Nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is formed by nitrosation of nicotine and has been identified as the most potent carcinogen in cigarette smoke. NNK cannot only induce DNA damage but also promotes the survival of human lung cancer cells. Protein kinase C (PKC) is an atypical PKC isoform and plays an important role in cell survival, but the downstream survival substrate(s) is not yet identified. Bad, a proapoptotic BH3-only member of Bcl2 family, is co-expressed with PKCβ in both small cell lung cancer and non-small cell lung cancer cells. We discovered that NNK potently induces multisite Bad phosphorylation at Ser-112, Ser-136, and Ser-155 via activation of PKCβ in association with increased survival of human lung cancer cells. Purified, active PKCβ can directly phosphorylate and inactivate this proapoptotic substrate(s) is not clear.

Because the specific depletion of PKCβ by RNA interference inhibits both NNK-induced Bad phosphorylation and promote apoptotic cell death. The β-adrenergic receptor inhibitor propranolol blocks both NNK-induced activation of PKCβ and Bad phosphorylation, indicating that NNK-induced Bad phosphorylation occurs at least in part through the upstream β-adrenergic receptor. Mechanistically, NNK-induced Bad phosphorylation prevents its interaction with Bcl-XL. Because the specific depletion of PKCβ by RNA interference inhibits both NNK-induced Bad phosphorylation and survival, this confirms that PKCβ is a necessary component in NNK-mediated survival signaling. Collectively, these findings reveal a novel role for PKCβ as an NNK-activated physiological Bad kinase that can directly phosphorylate and inactivate this proapoptotic BH3-only protein, which leads to enhanced survival and chemoresistance of human lung cancer cells.

Lung cancer is one leading cause of cancer deaths in both men and women, and the 5-year relative survival rate for all stages combined is only 15% (1). The World Health Organization reported that almost 1 billion men and 250 million women are daily smokers and cigarette smoking causes 90% of lung cancer cases and ~1.2 million deaths annually all over the world (1, 2). It is estimated that ~90% of male and 75–80% of female lung cancer deaths in the United States each year are caused by smoking (3, 4).

One piece of evidence for the connection between cigarette smoking and lung cancer is that lung cancer in women has increased by 600% since 1950 and has reached epidemic levels. This dramatic rise in lung cancer incidence is likely due to the increased prevalence of cigarette smoking, particularly in women over the same time period (5, 6).

There are more than 60 known carcinogens in cigarette smoke. Nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a nicotine-nitrosated derivative, has been identified as the most potent carcinogen and is thought to contribute significantly to smoking-related lung cancer (7). NNK can induce DNA damage (8), formation of DNA adducts, increased oxidative stress (9) as well as p53 and RAS mutation (7, 8). Because DNA damage and oxidative stress may potentially trigger cells to undergo apoptosis, the mechanism(s) that promotes cell survival is critical in NNK-initiated carcinogenesis. Recent studies indicate that NNK potently activates both phosphatidylinositol 3-kinase/AKT and MAPKs ERK1/2 in association with prolonged cell survival of human airway epithelial cells (10).

Protein kinase C (PKC) is a multigene family consisting of at least 12 distinct lipid-regulated protein-serine/threonine kinases that play pivotal roles in regulating cell proliferation, differentiation and survival (11). This family can be divided into three subtypes: the classic isoenzymes (PKCα, β, βII, and γ), which are Ca2+- and diacylglycerol-dependent; the novel isoforms (PKCδ, ε, η, θ, and μ), which are diacylglycerol-dependent but Ca2+-independent; and the atypical isoforms (PKCζ and λ), which possess only one zinc finger and lack the characteristic C2 domain, hence they are insensitive to both Ca2+ and diacylglycerol (11–13). PKCζ isoenzymes exhibit distinct tissue distribution and play a distinct role in various cellular events including cell survival, proliferation, and tumorigenesis (13, 14). For example, PKCζ, an atypical PKC isoform, presents predominantly in the lung and brain (15), suggesting a potential role in lung cancer development. Recent studies indicate that PKCζ can potently suppress apoptosis following treatment with chemotherapeutic drugs, but the downstream survival substrate(s) is not clear (16).

The Bcl2 family is comprised of at least 20 members that function as key regulators of cell survival and apoptosis (17). The

Received for publication, November 30, 2004, and in revised form, February 4, 2005
Published, JBC Papers in Press, February 10, 2005, DOI 10.1074/jbc.M413488200

This paper is available online at http://www.jbc.org
subfamily including Bcl2 and Bcl-XL inhibits apoptosis, whereas the Bax subfamily consisting of Bak and Bad as well as the BH3-only subfamily including Bad, Bid, Bok, Bik, and Bim promotes apoptosis (18). Functional studies have identified the importance of conserved Bcl2 homology domains (BH1, BH2, BH3, and BH4) in functionally related family members and the hydrophobic region in the C terminus is predicted to be the membrane-spanning domain that anchors these molecules to the outer mitochondrial membrane. Bcl2 and Bcl-XL have hydrophobic crevices on their surfaces that can bind to the BH3 domain of other family members (19, 20). Upon death stresses, the BH3-only proteins can couple death signals to mitochondria and bind and inactivate Bcl2/Bcl-XL via their BH3 domain (21). Bad, a BH3-only protein that lacks the typical hydrophobic C-terminal signal anchor, is a unique proapoptotic Bcl2 family member, because its function is tightly regulated by serine (Ser) phosphorylation at Ser-112, Ser-136, and Ser-155 (22). Thus, phosphorylation and dephosphorylation can switch the binding target of Bad to regulate its proapoptotic function. Although it is now clear that phosphorylation of Bad is able to abrogate its proapoptotic activity (27, 30), the signaling mechanism(s) by which Bad is regulated remains enigmatic.

Our previous study indicates that NNK-induced Bcl2 phosphorylation may be one of the mechanisms of NNK-enhanced cell survival (31). Because some lung cancer cells express low or undetectable levels of endogenous Bcl2 (32), it is possible that other Bcl2 family member(s), for example, Bad, may be involved in NNK-induced survival signal pathway(s). We and others (22, 33) recently discovered that nicotine can stimulate Bad phosphorylation in association with increased cell survival. In this study, we identified that Bad can function as a physiological PKCδ downstream substrate in the NNK-induced survival signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant, active PKCδ was purchased from Invitrogen Corporation. PKCδ, PKCβ, Bcl2, Bad, phosphospecific Bad, Bcl-XL, α-tubulin, and c-Src antibodies were purchased from Santa Cruz Biotechnol-ogy (Santa Cruz, CA). Recombinant Bad protein was purchased from Upstate (Charlottesville, VA). NIK was obtained from Toronto Research Chemicals (Toronto, Canada). Propranolol, etoposide (VP-16), and cisplatin, and c-Src antibodies were purchased from Santa Cruz Biotechnology, Inc. (Lexington, MA). Areas of protein co-localization appear yellow.

**Assay of PKCδ Activity in Vitro**—PKCδ was immunoprecipitated from cell lysates with an agarose-conjugated PKCδ antibody. Immunoprecipitated PKCδ was washed and resuspended in 50 μl of kinase assay buffer containing 50 mM Tris, pH 7.5, 10 mM MgCl2, 0.5 mM EGTA, 0.1 mM CaCl2, 10 μM ATP, 40 μg/ml phosphatidylylycerine, 10 μg of histone-1, and 10 μCi of [γ-32P]ATP. The reactions were incubated at room temperature for 30 min and terminated by the addition of SDS sample buffer and boiling prior to SDS-polyacrylamide gel electrophoresis. The activity of PKCδ was determined by autoradiography.

**Measurement of Intracellular c-Src Activity**—A549 cells were stimulated with NNK, harvested, and lysed in 0.5% Nonidet P-40 lysis buffer. The c-Src was immunoprecipitated from the lysates using a c-Src antibody. The complexes were washed three times with 500 μl of lysis buffer and twice with c-Src kinase assay buffer (20 mM HEPES, pH 7.0, 10 mM MnCl2, 0.05% Triton X-100). Then the immune complex beads were suspended in 45 μl of kinase assay buffer containing 1 μg of acid-treated enolase as described (34). The kinase reaction was initiated by the addition of 2 μCi of [γ-32P]ATP, and the reaction was incubated at 30°C for 10 min. The reaction was stopped by the addition of 50 μl of 2× SDS-PAGE sample buffer. Radiolabeled proteins were resolved by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and exposed to Kodak X-Omat film at −80°C for 24 h. The activity of c-Src was determined by autoradiography. The same filter was then probed by Western blot analysis using a c-Src antibody.

**Western Blot Analysis**—Cells were washed with 1× phosphate-buffered saline, plated on a glass slide, fixed with ice-cold methanol, and blocked with 10% donkey serum. Then, cells were incubated with a mouse antibody and rabbit PKCδ primary antibodies for 90 min. After washing, samples were incubated with rhodamine-conjugated anti-rabbit and fluorescein isothiocyanate-conjugated anti-mouse secondary antibodies for 60 min. Cells were washed with phosphate-buffered saline and observed under a fluorescent microscope (Zeiss). Pictures were taken and colored with the same exposure setting for each experiment. To determine subcellular regions of protein co-localization, individual red- and green-stained images derived from the same field were merged using Openlab 3.1.5 software from Improvision, Inc. (Lexington, MA). Areas of protein co-localization appear yellow.

**Immunofluorescent Staining**—A549 cells were washed with 1× phosphate-buffered saline, plated on a glass slide, fixed with ice-cold methanol, and blocked with 10% donkey serum. Then, cells were incubated with a mouse antibody and rabbit PKCδ primary antibodies for 90 min. After washing, samples were incubated with rhodamine-conjugated anti-rabbit and fluorescein isothiocyanate-conjugated anti-mouse secondary antibodies for 60 min. Cells were washed with phosphate-buffered saline and observed under a fluorescent microscope (Zeiss). Pictures were taken and colored with the same exposure setting for each experiment. To determine subcellular regions of protein co-localization, individual red- and green-stained images derived from the same field were merged using Openlab 3.1.5 software from Improvision, Inc. (Lexington, MA). Areas of protein co-localization appear yellow.

**RESULTS**

**NNK Induces Activation of PKCδ and Multisite Phosphorylation of Bad in Association with Increased Cell Survival**—PKCδ mRNA has been reported to predominantly express in brain and lung (15), suggesting that PKCδ may play a role in the development and/or chemoresistance of human lung cancer. Because PKCδ plays a role in supporting cell survival (35), and both small and non-small cell lung cancer cells express high levels of endogenous PKCδ (Fig. 1A), NNK may activate PKCδ to promote cell survival. To test this, A549 cells were treated with increasing concentrations of NNK as indicated. PKCδ was immunoprecipitated using an agarose-conjugated PKCδ antibody. Activity of PKCδ was measured by an immune complex kinase assay using purified histone-1 as a substrate as de-
subcellular distribution of PKCδ and Bad was examined by immunofluorescent staining. A mouse polyclonal antibody against human Bad, rabbit polyclonal PKCδ antibody, and fluorescein isothiocyanate-conjugated anti-mouse (green) or rhodamine-conjugated anti-rabbit (red) secondary antibodies were used so that cells could be simultaneously stained without cross-reaction. As shown in Fig. 2A, Bad is primarily co-localized with PKCδ in the cytoplasm of A549 cells. To test whether PKCδ can directly phosphorylate endogenous Bad, the Bad protein was immunoprecipitated from A549 cells and incubated with purified, active PKCδ in an in vitro kinase assay as described under “Experimental Procedures.” Phosphorylation of Bad was determined either by autoradiography or by Western blot using a phosphospecific Bad Ser-112 (lower), Ser-136 (S136), or Ser-155 (S155) antibody, respectively.

PKCγ Co-localizes with Bad in the Cytoplasm of Lung Cancer Cells and Active PKCγ Can Directly Phosphorylate Bad at Ser-112, Ser-136, and Ser-155 Sites in Vitro and in Vivo—To assess the ability of PKCγ to phosphorylate Bad, recombinant Bad protein was incubated with purified, active PKCγ in an in vitro kinase assay. Western blot analysis was performed to confirm and quantify Bad protein (lower). C, recombinant Bad protein was incubated with purified, active PKCδ for various times in an in vitro kinase assay. As shown in Fig. 2A, Bad is primarily co-localized with PKCδ in the cytoplasm of A549 cells. To test whether PKCδ can directly phosphorylate endogenous Bad, the Bad protein was immunoprecipitated from A549 cells and incubated with purified, active PKCδ in a kinase assay buffer containing [γ-32P]ATP as described under “Experimental Procedures.” Results indicate that active PKCδ directly phosphorylates Bad in vitro (Fig. 2B). In addition, active PKCδ can also directly phosphorylate recombinant Bad protein at Ser-112, Ser-136, and Ser-155 sites in a time-dependent manner (Fig. 2C). These findings suggest that PKCδ is a strong candidate for being the direct Bad kinase. To determine whether PKCδ may be a Bad kinase in vivo, a PKCδ/pAXneoRX expression construct was transfected into NCI-H157 cells that express relatively low levels of endogenous PKCγ. After transfection for 48 h, expression levels of exogenous PKCγ and Bad phosphorylation were analyzed by Western blotting using PKCγ or phosphospecific Bad antibodies, respectively. Results reveal that overexpression of PKCγ results in an increased Bad phosphorylation at Ser-112, Ser-136, and Ser-155 (Fig. 3). Importantly, phosphorylation sites are consistent with in vitro results (Fig. 2C). These findings provide both biochemical and genetic evidence...
that Bad is a novel physiological PKCα survival substrate.

**NNK Stimulates Activation of c-Src, Which Is a PKCα Upstream Kinase and the Src-specific Inhibitor PP2 Blocks NNK-induced PKCα Activation, Bad Phosphorylation, and Enhances Apoptosis**—Our data showed that NNK potently stimulates activation of PKCα in association with enhanced cell survival (Fig. 1). However, the upstream protein kinase(s) involved remains unclear. PKCα is insensitive to Ca++ due to the absence of the calcium-binding domain (13). Thus, NNK-induced activation of PKCα may occur through a calcium-independent mechanism. Because c-Src has been reported to directly induce tyrosine phosphorylation of PKCα at tyrosine 256, 271, and 325 sites along with activation of enzyme activity (34), and c-Src is ubiquitously expressed in both small and non-small cell lung cancer cells (Fig. 4A), we postulate that NNK may stimulate c-Src activity to activate PKCα. To test this, A549 cells expressing high levels of endogenous c-Src were treated with NNK for various times followed by immunoprecipitation of c-Src and measurement of its activity by an immune complex kinase assay with acid-treated enolase as a substrate as described under “Experimental Procedures.” Results reveal that NNK potently enhances c-Src activity in a time-dependent manner (Fig. 4B). To pharmacologically test whether c-Src functions upstream of PKCα/Bad in NNK survival signaling, A549 cells were treated with NNK in the absence or presence of increasing concentrations of the Src-specific tyrosine kinase inhibitor PP2 (34). Results show that PP2 inhibits NNK-induced PKCα activation in association with decreased Bad phosphorylation and enhanced cell death (Fig. 4, C and D). Collectively, these findings suggest that NNK-induced survival of human lung cancer cells may occur in a mechanism involving the c-Src/PKCα/Bad signal pathway.

**PKCα Inhibitor Staurosporine Inhibits NNK-induced Bad Phosphorylation and Enhances Apoptosis**—Staurosporine is a potent, cell-permeable inhibitor of PKCα that can block conventional, novel, and atypical PKC isoforms (38, 39). To test whether staurosporine affects NNK-stimulated Bad phosphorylation and cell survival, A549 cells were treated with NNK in the absence or presence of increasing concentrations of the Src-specific tyrosine kinase inhibitor PP2 (34). Results indicate that staurosporine potently blocks NNK-induced multisite Bad phosphorylation and promotes apoptotic cell death following treatment of cells with cisplatin or VP-16 (Fig. 5). These findings reveal that staurosporine-sensitive PKCα (i.e. PKCα) may be involved in NNK-induced Bad phosphorylation and survival of A549 cells.

**The β-adrenergic Receptor-specific Inhibitor Propranolol Potently Inhibits NNK-induced Bad Phosphorylation and Enhances Apoptosis**—NNK is a β-adrenergic receptor agonist which can stimulate DNA synthesis in lung adenocarcinoma (40). To test whether the β-adrenergic receptor is involved in NNK/c-Src/PKCα/Bad signaling, A549 cells were treated with NNK in the absence or presence of increasing concentrations of propranolol (a β-adrenergic receptor-specific inhibitor (22)). Results show that propranolol potently inhibits both NNK-induced PKCα activation and Bad phosphorylation (Fig. 6A). Importantly, propranolol abrogates NNK-induced cell survival following treatment with cisplatin or VP-16 (Fig. 6B). These results implicate NNK-induced Bad phosphorylation in a mechanism involving the upstream β-adrenergic receptor in pulmonary adenocarcinoma cells.

**NNK- or PKCα-induced Bad Phosphorylation Disrupts Bad/Bcl-XL Interaction**—The proapoptotic activity of Bad is regulated by serine phosphorylation. Dephosphorylated Bad can potently bind to Bcl-XL and quench the survival function of Bcl-XL (23). To test whether NNK-induced Bad phosphorylation inhibits Bad/Bcl-XL association, A549 cells were treated with increasing concentrations of NNK as indicated. A coimmunoprecipitation experiment was carried out using an agarose-conjugated Bad antibody. Bad-associated Bcl-XL (i.e.
bound Bel-XL) and Bad were analyzed by Western blotting using Bel-XL or Bad antibody, respectively. Results reveal that the treatment of cells with NNK results in Bad dissociation from Bel-XL in a dose-dependent manner, although NNK does not affect expression levels of Bel-XL (Fig. 7, A and B). Because our results indicate that NNK-induced Bad phosphorylation occurs through activation of PKCα, activated PKCα may directly phosphorylate Bad and disrupt Bad/Bel-XL binding. To test this, the Bad/Bel-XL complex was co-immunoprecipitated from A549 cells using an agarose-conjugated Bad antibody and incubated with purified, active PKCα in a kinase assay buffer containing γ-32P]ATP as described under “Experimental Procedures.” The phosphorylation of Bad was determined by autoradiography. Bad-associated Bel-XL (i.e. bound Bel-XL), non-bound Bel-XL, and Bad were analyzed as above. The results indicate that PKCα induces a time-dependent phosphorylation of Bad in association with decreased interaction with Bel-XL, which is characterized by a reduced amount of bound Bel-XL and enhanced level of non-bound Bel-XL (Fig. 7C). These findings suggest that PKCα can directly disrupt the Bad/Bel-XL complex in a mechanism that likely involves Bad phosphorylation.

Depletion of PKCα by RNAi Suppresses NNK-induced Bad Phosphorylation and Enhances Bad/Bel-XL Interaction as Well as Apoptosis—Our data strongly indicated that PKCα functions as a NNK-activated Bad kinase in human lung cancer cells (Figs. 1–3). To test whether PKCα is required for NNK-stimulated multisite Bad phosphorylation, a vector-based stable gene silencing approach was employed for specific depletion of PKCα from human lung cancer cells. The pSilencerTM 2.1-U6 hygro plasmids bearing the PKCα hairpin siRNA insert were transfected into A549 cells using LipofectamineTM 2000. The stable clones persistently producing PKCα siRNA were selected using hygromycin. The results indicated that cells expressing PKCα siRNA display more than a 95% reduction of PKCα protein expression (Fig. 8A). This silencing effect for PKCα is specific, because it does not affect expression of other PKCs (i.e. PKCβ or γ; Fig. 8A). A specific disruption of PKCα expression by RNAi blocks NNK-induced Bad phosphorylation in association with enhanced Bad/Bel-XL interaction and apoptotic cell death following treatment of cells with cisplatin or VP-16 in the absence or presence of NNK (Fig. 8). In addition, NNK can reduce Bad/Bel-XL interaction in cells expressing vector control but not in cells expressing PKCα siRNA (Fig. 8B). These findings indicate that PKCα is required for both NNK-induced Bad phosphorylation and dissociation of the Bad/Bel-XL complex. Because NNK has no additional survival effect on cells expressing PKCα siRNA, PKCα may be essential for NNK-induced survival of human lung cancer cells.

Bad May Be a Required Target for NNK-induced Survival and Chemoresistance of Human Lung Cancer Cells—Our results indicated that NNK can abrogate the proapoptotic activity of Bad by inducing its phosphorylation at multiple sites (i.e. Ser-112, Ser-136, and Ser-155) through activation of PKCα in human lung cancer cells. This suggests that Bad may be a potential target for treatment of patients with lung cancer. To test this, a vector-based stable silencing approach was employed for specific knockdown of the Bad gene as described under “Experimental Procedures.” The results indicated that depletion of Bad expression by RNAi enhances cell survival following treatment with cisplatin or VP-16 in the absence or presence of NNK (Fig. 9). This suggests that Bad may be a potential therapeutic target for patients with lung cancer.

**DISCUSSION**

Both nicotine and NNK have been found to prolong cell survival, which may be associated with increased chemoresistance of human lung cancer cells but our understanding of the molecular mechanism(s) is fragmentary (22, 31). PKC isoforms...
 Confirmation of PKC sites, indicating its potential direct role as a Bad kinase (Fig. 2).

**PKC Phosphorylates Bad**

**FIG. 7.** NNK-or PKCε-induced Bad phosphorylation disrupts Bad/Bcl-XL interaction. A, A549 cells were treated with increasing concentrations of NNK for 30 min. The cells were then harvested, washed, and lysed in detergent buffer. The levels of Bcl-XL in total lysate were analyzed by Western blot using a Bcl-XL antibody. B, A549 cells were treated with increasing concentrations of NNK for 30 min. A co-immunoprecipitation (IP) experiment was performed using an agarose-conjugated Bad antibody. Bad-associated Bcl-XL (i.e. Bound Bcl-XL) or total Bad was analyzed by Western blot using a Bcl-XL or a Bad antibody, respectively. C, the Bad/Bcl-XL complex was co-immunoprecipitated from A549 cells using an agarose-conjugated Bad antibody and incubated with purified, active PKCs in a kinase assay buffer in *vitro* for various times. The samples were centrifuged at 14,000 rpm for 5 min. The resulting supernatant and immunoprecipitates were subjected to SDS-PAGE. Phosphorylation of Bad was determined by autoradiography. Bad, bound Bcl-XL, and non-bound Bcl-XL were analyzed by Western blot using a Bad or Bcl-XL antibody, respectively.

that appear to be anti-apoptotic include PKCc, PKCβII, PKCe, and the atypical isoforms PKCθ and PKCγ (41). Previous studies reveal that the drug-resistant phenotype is associated with expression and/or activity of PKCs in lung cancer cell lines and lung carcinomas (41). PKCθ is an atypical PKC isomorph, and Northern blot analysis, using the full-length PKCθ cDNA as a probe, revealed that the PKCθ transcript presents predominantly in the lung and brain (15). Consistently, our data show that PKCθ is ubiquitously expressed in both human small cell and non-small cell lung cancer cells (Fig. 1A). Because NNK can potently activate PKCθ and enhances survival of human lung cancer cells (Fig. 1B), NNK-stimulated survival and/or chemoresistance may occur, at least in part, through activation of PKCθ. PKCθ has been reported to be an anti-apoptotic protein kinase (16), but the downstream survival effector(s) involved remains unknown. Because the decision phase for cell survival and cell death is largely regulated by the Bcl2 family of apoptotic regulators (42), a Bcl2 family member(s) may be the most attractive candidate for the substrate of PKCθ in NNK survival signaling. Importantly, Bad, a BH3-only proapoptotic protein, is widely expressed in various lung cancer cells, and NNK potently induces multistage Bad phosphorylation (i.e. Ser-112, Ser-136, and Ser-155), which is known to abrogate the proapoptotic activity of Bad (Fig. 1), suggesting that Bad may function as a survival target of PKCθ in human lung cancer cells.

Evidence reported here suggests that PKCθ may be a physiological Bad kinase, because PKCθ can co-localize with Bad in the cytoplasm and directly phosphorylate either endogenous or recombinant Bad *in vitro* at Ser-112, Ser-136, and Ser-155 sites, indicating its potential direct role as a Bad kinase (Fig. 2). Confirmation of PKCθ as a physiological Bad kinase was obtained *in vivo* from results of transfection studies demonstrating that PKCθ, when overexpressed in NCI-H157 cells, resulted in enhanced phosphorylation of Bad at all three sites, which is consistent with *in vitro* results (Fig. 3 versus Fig. 2C). Importantly, specific knockdown of PKCθ expression by RNAi can significantly inhibit NNK-stimulated Bad phosphorylation at these three sites (Fig. 8). These findings strongly indicate that PKCθ is a physiological NNK-activated Bad kinase.

PKCθ belongs to an atypical PKC isoenzyme category that differs significantly from other PKC family members in their regulatory domain in that it lacks both the calcium-binding domain and one of the two zinc finger motifs required for diacylglycerol binding (13). These domain variations result in a different requirement for activation. Because PKCθ is insensitive to both Ca^2+^ and diacylglycerol (13), other mechanisms, for example, phosphorylation or protein-protein interaction may be required for PKCθ activation. Recent studies reveal that c-Src not only induces tyrosine phosphorylation of PKCθ but also directly binds to PKCθ, which leads to its activation (34). Because NNK can induce activation of c-Src, which could then in turn phosphorylate PKCθ, and the Src-specific inhibitor PP2 blocks NNK-stimulated PKCθ activation (Fig. 4), these findings strongly suggest that c-Src most likely functions as a NNK-activated upstream PKCθ kinase.

High levels of β-adrenergic receptor are expressed in pulmo-
nary adenocarcinoma cells (43). NNK functions as a β-adrenergic receptor agonist, and its effect could be abrogated by propranolol (a β-adrenergic receptor inhibitor (44, 45)). Src, an upstream kinase of PKC <sub>β</sub> (34), has been found to play an active role in the agonist-induced activation of β-adrenergic receptors (46, 47). Our results reveal that the β-adrenergic receptor-specific inhibitor propranolol can block NNK-induced activation of PKC<sub>β</sub> in association with reduced Bad phosphorylation and enhanced apoptosis of A549 cells (Fig. 6), suggesting that the β-adrenergic receptor may be the major upstream receptor in NNK-mediated survival signaling. Thus, propranolol may potentially be developed as a therapeutic drug that specifically targets β-adrenergic receptors to enhance chemosensitivity in patients with lung cancer expressing high levels of β-adrenergic receptor, PKC<sub>β</sub> and Bad.

In addition to PKC<sub>β</sub>, previous reports and our data have demonstrated that NNK can also activate other known Bad kinases including MAPKs ERK1/2, AKT, and PKA (Refs. 10, 22, and 48 and data not shown). These various types of Bad kinases may cooperatively regulate the proapoptotic function of Bad through phosphorylation. However, distinct Bad kinases phosphorylate Bad at distinct sites. For example, ERK1/2 is a Bad Ser-112, AKT is a Bad Ser-136, and PKA is a Bad Ser-155 kinase, respectively (29, 49–51). By contrast, PKC<sub>β</sub> is a three-site Bad kinase that can phosphorylate Bad at Ser-112, Ser-136, and Ser-155 (Figs. 2 and 3). Thus, PKC<sub>β</sub> may play a more extensive and/or more important role in NNK survival signaling than that of other Bad kinases. This helps to explain why depletion of PKC<sub>β</sub> by RNAi potently blocks NNK-induced Bad phosphorylation and enhances cell death (Fig. 8).

Bad phosphorylation at Ser-112, Ser-136, and Ser-155 has been demonstrated to abrogate its proapoptotic function but the mechanism(s) is not fully understood (23, 25–27, 30). Our data showed that NNK not only induced Bad phosphorylation via activation of PKC<sub>β</sub> but also facilitated a dissociation of Bad/Bcl-XL heterodimers (Fig. 7B). As direct evidence for this potential mechanism, purified active PKC<sub>β</sub> can directly disrupt the Bad/Bcl-XL complex in vitro in a phosphorylation-dependent manner (Fig. 7C). This phosphorylated and unbound form of Bad is no longer able to quench the survival activity of Bcl-XL. Importantly, the specific knockdown of PKC<sub>β</sub> expression by RNAi enhances the Bad/Bcl-XL interaction in association with inhibition of Bad phosphorylation (Fig. 8). These findings reveal that PKC<sub>β</sub> may be required for NNK-induced Bad/Bcl-XL dissociation. Therefore, NNK-induced Bad phosphorylation resulting in inactivation of Bad via dissociation from Bcl-XL, which in turn contributes to cell survival (Fig. 10).

In summary, our findings have identified PKC<sub>β</sub> as a NNK-activated protein kinase that can directly phosphorylate the BH3-only proapoptotic protein Bad at Ser-112, Ser-136, and Ser-155. In addition to single-site Bad kinases (i.e. ERK1/2, AKT, and PKA), PKC<sub>β</sub> can apparently function as a three-site physiological Bad kinase. Thus, NNK-induced cell survival may occur, at least in part, through a novel signaling pathway involving β-adrenergic receptor/c-Src/PKC<sub>β</sub>/Bad (Fig. 10). PKC<sub>β</sub>-induced Bad phosphorylation can disrupt the Bad/Bcl-XL complex to abrogate the proapoptotic function of Bad, which may lead to enhanced survival and/or chemoresistance of human lung cancer cells.

Acknowledgment—We thank Dr. Alan P. Fields for kindly providing the human PKC<sub>β</sub> cDNA.

REFERENCES
1. American Cancer Society (2003) Cancer Facts & Figures, pp. 13–14, American Cancer Society Inc., Atlanta, GA
2. Hecht, S. S. (2003) Nat. Rev. Cancer 3, 733–744
3. Shipland, D. R. (1999) Environ. Health Perspect. 103, 131–142
4. Hecht, S. S. (1999) J. Natl. Cancer Inst. 91, 1194–1210
5. Zheng, T., Holford, T. R., Chen, Y., Ma, J. Z., Mayne, S. T., Liu, W., Flannery, J., and Boyle, P. (1996) Int. J. Cancer 68, 172–176
6. Kelly, A., Blair, N., and Peachack, T. F. (2001) J. Womens Health Gend. Based Med. 10, 515–518
7. Schuller, H. M. (2002) Nat. Rev. Cancer 2, 455–463
8. Coutler, J. F., Drum, R., Weinfeld, M., O’Connor, T. R., and Castonguay, A. (2003) J. Mol. Biol. 315, 539–557
9. Bhagwat, S. V., Vijayasrivaths, C., Raza, H., Mullick, J., and Avadhani, N. G. (1998) Biochem. Pharmacol. 56, 831–839
10. West, K. A., Brognard, J., Clark, A. S., Linnoila, I. R., Yang, X., Swain, S. M., Harris, C., Belinsky, S., and Dennis, P. A. (2003) J. Clin. Investig. 111, 81–90
11. Zhang, J., Anastasiadis, P. Z., Liu, Y., Thompson, E. A., and Fields, A. P. (2004) J. Biol. Chem. 279, 22118–22123
12. Mellor, H., and Parker, P. J. (1998) Biochem. J. 332, 281–292
13. Spitaler, M., Villunger, A., Grunicke, H., and Uberall, F. (2000) J. Biol. Chem.
Dumont, J. A., and Bitonti, A. J. (1994) *Biochem. Biophys. Res. Commun.* **204**, 264–272

Selbie, L. A., Schmitz-Peiffer, C., Sheng, Y., and Biden, T. J. (1993) *J. Biol. Chem.* **268**, 24296–24302

Murray, N. R., and Fields, A. P. (1997) *J. Biol. Chem.* **272**, 264–272

Selbie, L. A., Schmitz-Peiffer, C., Sheng, Y., and Biden, T. J. (1993) *J. Biol. Chem.* **268**, 24296–24302

Murray, N. R., and Fields, A. P. (1997) *J. Biol. Chem.* **272**, 27521–27524

Cory, S., and Adams, J. M. (2002) *Nat. Rev. Cancer* **2**, 647–656

Lin, B., Kumar, S., Lin, F., Liu, W., Han, Y., Cao, X., Dawson, M. I., Reed, J. C., and Zhang, X. (2004) *Cell* **116**, 527–540

Sattler, M., Liang, H., Nettesheim, D., Meadows, R., Harlan, J., Eberstadt, M., Yoon, H., Shuker, S., Chang, B., Minn, A., Thompson, C., and Fesik, S. (1997) *Science* **275**, 983–986

Huang, D. C., and Strasser, A. (2000) *Cell* **103**, 839–842

Jin, Z., Gao, F., Flagg, T., and Deng, X. (2004) *J. Biol. Chem.* **279**, 23837–23844

Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) *Cell* **87**, 619–628

Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) *Cell* **87**, 619–628

Hirai, I., and Wang, H. G. (2001) *Biochem. J.* **359**, 345–352

Masters, S. C., Yang, H., Datta, S. R., Greenberg, M. E., and Fu, H. (2001) *Mol. Pharmacol.* **60**, 1325–1331

Scheid, M. P., Schubert, K. M., and Duronio, V. (1999) *J. Biol. Chem.* **274**, 31108–31113

Tan, Y., Demeter, M. R., Ruan, H., and Comb, M. J. (2000) *J. Biol. Chem.* **275**, 25865–25869

Jin, Z., Gao, F., Flagg, T., and Deng, X. (2004) *J. Biol. Chem.* **279**, 40209–40219

Ikegaki, N., Katsumata, M., Minna, J., and Tsujimoto, Y. (1994) *Cancer Res.* **54**, 5425–5431

Trombino, S., Cesario, A., Margaritora, S., Granone, P., Motta, G., Falugi, C., and Russo, P. (2004) *Cancer Res.* **64**, 135–145

Wooten, M. W., Vandenplas, M. L., Seibenhener, M. L., Geetha, T., and Diaz-Meco, M. T. (2001) *Mol. Cell. Biol.* **21**, 8414–8427

Deng, X., Xiao, L., Lang, W., Gao, F., Ruvolo, P., and May, W. S., Jr. (2001) *J. Biol. Chem.* **276**, 23981–23988

Deng, X., Ruvolo, P., Carr, B., and May, W. S., Jr. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 1578–1583

Jamieson, L., Carpenter, L., Biden, T. J., and Fields, A. P. (1999) *J. Biol. Chem.* **274**, 3927–3930

Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M., and Tomita, F. (1996) *Biochem. Biophys. Res. Commun.* **135**, 397–402

Gescher, A. (2000) *Crit. Rev. Oncol. Hematol.* **34**, 127–135

Schuller, H. M., Tithof, P. K., Williams, M., and Plummer, H. (1999) *Cancer Res.* **59**, 4510–4515

Ding, L., Wang, H., Lang, W., and Xiao, L. (2002) *J. Biol. Chem.* **277**, 35305–35313

Adams, J. M., and Cory, S. (1998) *Science* **281**, 1322–1326

Park, P. G., Merryman, J., Oroff, M., and Schuller, H. M. (1995) *Cancer Res.* **55**, 3504–3508

Suemaru, K., Kawasaki, H., Oishi, R., Gomita, Y., and Tanizaki, Y. (1997) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **355**, 571–575

Uchida, S., Kawashima, K., and Lee, T. J. (2002) *Auton. Neurosci.* **96**, 126–130

Huang, J., Sun, Y., and Huang, X. Y. (2004) *J. Biol. Chem.* **279**, 21637–21642

Fan, G., Shumay, E., Malbon, C. C., and Wang, H. (2001) *J. Biol. Chem.* **276**, 13240–13247

Jull, B. A., Plummer, H. K., III, and Schuller, H. M. (2001) *J. Cancer Res. Clin. Oncol.* **127**, 707–717

Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotuh, Y., and Greenberg, M. E. (1997) *Cell* **91**, 231–241

Zhou, X., Liu, Y., Payne, G., Lutz, R. J., and Chittenden, T. (2000) *J. Biol. Chem.* **275**, 25046–25051

Fang, X., Yu, S., Eder, A., Mao, M., Bast, R. C., Boyd, D., and Mills, G. (1999) *Oncogene* **18**, 6635–6649