples were used for immunohistochemistry and for genomic real-time PCR, respectively. Twenty-one samples were used for immunohistochemistry and for genomic real-time PCR, respectively. Twenty-one samples were used for immunohistochemistry and for genomic real-time PCR, respectively.

Immunohistochemistry

Tissue microarrays (TMA) containing 125 primary esophageal tumors and the corresponding normal epithelia were created, and immunohistochemical analysis was done as described previously (9). TMAs were incubated with an anti-RelA antibody (sc-8008; Santa Cruz Biotechnology) or an anti-PLK1 antibody (Upstate). The results were separately evaluated by 2 independent observers. For RelA, the staining intensity was graded on the following scale: 0 (negative), 1 (weak-moderate), and 2 (strong). The evaluation criteria for PLK1 expression have been described previously (9).

Real-time PCR

The genomic copy number of RelA was assessed in 53 ESCC samples by TaqMan probe-based real-time PCR (assay ID: Hs02229145_cn; Applied Biosystems) on a LightCycler 480 Real-Time PCR system (Roche Applied Science) according to the manufacturer’s instructions. The RNase P H1 RNA gene was used as the standard reference (Applied Biosystems). The threshold cycle (Ct) for each gene was determined, and the average of 4 independent experiments was calculated. The relative copy number of the RelA gene normalized to the reference and relative to the calibrator is given by the formula \(2^{-\Delta\Delta Ct}\) using the comparative Ct method (13). Gene amplification was defined as a copy number \((2 \times 2^{-\Delta\Delta Ct}) > 3\).

Real-time reverse transcriptase PCR (RT-PCR) analysis of the PLK1 mRNA levels is described in the Supplementary Methods.

Cell culture, plasmids, and antibodies

The human ESCC cell line EC3 was established in culture in our laboratory. The human ESCC cell lines KYSE450 and KYSE410 were generously provided by Dr. Y. Shimada (Kyoto University, Kyoto, Japan). Cells were cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10% FBS. Other plasmids, siRNAs, and antibodies are described in the Supplementary Methods.

Assessment of anoikis

Cells were trypsinized and plated in 6-well polyhydroxyethylmethacrylate (polyHEMA) plates (which were prepared by applying 1.5 mL of a 10 mg/mL solution of polyHEMA in ethanol onto the plate and then allowing it to dry in a tissue culture hood). After 24 hours of growth in suspension, cells were harvested for apoptosis measurements using the Annexin V–FITC Apoptosis Kit (Sigma) or the Annexin V–R-phycocerythrin Apoptosis Kit (Southern Biotech).

Glutathione S-transferase pull-down and immunoprecipitation assays

See Supplementary Methods for details.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was conducted using the LightShift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer’s instructions. The oligonucleotide 5′-GCTGCGAGGGCCTCCCATTGTCGC-3′ (the NF-κB binding element is underlined) was labeled with biotin. For the competitive experiments, a 100-fold excess of unlabeled oligonucleotide was used. For supershift experiments, nuclear extracts were preincubated with anti-RelA antibody (sc-372x; Santa Cruz Biotechnology).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were conducted using the Magna ChIP G Kit (Millipore) according to the manufacturer’s instructions. The target protein was immunoprecipitated with either 2 μg of anti-RelA polyclonal antibody (ab7970; Abcam) or rabbit immunoglobulin G (IgG) as a negative control. The primer sequences are provided in Supplementary Table S2.
Luciferase reporter assay

ESCC cells were transiently transfected with 0.4 μg of luciferase reporter plasmid pGL3-Basic or expression plasmids (NF-κB-Luc, PLK1-WT-Luc, or PLK1-Mut-Luc). To correct for variations in transfection efficiency and cell number, 0.4 ng of the pRL-SV40 vector encoding the Renilla luciferase gene driven by the cytomegalovirus promoter (Promega) was cotransfected in each experiment. Twenty-four hours post-transfection, cell lysates were prepared, and both firefly and Renilla luciferase activities were quantified using the Dual-Luciferase Reporter Assay System (Promega). The data are presented as the ratio of firefly to Renilla luciferase activity.

Statistical analysis

The correlation between the expression levels of RelA and PLK1 was analyzed using the Spearman rank correlation test. The correlation between RelA protein expression and gene amplification was analyzed by Pearson’s \( \chi^2 \) test. Other statistical analyses were conducted using the Student’s \( t \)-test. Statistical significance was defined at \( P < 0.05 \).

Results

Detachment-induced upregulation of PLK1 inhibits anoikis of ESCC cells

In our previous study, we showed that several ESCC cell lines are relatively resistant to anoikis (14, 15). In this study, we found that detachment of ESCC cells upregulated both the mRNA and protein levels of PLK1 (Fig. 1B; Supplementary Fig. S1). In contrast, PLK1 expression was downregulated in the nonmalignant intestinal epithelial...
cell line IEC-6, which is highly susceptible to anoikis (Fig. 1A and B).

On the basis of the above observations, we hypothesized that changes in PLK1 expression level might be responsible for the susceptibility of ESCC cells to anoikis. To address this hypothesis, we first measured the ability of exogenous PLK1 to inhibit anoikis of esophageal cancer cells. Transient expression of PLK1 significantly promoted the survival of EC3, KYSE450, and IEC-6 cells cultured in suspension; similar results were obtained when PLK1 was stably expressed in these cells (Fig. 1C). To validate the anti-anoikis role of PLK1, we silenced endogenous PLK1 using specific siRNAs. As shown in Figure 2F and G (the first 3 groups), both siRNAs significantly suppressed PLK1 expression, which led to an increase in apoptotic cells upon detachment from the ECM. Similar

Figure 2. PLK1 regulates anoikis resistance through β-catenin in ESCC cells. A, after SDS-PAGE, bands present only in the PBD eluents were excised and digested with trypsin and subjected to LC/MS-MS. Amino acid sequences of 2 peptides corresponding to human β-catenin were identified. B, total 1 mg KYSE450 and KYSE410 cell lysates were prepared and immunoprecipitated with mouse monoclonal anti-PLK1 antibody or mouse IgG (as control). The immunocomplexes were resolved by 12% SDS-PAGE and immunoblotted with the indicated antibodies. After 48 hours of treatment by indicated siRNAs or plasmids, KYSE450 cells cultured in monolayer (C) and (D), or in suspension (E), for 16 hours were assayed for β-catenin, phosphorylated β-catenin, and PLK1 expression by Western blot analysis. β-Actin and ERK were used as loading controls. F, knockdown (KD) of PLK1 increased anoikis cells and forced transient expression of β-catenin markedly led to a decrease of apoptotic cells in PLK1-KD cells in anoikis assay. Columns, mean; error bars, SEM (n = 3); *, and #, P < 0.05 as compared with the corresponding PLK1-KD groups. G, after transfection for 48 hours, cells of each group were lysed and subjected to Western blot analysis with Flag or PLK1 antibodies.
results were noted when these cells were treated with a commonly used PLK1 inhibitor, BI-2536 (Fig. 1C). These data suggest that PLK1 is capable of inhibiting anoikis if its expression is induced in detached ESCC cells.

**PLK1 regulates anoikis resistance of esophageal cancer cells through β-catenin**

The polo-box domain of PLK1 was previously shown to mediate substrate recognition and targeting of PLK1 (16). To understand the molecular mechanisms by which PLK1 inhibits anoikis, glutathione S-transferase (GST) pull-down assays were utilized to identify PLK1-interacting proteins. Peptide mixtures extracted from the GST pull-down assays were separated using reverse-phase high-performance liquid chromatography (HPLC; Supplementary Fig. S2) and identified by tandem mass spectrometry (MS-MS). Two peptides (SAIVHLINQDDELATR and NEGTA-TYAAAVLFR) weighing approximately 90 kDAs were characterized, and the Mascot database search revealed that the 2 sequences corresponded to amino acids 134 to 151 and 648 to 661 of human β-catenin, respectively. Co-immunoprecipitation (Co-IP) experiments showed that β-catenin binds to endogenous PLK1 in esophageal cancer cells (Fig. 2A and B).

β-Catenin has been reported to promote anoikis resistance (17), and we confirmed that knockdown of β-catenin resulted in decreased survival of ESCC cells upon detachment (Supplementary Fig. S3). Interestingly, in both attached and detached cells, depletion of PLK1 or transfection of a kinase-dead PLK1 mutant (PLK1/K82M) led to reductions in β-catenin protein levels (Fig. 2C–E). In contrast, knockdown of β-catenin did not affect PLK1 expression (data not shown). Ectopic overexpression of β-catenin in PLK1-depleted cells partially restored their resistance to anoikis (Fig. 2F and G). Taken together, these data suggest that β-catenin contributes to PLK1-mediated anoikis resistance in ESCC cells. It is worth noting that the β-catenin mRNA levels did not change in PLK1-depleted cells (Supplementary Fig. S4), indicating that posttranslational modification of β-catenin was affected by PLK1 depletion.

**PLK1 depletion decreases β-catenin protein levels via the proteasomal degradation pathway**

Ubiquitination and proteasomal degradation of β-catenin is initiated by GSK-3β phosphorylation, and phospho-Ser/Thr residues of β-catenin are targets for β-TrCP and specific components of the ubiquitination apparatus (18–20). We examined the phosphorylation status of β-catenin with an antibody that detected phospho-Ser37 of β-catenin. PLK1 depletion led to an increase in phospho-β-catenin (Fig. 2D and E), which is consistent with the downregulation of β-catenin. No change was observed in GSK-3β and β-TrCP expression levels or activity following PLK1 depletion (data not shown).

Treatment of cells with the proteasome inhibitor MG-132 restored β-catenin levels in PLK1-depleted cells (Supplementary Fig. S5), indicating that downregulation of β-catenin in PLK1-depleted cells is proteasome dependent. Immunoprecipitation of cell lysates with an anti-β-catenin antibody revealed that β-catenin in PLK1-RNAi cells was ubiquitinated and migrated as a smear of protein bands with slower mobilities (Fig. 3A, lane 2). In addition, greater amounts of GSK-3β and β-TrCP co-immunoprecipitated with β-catenin (Fig. 3A and B). In contrast, β-catenin ubiquitination was undetectable in control cells (Fig. 3A, lane 1). These results indicate that
PLK1 depletion enhances the interaction between β-catenin and GSK-3β/β-Trcp and, thereby, promotes the degradation of β-catenin.

**RelA subunit of NF-κB upregulates PLK1 upon cell detachment**

Because PLK1-dependent pathways play an important role in the regulation of anoikis resistance, we explored the mechanisms underlying the transcriptional activation of PLK1 in detached ESCC cells.

Searching of online bioinformatics database (http://www.cbrc.jp/research/db/TFSEARCH.html) revealed a potential NF-κB–binding element (designated PLK1-NE) at −93 base pairs (bp) upstream of the transcription-initiation site within the PLK1 promoter region. As a first approach to investigate whether PLK1 is a transcriptional target of NF-κB, an EMSA was conducted with a 21-bp probe that encompassed the predicted PLK1-NE. As shown in Figure 4A, PLK1-NE showed strong binding activity to nuclear proteins and was supershifted by an antibody against RelA, a subunit of the NF-κB complex. Moreover, ChIP assays showed that RelA directly bound to the −151/+94 region of the PLK1 promoter, both in attached and detached cells. This interaction was significantly inhibited if the tumor cells were incubated with pyrrolidine dithiocarbamate (PDTC), a selective NF-κB inhibitor, indicating that the binding was specific (Fig. 4B).

We subsequently tested whether RelA regulated PLK1 transcription in suspended cells using a luciferase reporter assay. Consistent with the increase in PLK1 mRNA and protein levels upon cell detachment, PLK1 promoter activity was upregulated in suspended cells. More importantly, deletion of the PLK1-NE sequence abrogated PLK1 promoter activity. These results clearly indicate that PLK1-NE

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**Figure 4.** PLK1 is a transcription target of RelA. A, EMSA was conducted using nuclear extracts (NE) from EC3 cells and PLK1-NE labeled with biotin (Hot Probe). Competitive assays were conducted by adding 100-fold excess cold probe. Supershift analysis was conducted using anti-RelA antibody. B, after EC3 and KYSE450 cells were fixed and sonicated, cellular DNA–protein complex was immunoprecipitated by anti-RelA antibody or anti-rabbit IgG. BECN1 and GAPDH promoter were used as a positive control and a negative control, respectively. PCR products were resolved on a 1.5% agarose gel. C, deletion of PLK1-NE (GGGCGCTCCC) prevented NF-κB–mediated activation of PLK1 transcription activity during cell detachment. After 24 hours of transfection with the plasmids, detached EC3 cells were cultured for the indicated times, and the luciferase activity was measured as described in Materials and Methods. Each experiment was done in triplicate wells and repeated 3 times. Columns, mean; error bars, SEM; *, P < 0.05.
plays an important role in RelA-mediated promoter activation during cell detachment (Fig. 4C).

**RelA modulates anoikis resistance through the PLK1/β-catenin pathway**

To further explore the role of RelA in the regulation of PLK1 expression and anoikis resistance, 2 selective NF-κB inhibitors, PDTC and caffeic acid phenethyl ester (CAPE), were used to block the NF-κB signaling pathway. Both compounds significantly inhibited NF-κB–dependent transcription (Supplementary Fig. S6A). Notably, treatment of detached ESCC cells with PDTC or CAPE not only suppressed PLK1 promoter activity but also caused a significant downregulation of both PLK1 mRNA and protein levels (Fig. 5A and B). This, in turn, led to a reduction of β-catenin protein and enhanced anoikis of ESCC cells in a dose-dependent manner (Fig. 5A–D). It has been reported that RelA polyubiquitination was induced at the lysine 195 residue, and this ubiquitination event is critical for the degradation of RelA and termination of NF-κB activation (21). More importantly, ectopic expression of RelA K195R mutant, which is resistant to polyubiquitination and degradation, led to upregulation of both PLK1 mRNA and protein in suspended cells (Fig. 5E and F) but not in attached ESCC cells (data not shown). In addition, we found that NF-κB–dependent transcriptional activity was...
induced upon cell detachment (Supplementary Fig. S6C). These data suggest that RelA promotes anoikis resistance, mainly, by regulating PLK1/β-catenin expression. Interestingly, we also found that PLK1 promoter activity and expression were diminished upon PDTC treatment of attached cells (Supplementary Fig. S6B and D), indicating that RelA is also able to regulate PLK1 transcription in attached cells, but this process requires additional factors.

**Overexpression of RelA due to gene amplification is positively correlated with PLK1 dysregulation in ESCC tissues**

To extend our findings in vivo, we determined whether there is a correlation between the expression of RelA and PLK1 in resected human esophageal cancer specimens. In histologically normal tissues, RelA was undetectable in most epithelial cells. In contrast, esophageal tumor cells exhibited moderate (30.4%; 38 of 125) to intense (35.2%; 44 of 125) staining with an anti-RelA antibody (Fig. 6A). More importantly, RelA overexpression correlated with high levels of PLK1 expression. In 43 tumor samples without RelA expression, 74.4% (32 of 43) also showed PLK1 negative expression, and only 2.3% (1 of 43) expressed high levels of PLK1. However, in 44 tumor tissues with RelA overexpression, 54.5% (24 of 44) exhibited moderate to intense expression of PLK1 (Fig. 6C; Supplementary Table S1).

RelA is located on chromosome 11q in region 11q13, and gene amplification in this region has frequently been observed in human tumors, including those of the esophagus. To determine whether overexpression of RelA is associated with gene amplification, we used quantitative genomic real-time PCR to analyze RelA gene copy numbers. Amplification of RelA gene was observed in 35.8% (19 of 53) of ESCC patients, and the copy numbers of RelA gene were relatively high in tumors that overexpressed RelA protein, suggesting that gene amplification was responsible for RelA overexpression in some part of ESCC patients (Fig. 6B; Supplementary Table S2).

**Discussion**

Recent studies have indicated that detachment of epithelial cells triggers not only pro-apoptotic but also anti-apoptotic signals, and the equilibrium between these signals regulates anoikis (22–24). In suspended carcinoma cells, survival signals typically prevail, and anoikis induction is blocked, ultimately leading to tumor invasion and metastasis. Several anti-anoikis signals in cancer cells have been identified. For example, we and other researchers found that the activation of oncoproteins, including CTTN (14), calreticulin (15), Ras (25, 26), and β-catenin (17), suppressed anoikis of various types of cancer cells. In the present study, we found that detachment of ESCC cells...
trigged the upregulation of PLK1, a critical anti-apoptotic protein. Overexpression of PLK1 blocked anoikis in detached cells, and inhibition or depletion of PLK1 restored the sensitivity of ESCC cells to anoikis. Furthermore, it was noticed that PLK1 depletion did not affect the expression level of CTITN and calreticulin (Supplementary Fig. S7), indicating that these proteins still can inhibit anoikis in PLK1-depleted cells. This is consistent with our results that a subset of PLK1-depleted cells still survived after 24 hours of being cultured in suspension. Based on our previous findings that PLK1 overexpression inhibits the mitochondrial apoptotic pathway in ESCC cells, upregulation of PLK1 induced by cell detachment probably shifts the balance between the life and death signals toward survival and suppression of anoikis. However, the apoptosis-related molecules responsible for this process are unknown as yet and require further investigation.

Results from the GST pull-down and Co-IP assays suggest that β-catenin interacts with PLK1 in ESCC cells. The observation that PLK1 modulated β-catenin protein levels in attached and detached cells led to the hypothesis that β-catenin could be involved in the PLK1-dependent regulation of anoikis resistance. Ectopic expression of β-catenin rescued ESCC cells from detachment-induced anoikis downregulation due to PLK1 knockdown, indicating that β-catenin is an integral component and downstream signaling molecule in the PLK1-dependent anoikis-resistance pathway. The fact that PLK1 depletion did not affect β-catenin at the mRNA level but downregulated β-catenin at the protein level led us to speculate that posttranslational stabilization of β-catenin was regulated by PLK1. Indeed, the interactions between β-catenin and GSK-3β/β-Trcp were enhanced upon PLK1 knockdown, which promoted the ubiquitination and degradation of β-catenin. The importance of regulating β-catenin stability in cancer cells is supported by findings that GSK-3β-phosphorylation sites in β-catenin are commonly mutated in human colorectal cancers and other malignancies (27, 28). Furthermore, β-catenin is abnormally highly expressed in ESCC cells that overexpress end-binding protein 1 (EB1) and frequently rearranged in advanced T-cell lymphomas-1 (FRAT1), indicating that other events contribute to the dysregulation of β-catenin expression (29, 30). Thus, the interaction between PLK1 and β-catenin and the regulation of β-catenin by PLK1 identified in our study represents another mechanism by which β-catenin degradation is controlled in ESCC.

Arai and colleagues previously found that Ser-718 of β-catenin was specifically phosphorylated in M-phase by PLK1 (31). However, the study did not show that β-catenin protein degradation was regulated by PLK1, suggesting that this regulation is limited to certain types of tumor cells. We transfected PLK1 into ESCC cells and found that PLK1 was necessary, but not sufficient, for maintaining high protein levels of β-catenin. One possibility is that, in ESCC cells, PLK1 is already overexpressed (9) and it blocks proteasomal degradation of β-catenin; therefore, ectopic expression of PLK1 is somewhat redundant for this process and is not able to make the β-catenin protein more stable. Intriguingly, ectopic expression of a kinase-dead PLK1 mutant led to β-catenin downregulation, indicating that PLK1 kinase activity is required for β-catenin stabilization. Additional studies are required to clarify whether the regulation of β-catenin protein degradation is mediated by direct phosphorylation by PLK1 or through cooperation with other unidentified cellular signals.

The NF-κB family of transcription factors regulates a broad spectrum of biological responses, but its pro- and anti-apoptotic effects appear to be cell-type and context dependent (32–34). RelA has been shown to induce p53-dependent apoptosis, but it also efficiently counteracts TNF-α-induced apoptosis (35), suggesting that RelA promotes the expression of both anti- and proapoptotic molecules. In the present study, we showed the role of RelA in anoikis resistance of ESCC cells, which is consistent with previous reports that NF-κB delays anoikis of intestinal epithelial cells (24). Our results from EMSA, ChIP, and reporter assays suggest that PLK1 is a transcriptional target of RelA and that upregulation of PLK1 induced by cell detachment is RelA dependent. Using 2 different highly selective inhibitors of the NF-κB pathway, we showed that RelA protected ESCC cells from anoikis by modulating PLK1 and β-catenin expression. It is worth noticing that PDTC or CAPE treatment suppressed PLK1 promoter activity more strongly than deletion of PLK1-NE, suggesting that NF-κB has other binding sites in PLK1 promoter region or effectors downstream of the NF-κB pathway regulate PLK1 transcriptional activity. Other regulators of the NF-κB pathway, such as lipopolysaccharide (LPS), TNF-α, or interleukin (IL)-1β, had no effect on PLK1 promoter activity (data not shown). Thus, the regulation of PLK1 by RelA, identified in our study, is a novel mechanism of the complex NF-κB signaling network. Interestingly, recent studies have suggested that, through regulating NEMO and IκB kinase β, PLK1 inhibits NF-κB transcriptional activation induced by TNF-α or IL-1β, but not by RelA (36, 37). Altogether, these results suggest that there is no negative feedback loop between RelA and PLK1 regulation.

In the present study, we found that elevated protein levels of RelA were present in 65.6% of tumor samples, indicating that alterations in RelA expression are frequent events in ESCC. Analyses of both genomic copy number and protein expression of RelA in the same cases showed that gene amplification was one of the mechanisms underlying RelA overexpression. Our recent findings that only 37% of specimens with PLK1 protein upregulation exhibited gene amplification (9) suggest that transcriptional activation could be another mechanism of PLK1 overexpression in some esophageal carcinomas. Notably, a significant correlation between RelA and PLK1 overexpression was observed in ESCC specimens. In view of our finding that RelA is also required for PLK1 expression in attached cells, these data indicate that RelA is an important transcriptional regulator of PLK1 and is responsible for PLK1 overexpression in ESCC tissues.

In summary, we showed that upregulation of PLK1 triggered by cell detachment is regulated by RelA at the
transcriptional carcinoma level. Induction of PLK1 protects esophageal carcinoma cells from anoikis through modulation of β-catenin protein levels by inhibiting their degradation. Altogether, our study reveals novel mechanisms that underlie PLK1 overexpression in ESCC and the role of PLK1 in anoikis resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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