Identification of an Inactivating Cysteine Switch in Protein Kinase Cε, a Rational Target for the Design of Protein Kinase Cε–Inhibitory Cancer Therapeutics

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Abstract

Critical roles played by some protein kinases in neoplastic transformation and progression provide a rationale for developing selective, small-molecule kinase inhibitors as antineoplastic drugs. Protein kinase Cε (PKCε) is a rational target for cancer therapy, because it is oncogenic and prometastatic in transgenic mouse models. PKCε is activated by *sn*-1,2-diacylglycerol (DAG). Attempts to develop selective PKCε inhibitors that block activation by DAG or compete with ATP have not yet met with success, suggesting a need for new strategies. We previously reported that cystamine and a metabolic cystine precursor inactivate PKCε in cells in a thiol-reversible manner. In this report, we first determined that PKCε became resistant to inactivation by disulfides when Cys^{452} was replaced with alanine by site-specific mutagenesis of human PKCε or a constitutively active PKCε mutant. These results showed that the disulfides inactivated PKCε by thiol-disulfide exchange, either upon Cys^{452} S-thiolation or by rearrangement to an intra-protein disulfide. Mass spectrometric analysis of peptide digests of cystamine-inactivated, carbamidomethylated PKCε detected a peptide S-cysteaminylated at Cys^{452}, indicating that Cys^{452} S-cysteaminylation is a stable modification. Furthermore, PKCε inactivation by *N*-ethylmaleimide was Cys^{452} dependent, providing corroborative evidence that PKCε inhibitors can be designed by targeting Cys^{452} with small molecules that stably modify the residue. Cys^{452} is an active site residue that is conserved in only 11 human protein kinase genes. Therefore, the PKCε-inactivating Cys^{452} switch is a rational target for the design of antineoplastic drugs that selectively inhibit PKCε. (Cancer Res 2005; 65(22): 10478-85)

Introduction

Protein kinases play influential roles in aberrant cell signaling events involved in neoplastic transformation and malignant progression of epithelial and other types of cells. This is widely recognized as a strong rationale for developing selective, small-molecule inhibitors of protein kinases as antineoplastic drugs (1–4). This approach was recently validated as a modality of antineoplastic therapy by the efficacy of imatinib (Gleevec) against chronic myeloid leukemia (CML; ref. 1). Imatinib treatment induces CML remission by selectively inhibiting the protein-tyrosine kinase activity of BCR-ABL (1). In addition, gefitinib (Iressa) selectively inhibits the protein-tyrosine kinase activity of the epidermal growth factor receptor (EGFR) and is effective against a small fraction of non–small cell lung cancers that express various somatic EGFR mutations (3). Both imatinib and gefitinib inhibit their protein kinase targets by competing with the substrate ATP (1, 2). Similarly, selective inhibitors have been identified for other protein kinases in recent years by screening libraries of chemicals designed to compete with ATP (5, 6). However, this heavily mined approach has failed to identify selective inhibitors of some protein kinases that are rational targets for cancer therapy, such as protein kinase Cε (PKCε).

PKC is a family of 10 isozymes in the AGC group of serine/threonine protein kinases (7, 8). PKCε is a rational target for cancer therapy, based on its oncogenic activity in fibroblasts and epithelial cells (9–11) and prometastatic activity in transgenic mouse models of chemical carcinogenesis (12, 13). ATP-competitive inhibitors that have been identified for PKCε are nonselective; this strategy has also failed to yield selective inhibitors for most of the other PKC isozymes (8, 14). Similarly, allosteric binding site targeting has not yielded selective PKCε inhibitors. PKCε and seven other PKC isozymes are activated by phosphatidylserine-dependent binding of *sn*-1,2-diacylglycerol (DAG) to cysteine-rich Zn^{2+} fingers in the regulatory domain (8). Efforts to develop selective inhibitors of PKC isozymes by targeting the DAG-binding site have not met with success, and enthusiasm for this strategy has waned as other signaling protein families with homologous DAG-binding sites have been identified (e.g., RAS-GRPs and PKDs; refs. 15, 16).

In recent years, we have investigated regulatory responses of PKC isozymes to cysteine modification by cystamine and other disulfides (17–20). We initially analyzed purified human recombinant PKC isozymes and found that structurally diverse disulfides activated PKCδ, which is tumor suppressive (21), and inactivated PKCε, PKCα, and other PKC isozymes to various extents (18, 19). Similarly, treatment of cells with cystamine or a metabolic cystine precursor activated PKCδ and inactivated PKCε in a concentration-dependent and thiol-reversible manner (17, 18). These results identified covalent modification of one or more cysteine residues by thiol-disulfide exchange as a novel mode of PKCε inactivation.

In this report, we establish that Cys^{452} is a cysteine switch in human PKCε (hPKCε) that inactivates the kinase in cells upon modification by disulfides or *N*-ethylmaleimide (NEM). Cys^{452PKCε} is an active site residue (22) that is conserved in only 11 of the >400 genes that encode human protein kinases. Further narrowing the field, PKCδ conserves Cys^{452PKCε} but is resistant to inactivation by cysteine modification (17–20). Therefore, the design of small-molecule inhibitors that bind in the active site cavity in an orientation favoring reaction with the side chain of Cys^{452} may offer a new avenue for the development of antineoplastic drugs that selectively inhibit PKCε.

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Materials and Methods

Cell culture and reagents. COS-7 cells obtained from the American Type Culture Collection (Manassas, VA) were maintained in DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin in humidified air with 5% CO_2 at 37°C. The media, antibiotics, and serum were from Invitrogen (Carlsbad, CA).

Plasmids and mutagenesis. Murine PKCε (mPKCε) catalytic domain cDNA encoding a constitutively active PKCε mutant (11) and hPKCε cDNA were provided by Dr. I. Bernard Weinstein (Columbia University, New York) and Dr. A.C. Newton (University of California-San Diego), respectively. Sequence analysis confirmed that the cDNAs encoded the catalytic domain of mPKCε (residues 395-737; Genbank accession no. P16054) and hPKCε (residues 2-737; Genbank accession no. Q02156).

To generate cDNAs encoding site-specific mutants of mPKCε and hPKCε with alanine substituted for cysteine, we used the PCR-based, site-directed mutagenesis kit QuikChange (Stratagene, La Jolla, CA). pcDNA3-PKCε expression plasmids served as template DNA and contained the coding sequence for hPKCε with an NH_2-terminal FLAG-epitope tag or the coding sequence for the mPKCε catalytic domain with a COOH-terminal FLAG tag (mPKCε-CAT). Oligonucleotides were commercially synthesized and purified (Sigma-Genosys, The Woodlands, TX). Mutagenic primers were employed in conjunction with complementary primers to generate site-specific PKCε mutants. The following are mutagenic primers for hPKCε: 5′-GATGATGACGTGGACGCCACAATGACAGAG-3′ for C452A hPKCε generation, 5′-CCTTACCCAACTCTACGCCTGCTTCCAGACCAAG-3′ for C474A hPKCε, 5′-CCCAACTCTACTGCGCCTTCCAGACCAAGGAC-3′ for C475A hPKCε, 5′-GATGCAGAAGGTCACGCCAAGCTGGCTG-3′ for C546A hPKCε, 5′-GCTGACTTCGGGATGGCCAAGGAAGGGATTCTG-3 for C554A hPKCε, 5′-GACCACCACGTTCGCTGGGACTCCTGAC-3′ for C568A hPKCε, and 5′-CACAAGCGCCTGGGCGCTGTGGCATCGCAG-3′ for C652A hPKCε.

Introduction of the desired mutation unaccompanied by any fortuitous mutation was established for each site-specific PKCε mutant cDNA by sequencing the full-length cDNAs.

Transfection. COS-7 cells (1×10^6 per 10-cm dish) were expanded for 48 hours, switched to serum-free media for 30 minutes, and transfected with the designated pcDNA3-PKCε expression-plasmid or pcDNA3 (empty vector) for 5 hours at 37°C using LipofectAMINE PLUS (Invitrogen).

Cellular protein kinase Cε inactivation by disulfides. After incubation in sulfur amino acid–free DMEM supplemented with 10% dialyzed serum (Invitrogen) for 16 to 20 hours after transfection at 37°C, COS-7 cells were treated with cystamine, cystine dimethyl ester (CDME) or disulfiram (Sigma, St. Louis, MO) in the presence of 5% dialyzed serum for the time interval specified at 37°C (17, 18). The disulfides were not cytotoxic under the conditions employed in this report. PKCε was extracted from the cells after disulfide treatment as previously described (17, 18). Briefly, the cells were rinsed with PBS, harvested with Buffer A [20 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 1 mmol/L EGTA, 10 μg/mL leupeptin, 250 μmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L Na_3VO_4, 20 mmol/L NaF, 10 nmol/L microcystin], and lysed by sonication at 4°C. Lysates were cleared by centrifugation, and protein concentrations measured. To immunoprecipitate the PKCε transgene product, 400 μg cell lysate protein (1.2-1.5 mg/mL) was incubated with 5 μg FLAG M2 monoclonal antibody (mAb; Sigma) overnight at 4°C in IP buffer (17) and further incubated for 2 hours after the addition of protein A-Sepharose. Beads were washed thrice with 1 mL IP buffer and resuspended in 20 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 1 mmol/L EGTA (17).

DTT-reversible PKCε inactivation was measured as previously described (17, 18). Briefly, each immunoprecipitated PKCε sample was incubated in the absence and presence of DTT for 15 minutes at 30°C, and 25 μL aliquots were added to PKCε assays (final volume, 120 μL). PKCε assays contained [γ-^{32}P] ATP and [Ser^{25}]PKC 19–31 (Bachem Bioscience, King of Prussia, PA) as substrates and phosphatidylserine and *sn*-1,2-dioleoylglycerol (DAG), each at 30 μg/mL as lipid cofactors (17). DAG was omitted from mPKCε-CAT assays. Background activity was measured in control assays that contained the PKC inhibitor GF109203X (1 μmol/L; Alexis Laboratories, San Diego, CA). PKCε activity is calculated as total minus background (17).

Western blot analysis of FLAG-immunoprecipitated PKCε samples was done using a PKCε mAb from BD Biosciences (San Jose, CA) to detect the *wt/mut* hPKCε species and a PKCε mAb (sc-214) from Santa Cruz Biotechnology (Santa Cruz, CA) to detect the mPKCε-CAT species. Enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) was employed as the detection system.

Protein kinase Cε inactivation by *N*-ethylmaleimide. To measure Cys^{452}-dependent PKCε inactivation in NEM-treated cell lysates, *wt* hPKCε and C452A hPKCε transfectants were prepared and lysed as described in Cellular PKCε Inactivation by Disulfides, except that the cells were harvested with Buffer B [0.65 mL/plate; 50 mmol/L HEPES (pH 7.0), 1 mmol/L EDTA, 1 mmol/L EGTA, 10 μg/mL leupeptin, 250 μmol/L PMSF, 1 mmol/L Na_3VO_4, 20 mmol/L NaF, 10 nmol/L microcystin]. The cell lysates were briefly centrifuged, assayed for protein content, and normalized to 1 mg/mL protein. Next, the lysates were treated with NEM for 10 minutes at 30°C; the alkylation reaction was terminated by adding 2 mmol/L DTT (final concentration) to scavenge unreacted NEM. NEM-induced hPKCε inactivation was measured by immunoprecipitating hPKCε (*wt/mut*) with FLAG M2 mAb and analyzing the immunoprecipitated isozyme as described in Cellular PKCε Inactivation by Disulfides, except that the 15-minute incubation at 30°C was omitted.

To measure Cys^{452}-dependent PKCε inactivation in NEM-treated cells, *wt* and C452A hPKCε transfectants were cultured in DMEM supplemented with 10% serum for 16 to 20 hours at 37°C and treated with NEM for 20 minutes at 37°C. To terminate NEM treatment, the cells were rinsed with PBS/2 mmol/L DTT, harvested in Buffer B/2 mmol/L DTT at 4°C, and lysed. hPKCε was extracted from the cell lysates by FLAG immunoprecipitation and analyzed for NEM-induced inactivation by the methods used in the analysis of NEM-treated cell lysates.

Disulfide inactivation of the catalytic domain fragment of human protein kinase Cε. To prepare a constitutively active, catalytic domain fragment of hPKCε (hPKCε-CDF), hPKCε sulfhydryls were refreshed as previously described by incubating purified recombinant hPKCε (5 μg; Invitrogen) with DTT for 30 minutes at 4°C followed by removal of DTT by gel filtration (19). Half of the hPKCε sample was incubated with 1 μg/mL trypsin (12.7 BAEE units/μg; Sigma) for 5 minutes at 30°C, and the other half was incubated similarly but without trypsin; trypsinolysis was terminated with 1 mmol/L PMSF.

To measure cystine-induced hPKCε-CDF inactivation, the hPKCε-CDF and control hPKCε preparations, each at 6 μg hPKCε/mL, were incubated with 2 mmol/L cystine in 50 mmol/L Tris-HCl (pH 8.0), 200 mmol/L NaCl, 1 mmol/L EDTA, and 1 mmol/L EGTA for 20 minutes at 30°C and further incubated with/without 30 mmol/L DTT for 10 minutes at 30°C. hPKCε-CDF and hPKCε were assayed as described in Cellular PKCε Inactivation by Disulfides, except that assays contained 50 μg/mL histone III-S (Sigma) as phosphoacceptor substrate and 60 ng hPKCε or hPKCε-CDF.

Statistical analysis. Statistical analysis of the results was done with the Student's *t* test using SigmaPlot software.

Liquid chromatographic-tandem mass spectrometry. To identify the structural modification at Cys^{452} in cystamine-inactivated PKCε by liquid chromatographic-tandem mass spectrometry (LC-MS/MS), cystamine-inactivated PKCε was prepared as follows. First, sulfhydryls of purified human recombinant PKCε (10 μg; Invitrogen) were refreshed as described in Disulfide Inactivation of hPKCε-CDF, and hPKCε was incubated with 1 mmol/L cystamine in 50 mmol/L Tris-HCl (pH 8.0), 200 mmol/L NaCl, 1 mmol/L EDTA, and 1 mmol/L EGTA for 30 minutes at 30°C (750 μL). This was immediately followed by the addition of 20 mmol/L iodoacetamide (final concentration) and incubation in the dark for 30 minutes at 37°C. Excess cystamine and iodoacetamide were removed from the hPKCε sample by gel filtration chromatography at 4°C using a foil-wrapped, 2 mL G-25 Sephadex column equilibrated in 50 mmol/L Tris-HCl (pH 8.0). An aliquot of the cystamine-modified, carbamidomethylated hPKCε sample was digested by incubation with lysyl endopeptidase (Wako Chemicals, Richmond, VA) for 20 hours at 37°C (hPKCε/protease ratio = 5:1). A second hPKCε sample that was thermally denatured by incubation at 95°C for 7 minutes before digestion was also analyzed. Following digestion, the extracted peptides were cleaned with C18 reverse-phase ZipTips (Millipore,

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Billerica, MA) to remove glycerol and then concentrated by vacuum centrifugation to remove the organic solvent. In preparation for LC-MS/MS analysis, the peptides were desalted with a reverse-phase precolumn and a switching valve (Switchos, LC Packings/Dionex, Sunnyvale, CA). The peptides were separated in the LC-MS/MS analysis using a C18PepMap100 column (75 μm inner diameter × 15 cm, 3 μm particle size, 100 Å pore size; LC Packings/Dionex). The solvent system employed was (A) aqueous 2% acetonitrile with 0.01% trifluoroacetic acid (TFA) and (B) aqueous 80% acetonitrile with 0.01% TFA. The gradient was ramped from 2% B to 40% B over 10.5 minutes and from 40% B to 90% B over 2.5 minutes. Tandem mass spectra were acquired for two precursors from each MS^1 scan (mass-to-charge ratio, *m/z* 500-1,400) with an electrospray ion trap mass spectrometer (LTQ, ThermoElectron, San Jose, CA) set at 3.2 kV (spray voltage) and 25% normalized collision energy. Data were searched against the SwissProt database using Mascot (http://www.matrixscience.com) and allowing for variable cysteine modifications (carbamidomethylation or cysteaminylation) and methionine oxidation as well as one missed cleavage per peptide. Sequence assignments were verified by direct inspection of the tandem mass spectra.

Results

Cys^{452} is a disulfide-regulated, protein kinase Cε–inactivating cysteine switch. hPKCε is an 84-kDa polypeptide comprised of an NH_2-terminal regulatory domain containing 17 cysteine residues, an regulatory/catalytic domain–linking hinge region lacking cysteine, and a COOH-terminal catalytic domain containing seven cysteine residues (8). We recently established that cystine inactivates hPKCε by thiol-disulfide exchange in association with an [^{35}S] S-cysteinylation stoichiometry of ∼1 mol cysteine/mol hPKCε (19). Based on the abrogation of hPKCε activity by this mechanism, we hypothesized that cystine-induced hPKCε inactivation was triggered by S-cysteinylation of a critical cysteine residue in the vicinity of the active site.

To test the hypothesis, we initially investigated whether cystine could inactivate a constitutively active, tryptic hPKCε-CDF. The Western blot analysis in Fig. 1 shows that limited trypsinolysis of purified recombinant hPKCε (*lane 1*) produced an ∼50-kDa fragment that was immunoreactive with a catalytic domain–directed PKCε antibody (*lane 2*). The trypsinized hPKCε species was fully active in the absence of the regulatory domain–binding cofactors phosphatidylserine/DAG (Fig. 1, *white columns*), which stimulated hPKCε activity about 10-fold (*black columns*). This established that the trypsinized hPKCε preparation contained constitutively active hPKCε-CDF.

The analysis of disulfide-induced hPKCε-CDF inactivation in Fig. 1 shows that 2 mmol/L cystine profoundly inactivated hPKCε-CDF by a DTT-reversible mechanism (Fig. 1, *white columns*) that resembled cystine-induced hPKCε inactivation (Fig. 1, *black columns*). These results provide evidence that cystine inactivates hPKCε by undergoing thiol-disulfide exchange with one or more cysteine residues in the catalytic domain.

hPKCε and mPKCε express identical cysteine residues and share 95% amino acid sequence identity. We previously reported that the cell-permeable disulfides cystamine and CDME induce DTT-reversible mPKCε inactivation in COS-7 cells (17). mPKCε-CAT is a constitutively active truncation mutant of mPKCε, which consists of the catalytic domain and has a COOH-terminal FLAG-epitope tag (11). To corroborate the evidence shown in Fig. 1 that disulfides inactivate PKCε by covalently modifying cysteine residues in the catalytic domain, we investigated whether cystamine and CDME could induce mPKCε-CAT inactivation in COS-7 cells. COS-7 cells transfected with mPKCε-CAT were treated with the disulfides for 30 minutes at 37°C, and mPKCε-CAT was immunoprecipitated from the cell lysates with FLAG mAb. Western analysis with a catalytic domain–directed PKCε mAb verified that the amount of mPKCε-CAT (43 kDa) recovered by FLAG-immunoprecipitation was not altered by disulfide treatment (Fig. 2). Each immunoprecipitated mPKCε-CAT sample was assayed after a 15-minute incubation at 30°C in the absence and presence of 30 mmol/L DTT. Cystamine induced >90% inactivation of mPKCε-CAT in the cells in a concentration-dependent and DTT-reversible manner (Fig. 2*A*). In addition, 1 mmol/L CDME abrogated mPKCε-CAT activity in COS-7 cells, with measurable reversal of inactivation by DTT (Fig. 2*B*).

The robust reversal of cystamine-induced mPKCε-CAT inactivation by DTT (Fig. 2*A*) clearly indicated that covalent modification of cysteine residues in mPKCε-CAT by thiol-disulfide exchange was a major component of the inactivation mechanism in COS-7 cells. However, the reversal of inactivation by DTT was incomplete. Incomplete reversal could reflect the strength and stability of inactivating disulfide bonds introduced into mPKCε-CAT by thiol-disulfide exchange or an inhibitory mechanism independent of thiol-disulfide exchange. To evaluate whether an inhibitory mechanism other than thiol-disulfide exchange contributed to the loss of mPKCε-CAT activity in the disulfide-treated cells, the cells were also treated with cell-permeable sulfhydryl agents structurally related to cystamine and cystine. Figure 2*B* shows that neither 1 mmol/L *N*-acetylcysteine nor 1 mmol/L cysteamine attenuated mPKCε-CAT activity, contrasting with the strong inactivation achieved by the disulfides. Collectively, the results in

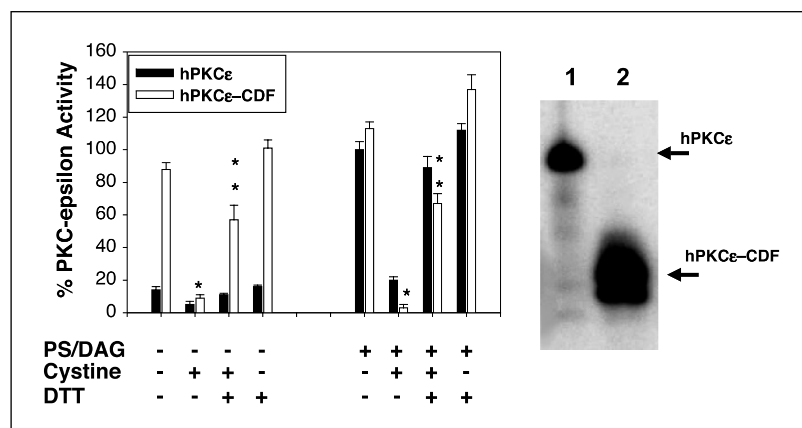

Figure 1. DTT-reversible inactivation of hPKCε-CDF by cystine. The ability of cystine to induce DTT-reversible inactivation of a constitutively active, tryptic hPKCε-CDF was analyzed. *Left,* hPKCε-CDF (*white columns*) and hPKCε (*black columns*) were incubated with/without 2 mmol/L cystine (20 minutes, 30°C), further incubated with/without 30 mmol/L DTT (10 minutes, 30°C), and assayed in the presence or absence of phosphatidylserine/DAG. *Columns,* mean PKCε activity of triplicate measurements; *bars,* SD. The activity of phosphatidylserine/DAG-activated hPKCε (1.3 pmol ^{32}P transferred/min) is defined as 100% activity. Single asterisk, $P < 0.01$, statistically significant difference versus the 2nd and 10th columns (above 4th and 12th columns, respectively). Double asterisk, $P < 0.01$, significant difference versus the 4th and 12th columns (above the 6th and 14th columns, respectively). *Right,* Western analysis of hPKCε (90 kDa; *lane 1*) and hPKCε-CDF (50 kDa; *lane 2*) with the catalytic domain–directed PKCε polyclonal antibody sc-214 (Santa Cruz Biotechnology). Reproduced in an independent analysis.

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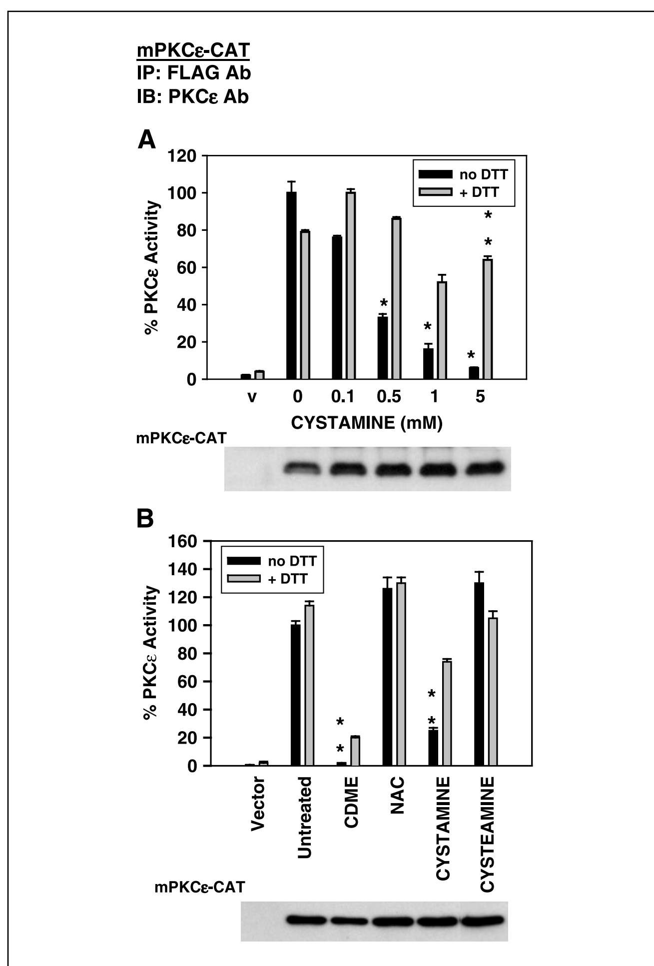

Figure 2. Inactivation of a constitutively active PKCε mutant in disulfide-treated cells. mPKCε-CAT transfectants were treated for 30 minutes at 37°C with the specified disulfide or thiol reagent at the concentrations indicated (*A*) or at 1.0 mmol/L (*B*) and lysed. mPKCε-CAT was immunoprecipitated from cell lysates with FLAG mAb, incubated with/without 30 mmol/L DTT, and assayed. *Columns,* mean mPKCε-CAT activity of triplicate measurements; *bars,* SD. 100% activity = the activity of mPKCε-CAT immunoprecipitated from untreated cells and assayed without DTT exposure. A mock analysis conducted with an empty vector transfectant. *A,* single asterisk, $P < 0.01$, significant difference versus the 100% activity value (3rd column); double asterisk, $P < 0.01$, significant DTT reversal of inactivation at 5 mmol/L cystamine (11th versus 12th column). *B,* double asterisks, $P < 0.01$, significant difference between the PKC activity values for CDME and NAC (5th versus 7th columns) and for cystamine and cysteamine (9th versus 11th columns). Western blot analysis of immunoprecipitated mPKCε-CAT, which migrated at 43 kDa, was done with a catalytic domain–directed PKCε mAb (sc-1681). Reproduced in an independent analysis.

Figs. 1 and 2 establish that disulfide inactivation of PKCε involves covalent modification of one or more cysteine residues in the catalytic domain.

To determine if any of the cysteine residues in the catalytic domain of PKCε can function as a disulfide-regulated, PKCε-inactivating switch, we generated cDNAs encoding site-specific hPKCε mutants with alanine substituted for cysteine that corresponded to each cysteine residue in the catalytic domain. hPKCε (full length) and the seven derived site-specific mutants exhibited similar activity and expression levels in COS-7 cells and migrated at 90 kDa in SDS PAGE (data not shown), validating comparison of their responses to disulfide in the cells. For this purpose, the cells were treated with CDME for 30 minutes at 37°C, and the *wt/mut* hPKCε species were immunoprecipitated from the cell lysates with FLAG mAb. Western blot analysis with a PKCε mAb (BD Biosciences) verified that CDME treatment did not alter the amount of the *wt/mut* hPKCε species recovered by immunoprecipitation (Fig. 3).

Each immunoprecipitated *wt/mut* hPKCε sample was assayed after incubation in the absence and presence of 10 mmol/L DTT. Both 5 and 10 mmol/L CDME induced >80% inactivation of *wt* hPKCε in COS-7 cells (Fig. 3, *black columns*), and exposure to DTT restored the original level of activity (Fig. 3, *gray columns*). Similarly, the five site-specific hPKCε mutants containing alanine substitutions at C474, C475, C546, C568, and C652 were profoundly inactivated by 5 and 10 mmol/L CDME in a DTT-reversible manner (Fig. 3). C554A hPKCε was also inactivated by 5 and 10 mmol/L CDME in a DTT-reversible manner, although it was not as sensitive as *wt* hPKCε. In contrast, C452A hPKCε was resistant to inactivation by 5 mmol/L CDME and modestly inactivated by 10 mmol/L CDME (*black columns*). Furthermore, C452A hPKCε was resistant to inactivation by disulfiram, a structurally dissimilar disulfide, at 100 and 200 μmol/L (45 minutes, 37°C). In contrast, *wt* hPKCε and C554A hPKCε were inactivated by 100 and 200 μmol/L disulfiram in a concentration-dependent and DTT-reversible manner, with >80% inactivation of C554A hPKCε achieved at 200 μmol/L disulfiram (Fig. 4*A*). Collectively, the results in Figs. 3 and 4*A* show a critical role for $Cys^{452hPKCε}$ in the inactivation of cellular hPKCε by disulfides disparate in structure and potency.

When C452A hPKCε was immunoprecipitated from lysates of disulfide-treated cells, its activity was increased by incubation with DTT (Figs. 3 and 4*A*). In an effort to eliminate this complication from our analysis, we generated the mutant C452A mPKCε-CAT, reasoning that elimination of the 17 regulatory domain-cysteine might abolish the effect. Figure 4*B* shows that C452A mPKCε-CAT was resistant to cystamine-induced inactivation in COS-7 cells, and that its activity was unaffected by exposure to DTT after immunoprecipitation from cystamine-treated cells. Taken together with the analysis of hPKCε in Figs. 3 and 4*A*, these straightforward results establish the identity of Cys^{452} as a disulfide-regulated PKCε-inactivating cysteine switch.

Stable conjugation of a small molecule at Cys^{452} inactivates protein kinase Cε. The requirement for Cys^{452} and the dispensability of other catalytic domain cysteine residues in the mechanism of disulfide-induced PKCε inactivation suggested that Cys^{452} S-thiolation (e.g., disulfide conjugation of cysteamine) was the inactivating modification. This supports the notion that small modifying groups at Cys^{452} would be sufficient to abrogate PKCε activity. However, the possibility that inactivation required rearrangement of S-thiolation modification(s) to intra-protein disulfides involving Cys^{452} was also consistent with the results. We therefore conducted mass spectrometric analysis of cystamine-inactivated hPKCε to identify the disulfide modification at Cys^{452}.

Exposure of purified recombinant hPKCε to 1 mmol/L cystamine for 30 minutes at 30°C, which induced 87 ± 1% inactivation, was immediately followed by the addition of 20 mmol/L iodoacetamide to prevent disulfide rearrangement in the cystamine-inactivated hPKCε sample. The sample was then digested with lysyl endopeptidase for LC-MS/MS analysis of the Cys^{452}-containing peptide fragment. LC-MS/MS detected the peptide DVILQDDDVDCTMTEK (hPKCε 442-457) with an *S*-cysteaminyl modification at Cys^{452}. The *m/z* ratios of the parent ion and the Cys^{452}-containing fragments in the y ion series (y_6-y_{14}) and the b ion series (b_{13} and b_{15}) in the

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tandem mass spectrum were consistent with S-cysteaminylation (+75 Da) at Cys^{452} and clearly distinguishable from carbamidomethylation of the residue (+57 Da), because the database search errors are limited to ±2 Da for parent ions and ±0.8 Da for fragments (Fig. 5). In addition, the residue mass for S-cysteaminylated cysteine was observed to be the difference in *m/z* between the y_6 and y_5 fragment ions, consistent with the assignment of Cys^{452} as the modified amino acid (Fig. 5). S-cysteaminylated hPKCε 442-457 was similarly detected when cystamine-modified, carbamidomethylated hPKCε was thermally denatured before digestion (data not shown), establishing the stability of the disulfide modification. These results provide structural evidence that cystamine inactivates hPKCε by introducing a stable S-cysteaminylation modification at Cys^{452}.

We next sought to corroborate the mass spectrometric evidence that conjugation of a small modifying group at Cys^{452} inactivates PKCε. We reasoned that one way to accomplish this would be to show resistance of C452A hPKCε to inactivation by a small sulfhydryl-alkylating agent, because alkylation of Cys^{452} in *wt* PKCε would be an inactivating modification that could not rearrange. For this purpose, we investigated the effects of NEM on *wt* hPKCε versus C452A hPKCε activity.

We first examined the effects of treating COS-7 cell lysates with NEM on *wt* hPKCε and C452A hPKCε activity. In these experiments, cell lysates were treated with NEM for 10 minutes at 30°C; 2 mmol/L DTT was added to terminate the alkylation reaction; and the *wt/mut* hPKCε species were extracted by immunoprecipitation with FLAG mAb and assayed. NEM treatment had no effect on the recovery of *wt* hPKCε and C452A hPKCε by immunoprecipitation (Fig. 6*A*). NEM (0.2-1.0 mmol/L) potently inactivated *wt* hPKCε in a concentration-dependent manner with a maximal effect of ∼80% inactivation (Fig. 6*A, black columns*), whereas C452A hPKCε was resistant to inactivation by NEM across this concentration range (*white columns*).

Cys^{452}-dependent hPKCε inactivation in NEM-treated cell lysates supported the notion that Cys^{452} can be exploited as a target for cancer therapeutics that inhibit hPKCε by permanent modification of the sulfhydryl. To test the principle that an alkylating drug could inactivate cellular hPKCε by a Cys^{452}-dependent mechanism under homeostatic conditions, *wt* hPKCε and C452A hPKCε transfectants cultured in DMEM supplemented with 10% serum were treated with NEM (1.25-2.0 mmol/L) for 20 minutes at 37°C, lysed in HEPES buffer containing 2 mmol/L DTT to quench unreacted NEM, immunoprecipitated, and assayed. Figure 6*B* shows that C452A hPKCε was resistant to inactivation by 1.25 mmol/L NEM (*white columns*), whereas *wt* hPKCε was inactivated >70% (*black columns*). These results indicate the availability of Cys^{452} in hPKCε for targeting by alkylating drugs in the cellular milieu.

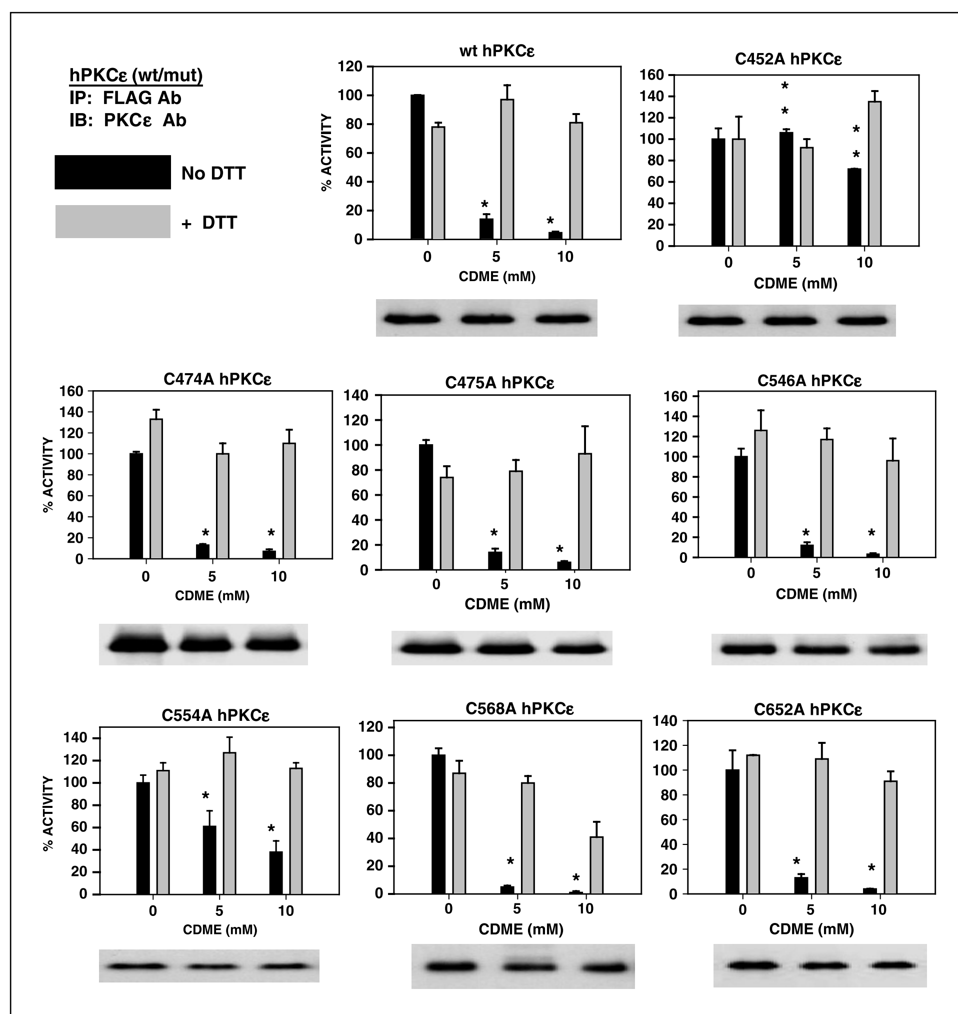

Figure 3. Resistance of C452A hPKCε to inactivation in disulfide-treated cells. COS-7 cells transfected with *wt* or *mut* hPKCε were treated with CDME for 30 minutes at 37°C and lysed. hPKCε species (*wt/mut*) were immunoprecipitated from the lysates with FLAG mAb, incubated for 15 minutes at 30°C without (*black columns*) or with 10 mmol/L DTT (*gray columns*), and assayed. *Columns,* mean PKCε activity of triplicate measurements; *bars,* SD. 100% activity = the activity of the hPKCε species (*wt* or *mut*) immunoprecipitated from untreated cells and assayed without DTT exposure. Single asterisks, $P < 0.01$, significant inactivation versus the 100% control value. Double asterisks, $P < 0.01$, significant resistance of C452A hPKCε to inactivation, based on the comparison of equivalent columns in the *wt* and C452A hPKCε. Western blot analysis of immunoprecipitated hPKCε was done with BD Biosciences PKCε mAb; *wt* and *mut hPKCε* species comigrated at 90 kDa. Reproduced in an independent analysis.

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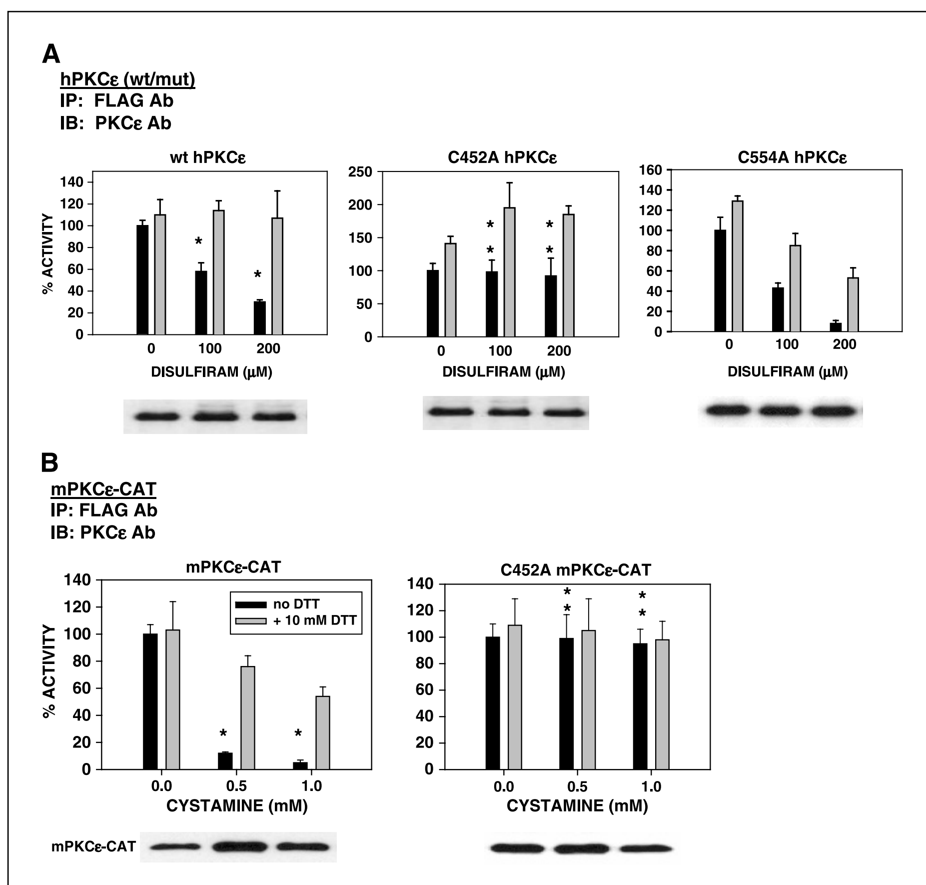

Figure 4. *A,* resistance of C452A hPKCε to inactivation in disulfiram-treated cells. The effects of disulfiram treatment on the activity of *wt* hPKCε, C452A hPKCε, and C554A hPKCε were ascertained as described in Fig. 3 legend, except that the cells were treated with 100 and 200 μmol/L disulfiram for 45 minutes at 37°C. Reproduced in an independent analysis. *B,* resistance of C452A mPKCε-CAT to inactivation in disulfide-treated cells. The effects of cystamine treatment (30 minutes, 37°C) on the activity of mPKCε-CAT and C452A mPKCε-CAT in COS-7 cells were compared. Western blot analysis was done as described in Fig. 2 legend; nonmutated and C452A mPKCε-CAT comigrated at 43 kDa. Single asterisks, $P < 0.01$, significant inactivation versus the 100% control value. Double asterisks, $P < 0.01$, significant resistance of the C452A mutant versus PKCε to inactivation, based on the comparison of equivalent columns in the C452A mutant versus *wt* hPKCε (*A*) or mPKCε-CAT (*B*). For other details, see Fig. 3 legend. Reproduced in an independent analysis.

Eleven human protein kinase genes conserve protein kinase Cε residue Cys^{452}. The recent elucidation of the crystal structure of the PKCθ catalytic domain has established that the side chain of Cys^{452hPKCε} is in the active site cavity of PKC isozymes (22). The significance of this to pharmacologic targeting of PKCε is that it offers active site binding as a strategy to selectively target Cys^{452hPKCε} versus cysteine residues on the surfaces of other proteins or in binding pockets other than protein kinase active

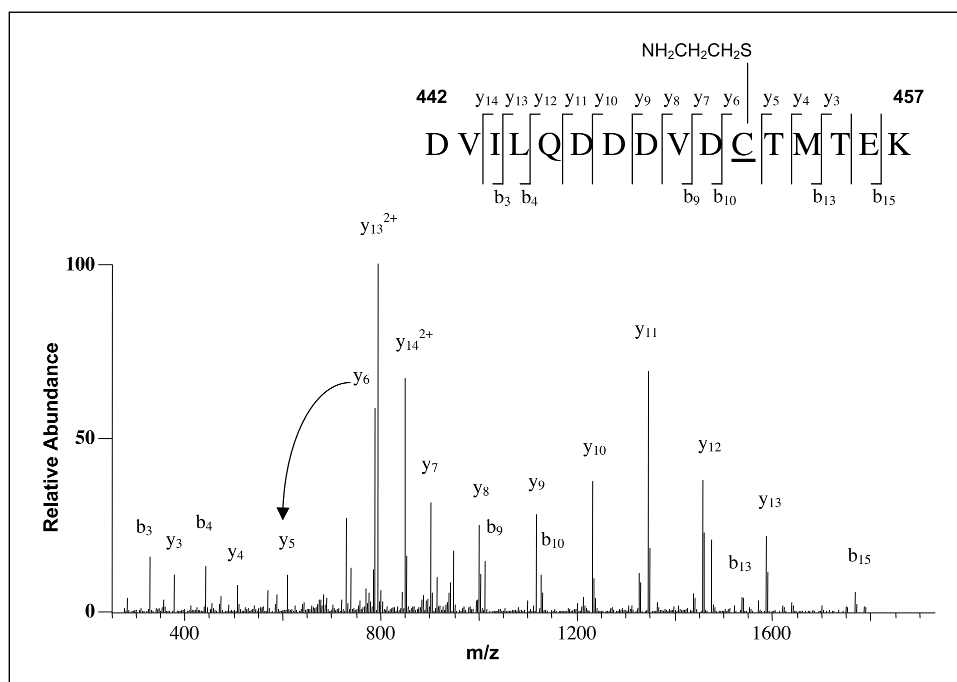

Figure 5. Tandem mass spectrum of hPKCε 442-457 S-cysteaminylated at Cys^{452}. Purified recombinant hPKCε was inactivated by cystamine, carbamidomethylated, and digested with lysyl endopeptidase. *Arrow,* y-type fragment ions separated by the residue mass corresponding to S-cysteaminylated cysteine. Reproduced in multiple experiments. In addition, this modified peptide was also observed in tryptic digests and even when the protein was thermally denatured before enzymatic digestion.

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sites. To assess whether Cys[452] targeting could serve as a strategy to selectively inactivate PKCε versus other human protein kinases, we searched the human protein kinase sequence database for protein kinases that conserve Cys[452hPKCε].

The proximity of Cys[452] to Glu[456] in the sequence of hPKCε provided a rational basis to investigate the conservation of Cys[452hPKCε] in human protein kinases, because Glu[456hPKCε] is nearly invariant in human protein kinases (23). Furthermore, Cys[452hPKCε] and Glu[456hPKCε] both occur in subdomain III, which is one of the 12 conserved catalytic domain subdomains in human protein kinases that fold into a common catalytic core (23). Using a comprehensive database of aligned human subdomain III sequences (7), we eliminated sequences corresponding to catalytically dead kinases and analyzed the remaining 443 sequences for CXXXE, where E denotes E456[hPKCε]. The search revealed that conservation of Cys[452hPKCε] is restricted to PKCε and 10 other genes that encode human protein kinases. The genes conserving Cys[452hPKCε] encode DAG-responsive PKC isozymes (PKCα, PKCβ, PKCγ, PKCδ, PKCε, PKCθ, PKCη), myotonic dystrophy protein kinase (DMPK), and myotonic dystrophy kinase-related Cdc42-binding protein kinase (MRCK) isozymes (MRCKα, MRCKβ, and MRCKγ; ref. 7). The rare conservation of C452[hPKCε] in protein kinases and its residence in the active-site cavity may allow the design of a new class of antineoplastic drugs that selectively inhibit PKCε by binding at the active site and permanently modifying the side chain of Cys[452].

Discussion

In this report, we show that Cys[452] is a PKCε-inactivating cysteine switch that abrogates PKCε activity when small molecules are conjugated to its side chain, whether by disulfide linkage (i.e., S-cysteaminylation) or alkylation (i.e., modification by NEM). The crystal structure of PKCθ, which is the only PKC isozyme with a solved three-dimensional catalytic domain structure, places the side chain of Cys[452hPKCε] in the active site cavity of PKC isozymes (22). This suggests a rational strategy for the development of antineoplastic drugs that selectively inhibit PKCε by permanent modification of Cys[452]. First, selectivity for protein kinases versus other classes of proteins could be achieved with agents that bind protein kinase active sites. Second, selectivity for the limited number of protein kinases that conserve Cys[452hPKCε] could be achieved with agents designed to bind the active site with an affinity below the threshold for inhibition by reversible binding, in an orientation that points the reactive group towards the nucleophilic side chain of Cys[452hPKCε] and facilitates inactivation by permanent modification of the sulfhydryl. Third, with respect to the issue of specificity for PKCε versus other PKC isozymes, PKCζ and PKCι do not conserve Cys[452hPKCε], and PKCδ conserves the residue but is resistant to inactivation by cysteine modification (17–20). Thus, at most, six PKC isozymes other than PKCε could be inactivated by a Cys[452hPKCε]-targeting mechanism. Furthermore, distinctions between some of these isozymes and PKCε are already evident; for example, PKCε is more sensitive than PKCα to inactivation by cystine but less sensitive than PKCα to inactivation by disulfiram (18, 19).

Our results distinguish the mechanism of PKCε inactivation by disulfide modification from cyclic AMP-dependent protein kinase (PKA) inactivation by S-glutathionylation, because the latter is mediated by Cys[199PKA] (24), which is homologous to Cys[568] in PKCε. Cys[568hPKCε] is located in the activation loop, which functions as a peptide substrate binding surface in protein kinase active sites

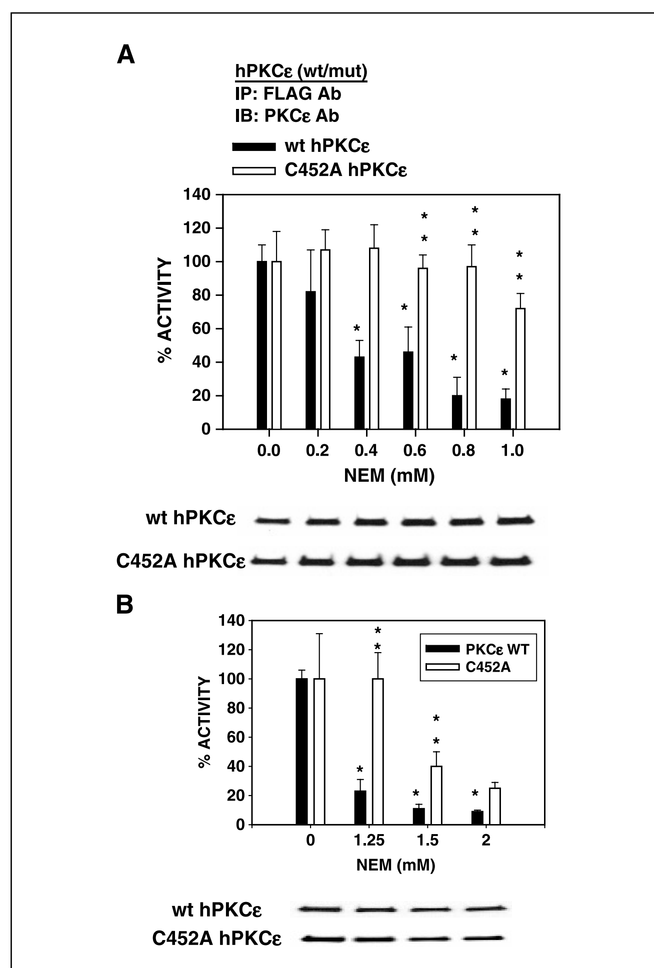

Figure 6. Resistance of C452A hPKCε to inactivation by *N*-ethylmaleimide. *A,* analysis of hPKCε inactivation in NEM-treated cell lysates. Cell lysates of *wt* and C452A hPKCε transfectants were treated with NEM for 10 minutes at 30°C; alkylation was terminated with 2 mmol/L DTT. hPKCε (*wt/mut*) was FLAG immunoprecipitated and assayed; 100% activity = the activity of hPKCε (*wt* or *mut*) immunoprecipitated from untreated cell lysates. For other details, see Fig. 3 legend. *B,* analysis of hPKCε inactivation in NEM-treated cells. *wt* and C452A hPKCε transfectants cultured in DMEM supplemented with 10% serum were treated with NEM for 20 minutes at 37°C and lysed in HEPES buffer containing 2 mmol/L DTT. hPKCε (*wt/mut*) was immunoprecipitated and analyzed as described in (*A*). Single asterisks, $P < 0.01$, significant inactivation versus the 100% control value. Double asterisks, $P < 0.01$, significant resistance of C452A hPKCε to inactivation compared with *wt* hPKCε. Reproduced in an independent analysis.

(22, 23). Cys[452hPKCε] is a component of helix αC, which forms another surface in the architecture of protein kinase active sites. Helix αC faces the activation loop and is crucial for the proper positioning of key catalytic residues in protein kinase active sites (22, 23). Thus, drugs designed to bind the active site of PKCε in an orientation that points a reactive group towards Cys[452hPKCε] would not be positioned for reaction with Cys[568hPKCε] and vice versa. This supports the notion that selective PKCε inhibitors may be designed by targeting Cys[452hPKCε].

The involvement of protein S-glutathionylation in the regulation of signaling and metabolic pathways is well established (reviewed in ref. 25). For example, angiotensin II activates Ras by inducing S-glutathionylation of the small GTPase in smooth muscle cells (26), and ischemia-reperfusion inactivates glyceraldehyde-3-phosphate dehydrogenase in isolated rat hearts by inducing

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S-glutathionylation of this critical glycolytic enzyme (27, 28). Interestingly, recent studies suggest that S-cysteaminylation may also regulate protein function *in vivo*.

Cysteamine is produced as a byproduct of pantothenic acid recycling in a broad spectrum of mammalian tissues (29, 30), where it partially converts to cystamine (31). Recent findings in a pantetheinase knockout mouse model, which was characterized by tissues deficient in cysteamine/cystamine, support the notion that S-cysteaminylation regulates protein function under homeostatic conditions *in vivo* (29). For example, γ-glutamylcysteine synthetase (γGCS) is inactivated by thiol-disulfide exchange with cystamine (32). In pantetheinase knockout mice, the γGCS activity level of the cysteamine/cystamine-deficient hepatic tissue is elevated, and oral administration of cystamine attenuated the γGCS activity level to that of *wt* mice (29). Thus, the finding in this report that Cys^{452} S-cysteaminylation profoundly inactivates PKCε raises the intriguing question of whether PKCε is regulated by cystamine *in vivo*.

Acknowledgments

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Identification of an Inactivating Cysteine Switch in Protein Kinase C ε, a Rational Target for the Design of Protein Kinase C ε–Inhibitory Cancer Therapeutics

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