Aurothiomalate Inhibits Transformed Growth by Targeting the PB1 Domain of Protein Kinase C\textsubscript{t}\textsuperscript{*S}

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We recently identified the gold compound aurothiomalate (ATM) as a potent inhibitor of the Phox and Bem1p (PB1)-PB1 domain interaction between protein kinase C (PKC)\textsubscript{t} and the adaptor molecule Par6. ATM also blocks oncogenic PKC\textsubscript{t} signaling and the transformed growth of human lung cancer cells. Here we demonstrate that ATM is a highly selective inhibitor of PB1-PB1 domain interactions between PKC\textsubscript{t} and the two adaptors Par6 and p62. ATM has no appreciable inhibitory effect on other PB1-PB1 domain interactions, including p62-p62, p62-NBR1, and MEKK3-MEK5 interactions. ATM can form thio-gold adducts with cysteine residues on target proteins. Interestingly, PKC\textsubscript{t} (and PKC\textsubscript{\textalpha}) contains a unique cysteine residue, Cys-69, within its PB1 domain that is not present in other PB1 domain containing proteins. Cys-69 resides within the OPR, PC, and AID motif of PKC\textsubscript{t} at the binding interface between PKC\textsubscript{t} and Par6 where it interacts with Arg-28 on Par6. Molecular modeling predicts formation of a cysteine-aurothiomalate adduct at Cys-69 that protrudes into the binding cleft normally occupied by Par6, providing a plausible structural explanation for ATM inhibition. Mutation of Cys-69 of PKC\textsubscript{t} to isoleucine or valine, residues frequently found at this position in other PB1 domains, has little or no effect on the affinity of PKC\textsubscript{t} for Par6 but confers resistance to ATM-mediated inhibition of Par6 binding. Expression of the PKC\textsubscript{t} C69I mutant in human non-small cell lung cancer cells confers resistance to the inhibitory effects of ATM on transformed growth. We conclude that ATM inhibits cellular transformation by selectively targeting Cys-69 within the PB1 domain of PKC\textsubscript{t}.

We recently demonstrated that PKC\textsubscript{t} is an oncogene in human lung cancer (1). PKC\textsubscript{t} expression is elevated in non-small cell lung cancer (NSCLC) cell lines and primary tumors, and PKC\textsubscript{t} expression predicts poor clinical outcome (1). PKC\textsubscript{t} expression is driven in a significant subset of NSCLC tumors by tumor-specific amplification of the PKC\textsubscript{t} gene (PRKCI) that resides on chromosome 3q26 (1), the most frequent site of tumor-specific amplification in NSCLC cancers (2, 3). PKC\textsubscript{t} stimulates a pro-oncogenic PKC\textsubscript{t}/Par6 → Rac1 → Mek1/2 → Erk1/2 signaling pathway that is required for transformed growth of NSCLC cells \textit{in vitro} and tumor formation \textit{in vivo} (4). Expression of either a kinase-deficient PKC\textsubscript{t} mutant (kdPKC\textsubscript{t}) or the Phox and Bem1p (PB1) domain of PKC\textsubscript{t} inhibits oncogenic PKC\textsubscript{t} signaling and blocks transformed growth, indicating the involvement of both PKC\textsubscript{t} kinase activity and PB1 domain interactions in oncogenic PKC\textsubscript{t} signaling (4). PKC\textsubscript{t} mediated transformation requires activation of the downstream effector, Rac1, which is coupled to PKC\textsubscript{t} through PB1-PB1 domain interactions between PKC\textsubscript{t} and the adaptor molecule Par6 (4). Our studies provided the first compelling evidence that PKC\textsubscript{t} is an oncogene in human cancer (1). Subsequent reports have indicated that PKC\textsubscript{t} serves as both a prognostic marker (5, 6) and an oncogene in ovarian cancer (6). However, it remains to be determined whether PKC\textsubscript{t} mediates transformed growth of ovarian cancer cells by a similar signaling mechanism.

Based on our results, we reasoned that a small molecule chemical inhibitor of the PKC\textsubscript{t}-Par6 interaction would have potential as a chemotherapeutic agent in the treatment of NSCLC. Therefore, we designed and implemented a novel high throughput drug screening assay based on fluorescence resonance energy transfer to identify compounds that can disrupt the PB1-PB1 domain-mediated binding of PKC\textsubscript{t} to Par6 (7). We used our assay to screen a commercial library consisting of a diverse set of chemical compounds approved for human use. From our screen, we identified several known drugs that potently inhibit PKC\textsubscript{t}-Par6 interactions \textit{in vitro} (7). Two of the most potent inhibitors identified were the gold compounds aurothiomalate (ATM) and aurothioglucose (ATG). These compounds inhibit the PB1-PB1 domain interaction between PKC\textsubscript{t} and Par6 in a dose-dependent fashion and exhibit potent anti-tumor effects \textit{in vitro} and \textit{in vivo} in pre-clinical models of

- glucose: OPCA, OPR, PC, and AID; aPKC, atypical protein kinase C; GST, glutathione S-transferase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; MEKK, MEK kinase; HA, hemagglutinin; YFP, yellow fluorescent protein; NEM, N-ethylmaleimide; PBS, phosphate-buffered saline.
NSCLC (7). Because ATM is currently approved for clinical use in the treatment of rheumatoid arthritis, this compound is a particularly attractive candidate for clinical development as a novel, mechanism-based anti-tumor agent and is the focus of this study.

The PB1 domain is a highly conserved protein-protein interaction domain that is present on a family of signaling proteins. Unique interaction codes exist that specify the ability of PB1 domains to interact with each other (8). Therefore, we wished to determine whether ATM exhibits selectivity toward specific PB1-PB1 domain interactions or is a broad specificity inhibitor of PB1-PB1 domain interactions. We also sought to elucidate the molecular mechanism of ATM-mediated inhibition of PB1-PB1 domain interactions involving PKC family members.

In this study, we first identified the molecular mechanism by which ATM selectively inhibits the PB1 domain of PKC family members: genetic oligonucleotides, carrying the mutation was amplified using the following mutagenesis: C69R, 5'-GGAAAGGAGAAGCCGTCAGATGTACCTCAGTTGGA-3'. In the first step, closed double-stranded mutant DNA was produced using the generic oligonucleotide, 5'-GGGTCTCCCTATAGTGGTCTGATTAAATTCGCGGGG-3'. All constructs were sequenced to confirm their identities.

**Expression and Purification of Proteins**—Cm' plasmids, carrying the αB crystallin gene, were co-transformed into Escherichia coli BL21(DE3) along with Par6α cDNA-PinPoint Xa-3. Transforms were selected on LB plates containing 35 μg/ml chloramphenicol and 50 μg/ml ampicillin. Biotinylated human Par6 protein was expressed in E. coli BL21 (DE3) modified Terrific Broth (10) at 22 °C (220 rpm), and cells were harvested 48 h after A600 reached ~0.8. Biotinylated human Par6 protein was isolated from inclusion bodies using B-PER Reagent (Pierce) as described previously (7). Cm' plasmids carrying the αC crystallin gene and PKCα(1–113)/YFP-N1-pRSET were co-transformed into E. coli BL21 (DE3), and transforms were grown in LB containing 35 μg/ml chloramphenicol and 100 μg/ml ampicillin at 22 °C (220 rpm), and cells were harvested 48 h after A600 reached ~0.8. Soluble His-tagged PKCα/YFP proteins were isolated using the B-PER His8 purification kit (Pierce) and dialyzed against Tris buffer (50 mM Tris (pH 8.0), 135 mM NaCl, 10% glycerol). In some experiments, His-tagged PKCα/YFP protein was incubated with 100 μM N-ethylmaleimide (NEM) at room temperature for 20 min and dialyzed against Tris buffer (50 mM Tris (pH 8.0), 135 mM NaCl, 10% glycerol) containing 2 μM urea prior to performing PKCα-Par6 binding as described previously (7).

**GST Pulldown Assays of PB1-PB1 Domain Interactions**—GST fusion proteins were produced in E. coli strain BL21(DE3)pLysS (Novagen) and purified from cell extracts using glutathione-Sepharose 4 Fast Flow beads (Amersham Biosciences). 35S-Labeled Myc-tagged proteins were co-transcribed/translated in vitro using the TnT T7 coupled reticulocyte lysate system (Promega). For each GST pulldown experiment, 5–10 μl of in vitro translated Myc-tagged protein was diluted in 100 μl of NETN buffer (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.5% Nonidet P-40, 6 mM EDTA, 6 mM EGTA) containing 1 tablet per 10 ml of Complete Mini, EDTA-free protease inhibitor mixture (Roche Diagnostics). The diluted in vitro translated proteins were preincubated with empty glutathione-Sepharose 4 Fast Flow beads and the indicated amount of ATM for 30 min on a rotating wheel at 4 °C. Glutathione-Sepharose beads with immobilized GST fusion protein were added to the supernatant, and the samples were incubated for 60 min on a rotating wheel at 4 °C. The beads were washed five times with NETN buffer containing the indicated amount of ATM and boiled in 30 μl of Laemmli sample buffer. The samples were resolved in a 10% SDS-polyacrylamide gel, and the gel was Coomassie-stained and vacuum-dried. 35S-Labeled proteins were detected on a bio-imaging analyzer (BAS-5000, Fujifilm). Quantification of gel band intensities was done using the program Image Gauge (Fujifilm).

**Immunoprecipitation of In Vitro Translated p62**—pDestHA-p62 or pDestHA-p62R21A (500 ng) was transcribed/translated in vitro in a total volume of 25 μl using the TnT T7 coupled reticulocyte lysate system according to the manufacturer's pro-
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toc (Promega). Before transcription/translation in vitro, the indicated amount of ATM was added to the reticulocyte lysate. Expression of p62 from these vectors results in two products, HA-tagged p62 and untagged p62. After transcription/translation in vitro, 20 μl of the extract was diluted in 200 μl of ice-cold NETN buffer (with protease inhibitor mixture and the indicated amount of ATM). The samples were preincubated with a slurry of CL-4B Protein A-Sepharose beads in NETN buffer for 20 min at 4 °C on a rotating wheel and then incubated with 0.1 μg of anti-HA monoclonal antibody (12CA5) for 1 h and for another 30 min in the presence of bovine serum albumin-saturated Protein A-Sepharose beads in NETN buffer. The beads were washed five times in NETN containing the indicated amounts of ATM or ATG. The beads were resuspended in 15 μl of 2× SDS-polyacrylamide gel load buffer and boiled for 5 min. The samples were resolved by SDS-PAGE, and 35S-labeled proteins were detected on a bio-imaging analyzer (BAS-5000, Fujiﬁlm).

PKC-Par6 Binding Assay—Recombinant biotinylated human Par6 protein was covalently bounded to streptavidin-coated 96-well microtiter plates (Nunc, Roskilde, Denmark) by incubation at room temperature for 2 h as described previously (7). Plates were washed twice with PBS/Tween 20 and then once with PBS. After washing, puriﬁed PKCα/YFP was added to each well along with ATM to achieve the desired ﬁnal concentration as indicated in the ﬁgures, and binding was determined as described previously (7). Wells were then washed with PBS two times, and bound PKCα/YFP was determined by measuring ﬂuorescence intensity using a Typhoon 9410 imager (GE Healthcare). Binding analysis of wild-type and C69I and C69V/PKCα mutants was performed in the presence of increasing amounts of PKCα protein as indicated in the ﬁgures. Scatchard analysis was used to calculate apparent Kd values for binding to Par6.

Cell Transfections, PKCα Kinase Assay, and Transformation Assays—Human H1703 NSCLC and HEK 293 cells were obtained from American Type Culture Collection and maintained in adherent culture as described previously (4). H1703 cells were stably infected with pBabe retroviruses containing either FLAG-tagged wild-type human PKCα, a human PKCα C69I mutant, or empty pBabe virus using puromycin selection as described previously (1, 11). Cell lysates were analyzed by immunoblot analysis for FLAG-tagged transgenic PKCα, endogenous PKCα, and actin as described previously (4). Lysates from HEK 293 cells transfected with wild-type PKCα, PKCα C69I, or kinase-deﬁcient PKCα (kdPKCα) were subjected to immunoprecipitation kinase assay using anti-FLAG antibody as described previously (11, 12). H1703 cell transfectants were plated in soft agar in the absence or presence of ATM at the concentrations indicated in the ﬁgure legends and assessed for soft agar colony formation as described previously (1, 4). Plates were analyzed at each ATM concentration, and results are expressed as the mean colony number ± S.E. (n = 5).

Molecular Modeling—Molecular modeling studies were conducted on a Linux work station with the software package Gold (13). The initial three-dimensional structures were obtained using SYBYL version 7.0 (Tripos Inc.) and energy-minimized by using the MAXIMIN routine of SYBYL.

RESULTS

ATM Is a Highly Selective Inhibitor of PB1-PB1 Domain Interactions Involving PKCα—We recently identiﬁed the gold compounds ATM and ATG as inhibitors of the PB1-PB1 domain interaction between PKCα and the adaptor molecule Par6 (7). Our results demonstrated that not only did these compounds inhibit PKCα-Par6 interactions, but they also inhibited PKCα signaling to Rac1, a downstream effector of the PKCα-Par6 complex and blocked transformed growth of NSCLC cells in vitro and tumorigenicity in vivo (7). Our results suggested that the inhibitory effects of ATM on transformed growth are mediated through disruption of PB1-PB1 domain interactions. However, it remained to be determined whether the growth inhibitory effects of ATM were mediated exclusively through inhibition of PKCα-dependent PB1-PB1 domain interactions or whether ATM inhibits other PB1-PB1 domain interactions that might contribute to its cellular effects. To address this question, we developed highly speciﬁc pull-down assays for major PB1-PB1 domain interactions using GST fusion proteins and 35S-labeled human PKCα and GST-tagged p62. Because p62 can polymerize via PB1-PB1 domain interactions involving both the OPCα and basic cluster motifs within its PB1 domain (8), we utilized a mutant p62 in which Asp-69 within the OPCα motif was mutated to alanine (p62D69A). The D69A mutation makes p62 incapable of forming homopolymers or homodimers but preserves its ability to bind to PKCα via its basic cluster motif (8). In the absence of ATM, PKCα binds GST-p62 and is recovered in the GST pulldown (Fig. 1A). However, in the presence of ATM, signiﬁcant inhibition of PKCα binding is observed (Fig. 1, A and B). The mouse homolog of PKCα, PKCα, also binds p62 via the conserved OPCα motif. Binding of PKCα to p62 is similarly inhibited in the presence of ATM (Fig. 1C).

Because ATM inhibits binding of PKCα to Par6 (7), our present results suggest that ATM may target either the OPCα domain of PKCα or the basic cluster domains of both Par6 and p62. To distinguish between these two possibilities, we next assessed the ability of ATM to inhibit PB1-PB1 domain interactions involving p62. For this purpose, we developed two separate assays to measure p62 dimerization and polymerization, respectively. To measure p62 dimerization, we used a pulldown assay in which we incubate GST-p62D69A immobilized on glutathione-Sepharose beads with in vitro translated, 35S-labeled p62R21A. These two mutants are incapable of self-dimerization because of the disruption of the OPCα motif (D69A) and basic cluster motif (R21A), respectively (8). However, when combined they are capable of dimerizing with each other (Fig. 2A), making this a highly speciﬁc assay for p62 dimerization. Interestingly, addition of ATM had no appreciable effect on the ability of these proteins to dimerize (Fig. 2, A and B). Similar results were obtained in a related p62 polymerization assay in which wild-type p62 and HA-tagged wild-type p62 were incubated together followed by pulldown using protein A beads and
FIGURE 1. ATM inhibits the PB1 domain interaction between PKCα/λ and p62. A, the PB1 domain interaction between human PKCα and p62 was assayed by GST pull-down experiments in the absence or presence of ATM. Full-length p62 harboring the D69A mutation fused to GST and immobilized on glutathione-Sepharose beads was incubated with full-length PKCα produced by in vitro transcription and translation in the presence of [35S]methionine. Five- to 10-μl portions of in vitro translated protein were preincubated with glutathione-Sepharose beads for 30 min before incubation with beads with bound GST-p62D69A for 60 min. ATM (10 or 50 μM) was present from the preincubation step. The beads with bound proteins were washed five times and subjected to SDS-PAGE analysis in 10% SDS-polyacrylamide gels. Ten % of the in vitro translated proteins were run on the same gel to visualize the signal from 10% of input. The upper panels show the autoradiographs of the gels and the lower panels the same gels stained with Coomassie to visualize the GST-p62D69A and GST control proteins used in the pulldown experiments. The GST control was run on a separate lane in the same gel.

CAT indicates catalytic domain of PKCα/λ.

B, quantitative representation of the interaction data shown in A. A Fuji Bio-imaging analyzer (BAS5000) was used to quantitate [35S]-labeled proteins in the SDS-polyacrylamide gels employing Image Gauge version 4.0 software. The amount of [35S]-labeled PKCα pulled down by GST-p62D69A was set to 100%. C, quantitative representation of the interaction data obtained in similar GST pull-down experiments where murine PKCα was used instead of human PKCα. The data shown in B and C represent the mean with standard deviations of triplicate experiments and are representative of two other independent experiments.

FIGURE 2. ATM does not inhibit PB1 domain-mediated dimerization or polymerization of p62. A, dimerization of p62 was assessed using GST pull-down assays in the presence or absence of ATM by monitoring binding of in vitro translated, [35S]-labeled p62R21A to GST-p62D69A immobilized on glutathione-Sepharose beads. The two mutants were used because they are unable to polymerize. The experiments were performed as described in the legend to Fig. 1.

B, quantitative analysis of p62 dimerization in the presence of the indicated concentration of ATM (n = ± S.E.). C, the effect of ATM on polymerization of p62 was analyzed by immunoprecipitation of in vitro translated, [35S]-labeled HA-tagged p62. The fact that 40% of the in vitro translated p62 protein lacks the N-terminal HA tag due to translational initiation from the native ATG codon was exploited in a polymerization assay for p62. Immunoprecipitations were performed using an anti-HA antibody. Immunoprecipitated (IP) and co-precipitated proteins were resolved by SDS-PAGE and detected by autoradiography (upper panel). The in vitro translated, [35S]-labeled proteins used as input are shown in the lower gel panel (20% of the input run on the gel). The lack of polymerization/self-interaction of the p62R21A mutant is shown in the lower panel. The results shown are representative of three independent experiments.
an anti-HA antibody (Fig. 2C). In this latter assay, the presence of untagged p62 in the precipitate indicates p62 polymerization. In the presence of an inhibitor, the amount of untagged p62 will decrease relative to the amount of HA-tagged p62 precipitated. However, no demonstrable change in polymerization was seen in the presence of ATM (Fig. 2C). As a control, HA-tagged p62R21A and untagged p62R21A mutants were incubated together. As expected, these mutants are incapable of polymerizing, and as a result, no untagged p62 is found in the precipitate (Fig. 2C). Our results indicate that ATM does not significantly inhibit p62-p62 interactions. Thus it is unlikely that ATM targets either the basic cluster or OPCA motif of p62, but rather our data are consistent with an effect of ATM on the OPCA motif of PKC/H9259.

To further probe the selectivity of ATM, we assessed the ability of ATM to inhibit PB1-PB1 domain interactions between p62 and NBR1. In this interaction, p62 uses a basic cluster motif to bind to the OPCA motif of NBR1 (8). Using a pulldown binding assay analogous in design to our p62 dimerization assay, we observed efficient binding and pulldown of the 35S-labeled NBR1 protein with GST-p62D69A (Fig. 3A). Just as was seen with p62 dimerization, addition of ATM to the binding assay had no demonstrable inhibitory effect on p62-NBR1 binding (Fig. 3, A and B). Finally, we assessed whether ATM could inhibit the binding of yet another PB1-PB1 domain interaction. In this case, we designed a specific pulldown assay to measure the binding of MEKK3 to MEK5 similar to that used in our other binding assays (Fig. 4A). ATM had no inhibitory effect on MEKK3-MEK5 binding (Fig. 4, A and B). Taken together, our results demonstrate that ATM exhibits remarkable selectivity for PB1-PB1 domain interactions involving PKC/H9259, suggesting that ATM targets the PB1 domain of PKC/H9259. Given these observations, we next investigated the molecular mechanism underlying ATM selectivity.

Potential Role of Cysteine Residues in PKC/H9259-Par6 Binding and ATM Action—Despite extensive clinical use in the treatment of rheumatoid arthritis, the mechanism of action of gold compounds such as ATM is largely unknown. ATM exhibits anti-inflammatory properties thought to be mediated through NFκB inhibition; however, the molecular mechanism and the target(s) of this effect are currently unknown (15–17). Based on gold chemistry, a proposed mechanism for ATM involves formation of thiogold-cysteine adducts on target proteins. For example, ATM inhibits thioredoxin reductase activity by forming a thiogold adduct with a critical active-site cysteine residue.
Because ATM is reactive with protein sulfhydryl groups, we explored the possible involvement of cysteines in ATM-mediated inhibition of PKCε-Par6 binding. We first assessed the effect of the sulfhydryl blocking reagent NEM on PKCε-Par6 binding. Pretreatment of PKCε with 100 μM NEM led to significant inhibition of PKCε-Par6 binding comparable with that observed in the presence of ATM (Fig. 5A). These results suggested a possible role for one or more cysteine residues in PKCε and/or Par6 in PKCε-Par6 binding. To determine whether cysteines are also involved in ATM action, we assessed the effect of the sulfhydryl-reducing agent dithiothreitol (DTT) on the inhibitory effect of ATM on PKCε-Par6 binding. C, the crystal structure of the PKCε-Par6 complex reveals that Cys-69 (yellow) of PKCε resides in the binding interface between PKCε (green) and Par6 (blue) where it interacts with Arg-28 (red) on Par6. D, sequence alignment of the OPCA motifs of 10 human PB1 domain-containing proteins reveals that Cys-69 is unique to the OPCA motif of PKCε and PKCa (arrow).

Cystein-69 in PKCε Is a Critical Determinant of PKCε-Par6 Binding and ATM Action—Given the possible involvement of cysteines in PKCε-Par6 binding and ATM action, we assessed whether the PB1 domains of PKCε and/or Par6 contain any cysteine residues that might participate in PKCε-Par6 binding and serve as a target for ATM-mediated inhibition. The published crystal structure of the PKCε-Par6 complex reveals two highly conserved motifs within the PB1 domains of PKCε and PKCa that mediate binding between these proteins (20). PKCCa utilizes its OPCA motif to bind Par6, whereas Par6 utilizes its basic cluster motif to interact with the OPCA motif on PKCa. The crystal structure of the PKCε-Par6 PB1 domain complex reveals the presence of a cysteine residue on PKCε, Cys-69, at the binding interface between PKCε and Par6 where it interacts with Arg-28 within the basic cluster motif of Par6 (Fig. 5C). Arg-28 has been shown previously by site-directed mutagenesis to be important for PKCε-Par6 binding (20); however, the importance of Cys-69 has not been explored. Interestingly, sequence alignment of 10 known human PB1 domains revealed that PKCε (and PKCa) uniquely contains a cysteine residue, Cys-69, within the OPCA motif not present in the OPCA motifs of other PB1 domains (Fig. 5D).

To assess the role of Cys-69 in PKCε-Par6 binding and ATM action, we generated PKCa mutants in which Cys-69 was changed to isoleucine (C69I) or valine (C69V), two amino acids frequently found at this position in other PB1 domains (Fig. 5D). We also made a substitution of Cys-69 to the structurally similar serine (C69S). Each PKCa mutant was expressed in bacteria and analyzed for Par6 binding as described previously (7). Circular dichroism revealed no global structural changes in PKCa as a result of these amino acid substitutions (data not shown). However, distinct differences in the ability of these mutants to bind Par6 were observed. The C69S mutant was unable to bind Par6 (data not shown). In contrast, wild-type PKCε, PKCa C69I, and PKCa C69V all bound Par6 (Fig. 6A–C). Scatchard analysis of PKCε-Par6 binding revealed that wild-type PKCε (Fig. 6A), PKCa C69I (Fig. 6B), and PKCa C69V (Fig. 6C) exhibited very similar apparent Kd values for Par6 in the 200–300 nM range demonstrating that these amino acid substitutions had no apparent effect on the affinity of PKCε for Par6. However, although ATM inhibited binding of wild-type PKCε to Par6 with an apparent IC50 of ~1 μM as expected (7), little or no inhibition was observed with either PKCaC69I or PKCaC69V in the presence of up to 100 μM ATM (Fig. 6D). These results demonstrate that Cys-69 within the OPCA motif of PKCa plays an important role in binding to Par6, that substitution of Cys-69 to conserved residues found at this position in other PB1 domains supports binding of PKCa to Par6, and that Cys-69 is a critical target of ATM responsible for mediating the inhibitory effects of ATM on Par6 binding. Interestingly, Cys-69 is conserved in mouse PKCa, consistent with the fact that ATM inhibits the interaction between PKCa and p62 (Fig. 1C).
ATM Inhibits Transformed Growth by Targeting Cys-69 on PKCγ—Our results demonstrate that ATM selectively inhibits PB1 domain interactions involving PKCγ by targeting Cys-69 within the OPCA motif of PKCγ. Our previous studies demonstrated that expression of either dominant negative, kinase-deficient PKCγ (kdPKCγ) or the PB1 domain of PKCγ blocks transformed growth of human NSCLC cells in vitro (4) and that ATM inhibits the transformed growth of NSCLC cells (7). Because ATM selectively inhibits PB1-PB1 domain interactions involving PKCγ by targeting Cys-69 in vitro, we wished to assess whether this mechanism accounts for the inhibitory effects of ATM on transformed growth. Thus, we generated NSCLC cells that stably express either FLAG-tagged wild-type PKCγ, PKCγ-C69I, or kinase-deficient PKCγ in HEK 293 cells and subjected cell lysates to immunoprecipitation-kinase assays for PKCγ HEK 293 cells and subjected cell lysates to immunoprecipitation kinase assays for PKCγ. Immuno blot analysis using anti-FLAG antibody confirmed expression of the transfected proteins (Fig. 7A). To assess whether the C69I mutation had any effect on the catalytic activity of PKCγ, we expressed FLAG-tagged wild-type PKCγ, PKCγ-C69I, or kinase-deficient PKCγ in HEK 293 cells and subjected cell lysates to immunoprecipitation-kinase assays for PKCγ as described previously (12). PKCγ-C69I exhibited robust kinase activity with a specific activity comparable to wild-type PKCγ indicating that this amino acid substitution did not affect PKCγ kinase activity (Fig. 7B). A kinase-deficient PKCγ allele (kdPKCγ) served as a negative control and showed little or no kinase activity as expected (12). We next assessed the sensitivity of NSCLC cells expressing wtPKCγ, PKCγ-C69I, or empty pBabe to ATM-mediated inhibition of colony formation in soft agar (Fig. 7C). As expected, ATM exhibited potent, dose-dependent inhibition of the transformed growth of cells expressing either wild-type PKCγ or harboring the control plasmid pBabe with an apparent IC_{50} of ~2 μM (Fig. 7C). Cells expressing PKCγ-C69I exhibited transformed growth comparable with that of wild-type PKCγ and pBabe cells in the absence of ATM. PKCγ-C69I cells formed 50 ± 6 colonies, wtPKCγ cells 52 ± 19 colonies, and pBabe cells 58 ± 10 colonies (mean ± S.D., n = 5). However, PKCγ-C69I cells were resistant to the inhibitory effects of ATM on transformed growth (Fig. 7C). Taken together these results demonstrate that the PKCγ-C69I mutant is catalytically competent and supports transformed growth but confers resistance to the inhibitory effects of ATM on transformed growth. We conclude that ATM inhibits transformed growth by targeting Cys-69 within the OPCA motif of the PB1 domain of PKCγ. These results are consistent with the mechanism of ATM-mediated inhibition of PKCγ-Par6 interactions elucidated in this report. Molecular docking of ATM onto the crystal structure of the PKCγ PB1 domain predicts formation of a cysteine-aurothiomalate adduct at Cys-69 that protrudes into the binding cleft between PKCγ and Par6 that is likely to cause steric hindrance to Par6 binding (Fig. 7D).

DISCUSSION

The atypical protein kinase C (aPKC) isoforms PKCζ and PKCγ define a structurally and functionally distinct subclass of PKCs. Unlike other PKCs, the catalytic activity of aPKCs is not dependent upon diacylglycerol, calcium, or phosphatidylserine (21, 22). This functional divergence is because of the presence of a unique N-terminal regulatory domain on aPKCs that lacks calcium, phospholipid, and diacylglycerol-binding motifs (22). Although aPKC activity can be regulated by 3-phosphoinositides (23) and through phosphorylation by the phosphoinositide-dependent kinase, PDK1 (24–26), recent evidence indicates that protein-protein interactions mediated through the N-terminal PB1 domain play a prominent role in regulating the activity of aPKCs (22, 27–31). aPKCs are thought to be coupled to such diverse cellular functions as the establishment of cell polarity (32), cell proliferation (4, 11), and cell survival (12, 31, 33, 34) through direct PB1-PB1 domain interac-
Results represent the mean presence of the indicated concentrations of ATM. Soft agar colonies were assessed as described previously (4).

Expression of the PB1 domain of PKC blocks transformed growth and tumorigenicity (1, 4). PKC normally occupied by Par6 in the PKC molecular weight GTPases, Rac1 or cdc42 (29, 30, 32, 35–38). A complex consisting of Par6, aPKC, and either of the small domain-containing proteins have been identified that bind to interactions between aPKC and adaptor molecules (35). Several PB1 domain-containing proteins have been identified that bind to aPKC via PB1-PB1 domain interactions (8, 14, 35). One such protein, Par6, binds aPKC and links it to cell polarity by forming a complex consisting of Par6, aPKC, and either of the small molecular weight GTPases, Rac1 or cdc42 (29, 30, 32, 35–38). Similarly, the signal adaptor protein p62 binds aPKC via PB1-PB1 domain interactions, thereby linking it to Nfkb activation and cell survival signaling (27, 28, 39–41).

We recently demonstrated that PKC is an oncogene in NSCLC and elucidated an oncogenic PKC signaling pathway that is required for transformed growth (1, 4). PKC activity is required for transformation, because expression of a kinase-deficient, dominant negative PKC allele in NSCLC cells blocks transformed growth and tumorigenicity (1, 4). Expression of the PB1 domain of PKC also blocks transformation, indicating the importance of this region of PKC in oncogenic signaling (4). Furthermore, dissection of oncogenic PKC signaling revealed that PKC-dependent transformation requires activation of the small molecular weight GTPase Rac1, which in turn activates Mek1/2-Erk1/2 signaling (4) that is required for transformed growth.

Based on these observations, we concluded that PKC is a critical oncogene in NSCLC required for transformed growth and that PKC is an attractive target for therapeutic interven-

allele in NSCLC cells confers resistance to the inhibitory effects of ATM on transformed growth, demonstrating that Cys-69 on PKC\(\alpha\) is a critical target for ATM action in NSCLC cells.

Our finding that ATM inhibits both PKC\(\alpha\)-Par6 and PKC\(\alpha\)-p62 interactions in vitro raises the possibility that the inhibitory effects of ATM on transformed growth may be mediated by disruption of either or both of the PKC\(\alpha\)-Par6 and PKC\(\alpha\)-p62 signaling complexes. We recently demonstrated that PKC\(\alpha\)-mediated transformation of NSCLC cells is dependent upon Rac1, a key effector of the PKC\(\alpha\)-Par6 complex (4), and that ATM blocks Rac1 activity in NSCLC cells (4). In contrast, neither genetic disruption nor pharmacologic inhibition of PKC\(\alpha\) signaling with ATM affects NF\(\kappa\)B activity in NSCLC cells (4). Taken together, these data strongly indicate that the primary mechanism by which ATM inhibits transformed growth of NSCLC cells is by targeting Cys-69 on PKC\(\alpha\) thereby inhibiting the PKC\(\alpha\)-Par6-Rac1 signaling complex. An important question for future exploration is to determine whether PKC\(\alpha\)-dependent transformation invariably proceeds through the PKC\(\alpha\)-Par6-Rac1 signaling complex in other tumor types or whether the PKC\(\alpha\)-p62-NF\(\kappa\)B pathway may contribute to PKC\(\alpha\)-mediated transformation in some tumor types. We are actively investigating the role of these two signaling pathways in tumor cells in which PKC\(\alpha\) serves as an oncogene particularly in ovarian cancer and myelogenous leukemia cells. We also are assessing whether ATM may be an effective anti-tumor agent in other tumor types.

In addition to the obvious implications of our data on the use of ATM as a mechanism-based, targeted anti-tumor agent, our data also may shed light on the mechanism of ATM in the treatment of rheumatoid arthritis. ATM has been used clinically in the treatment of rheumatoid arthritis for decades, yet its mechanism(s) of action are still unresolved. Recent studies indicate that the anti-inflammatory effects of ATM are mediated through inhibition of NF\(\kappa\)B signaling (16). Interestingly, the inhibitory effects of ATM on NF\(\kappa\)B signaling are observed at concentrations in the 10–100 \(\mu\)M range, higher than the IC\(_{50}\) of ATM on transformed growth of NSCLC cells. Several potential targets for ATM-mediated inhibition of NF\(\kappa\)B have been proposed, including I\(\kappa\)K (17), thioredoxin reductase (42), and NF\(\kappa\)B (43). In each case, ATM is thought to act through modification of critical cysteine residues within these proteins in a fashion analogous to the mechanism we have identified for inhibition of oncogenic PKC\(\alpha\) signaling. Because the PKC\(\alpha\)-p62 interaction has been implicated in the activation of NF\(\kappa\)B signaling, it is possible that inhibition of this interaction may contribute to the anti-inflammatory actions of ATM in rheumatoid arthritis. Indeed, our results suggest a possible unifying molecular mechanism that could explain both the anti-tumor and anti-inflammatory effects of ATM through targeting of the PBI1 domain of PKC\(\alpha\).

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