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p38 kinase mediates nitric oxide-induced apoptosis of chondrocytes through the inhibition of protein kinase C ζ by blocking autophosphorylation

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Abstract

This study investigated the molecular mechanisms underlying inhibition of protein kinase C (PKC) ζ by p38 kinase during nitric oxide (NO)-induced apoptosis of chondrocytes. Coimmunoprecipitation experiments showed that activation of p38 kinase following addition of an NO donor resulted in a physical association between PKCζ and p38 kinase. Direct interaction of p38 kinase with PKCζ was confirmed *in vitro* using p38 kinase and PKCζ recombinant proteins. p38 kinase interacts with the regulatory domain of PKCζ and its association blocked PKCζ autophosphorylation. Micro LC–MS/MS analysis using recombinant proteins indicated that the interaction of p38 kinase with PKCζ blocked autophosphorylation of PKCζ on Thr-560, which is required for PKCζ activation. Collectively, our results demonstrate a novel mechanism of PKCζ regulation: following activation by the production of NO, p38 kinase binds directly to the PKCζ regulatory domain, preventing PKCζ autophosphorylation on Thr-560, thereby inhibiting PKCζ activation.

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Abbreviations: aPKC, atypical PKC; ATF, activating transcription factor; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione *S* transferase; MAP kinase, mitogen-activated protein kinase; NO, nitric oxide; Par-4, prostate androgen response-4; PDK-1, 3-Phosphoinositide-dependent protein kinase-1; PI3-K, phosphoinositide 3-OH kinase; PKC, protein kinase C; PS-PKC, pseudosubstrate region of human PKCζ 113-125; RT-PCR, reverse transcription-polymerase chain reaction; SNP, sodium nitroprusside; TUNEL, terminal deoxynucleotidyl transfer-mediated nick end labeling

Introduction

Protein kinase C (PKC) comprises at least 10 structurally related phospholipid-dependent protein kinases. Each PKC isoform is a single polypeptide with an amino-terminal regulatory domain and a carboxyl-terminal catalytic domain. PKC isoforms can be grouped into three subclasses on the basis of their regulatory properties. The 'conventional' PKCs (α, βI, βII and γ) require Ca^{2+} and diacylglycerol for activation, whereas 'novel' PKCs (δ, ε, θ and η) are activated by diacylglycerol but are Ca^{2+} independent. Unlike conventional and novel PKC isoforms, 'atypical' PKCs (aPKC) ζ and λ/ι are activated independent of either Ca^{2+} or diacylglycerol.

Although the regulatory mechanisms for atypical PKC activation remain largely unknown, it has been shown that phosphorylation of PKC and its interaction with other regulatory proteins plays an important role.[1–3] The interaction of PKC isoforms with regulatory proteins determines subcellular localization as well as function. A variety of PKC binding proteins have been identified, including proteins that interact with active PKC, anchoring proteins and substrates.[2–4] In the case of PKCζ, binding of Par-4 to the regulatory domain inhibits PKCζ activity.[4] Growing evidence indicates that phosphorylation is also a crucial event in regulation of PKC. PKC phosphorylation is a processing event that regulates maturation of the enzyme, in addition to regulating activation.[2,5] PKCζ is phosphorylated at Thr-410 in the activation loop by phosphoinositide-dependent protein kinase-1 (PDK-1), and this phosphorylation is required for activity. PKCζ is also phosphorylated at Thr-560, which is an autophosphorylation site.[5–8] Recently, it has been shown that phosphorylation at these sites controls not only PKCζ activity but also its turnover.[9]

Chondrocyte apoptosis is believed to contribute to the pathogenesis of arthritis and cartilage destruction.[10–12] Previous studies from our group indicate that direct production of nitric oxide (NO) via addition of the NO donor sodium nitroprusside (SNP) to primary culture articular chondrocytes causes apoptosis.[13,14] PKCζ functions as a survival signal during NO-induced apoptosis of articular chondrocytes since NO-induced chondrocyte apoptosis requires inhibition of PKCζ.[15–19] The PKCζ inhibition occurs as a result of NO-induced p38 kinase activation and the inhibition of PKCζ activity is followed by proteolytic cleavage via the caspase-3-dependent pathway.[15] Our previous observation[15] that inhibition of caspase-3 did not rescue the inhibition of PKCζ activity although cleavage of PKCζ was significantly abrogated clearly indicated that inhibition of PKCζ activity precedes its cleavage by caspase-3.

The aim of the present study was to investigate the molecular mechanisms underlying PKCζ inhibition by p38 kinase during NO-induced chondrocyte apoptosis. We report here a novel mechanism of PKCζ inhibition by p38 kinase. We

demonstrated that following activation, p38 kinase directly interacts with the PKCζ regulatory domain, thereby inhibiting the autophosphorylation required for PKCζ activation.

Results

p38 kinase-dependent inhibition of PKCζ during NO-induced apoptosis of articular chondrocytes

Consistent with previous observations,[15] we found addition of the NO donor, SNP, to primary monolayer cultures of rabbit articular chondrocytes transiently activated p38 kinase (Figure 1a, upper panel). NO production also caused a decrease in protein level and activity of PKCζ. The decrease in PKCζ protein level was independent of its transcript level as determined by RT-PCR (Figure 1b, lower panel). This is consistent with our previous demonstration that NO inhibits PKCζ activity and stimulates subsequent proteolysis by caspase-3.[15] Inhibition of NO-induced p38 kinase activation by the specific inhibitor SB203580[20] blocked the decrease in PKCζ protein level and activity (Figure 1b), and also blocked

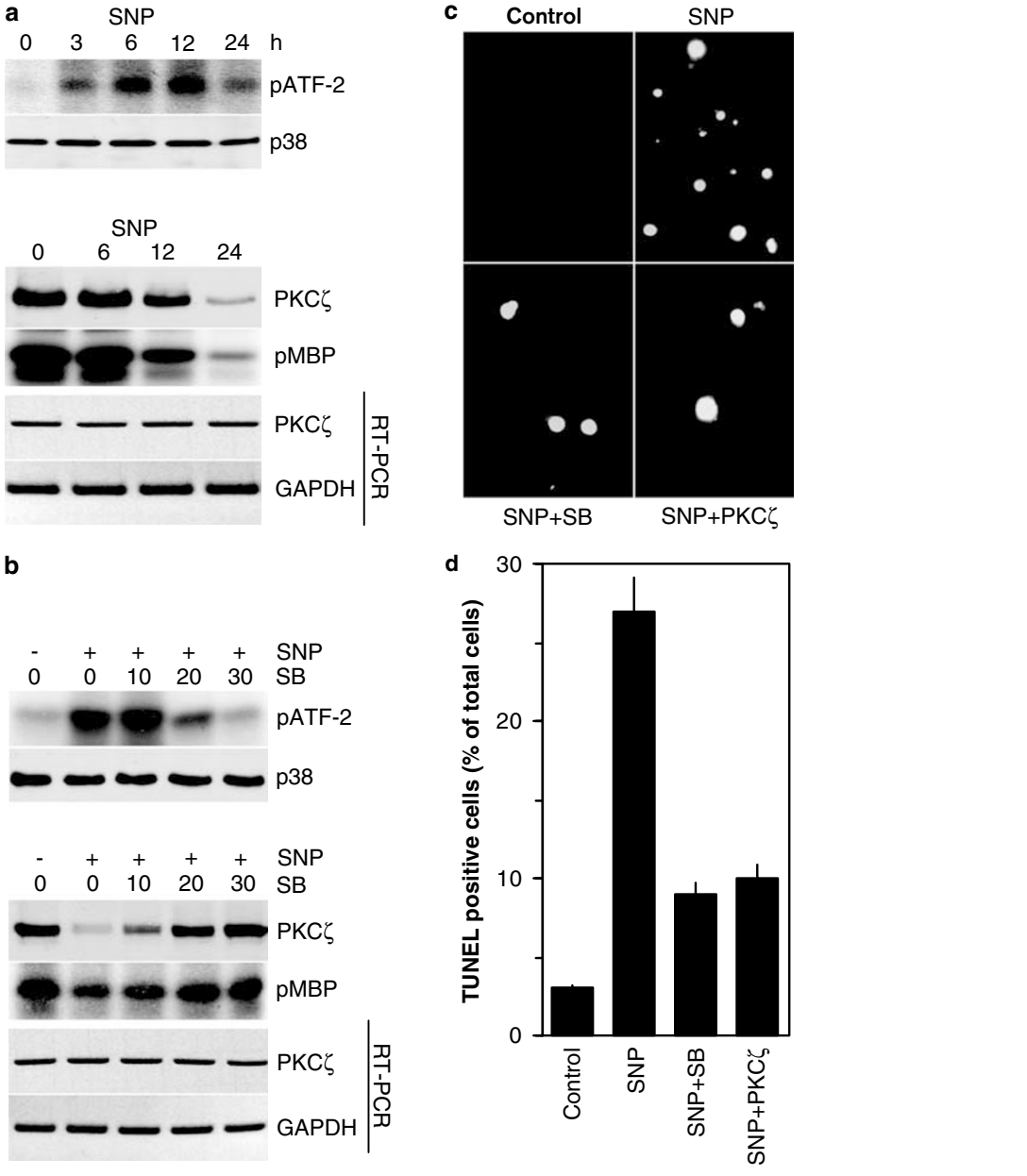

Figure 1 NO-induced activation of p38 kinase inhibits expression and activity of PKCζ in chondrocytes. (**a**, **b**) Articular chondrocytes were treated with 1 mM SNP (NO donor) for the indicated time period (**a**) or 1 mM SNP for 12 h (upper panel) or 24 h (lower panel) in the absence or presence of the indicated concentrations (μM) of SB203580 (**b**). Levels of p38 kinase and PKCζ proteins were determined by Western blot analysis, and the activities were determined by immune complex kinase assays using ATF-2 and MBP as p38 kinase and PKCζ substrates, respectively. mRNA levels of PKCζ and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined by RT-PCR. (**c**, **d**) Chondrocytes were infected with control adenovirus or adenovirus carrying cDNA for wild-type PKCζ. Infected cells were cultured in complete medium for 24 h and treated with 1 mM SNP for an additional 24 h. Apoptotic cells were identified by TUNEL assay (**c**), and quantified by flow cytometry (**d**). The data in (**a**–**c**) represent a typical result from at least four independent experiments, and in (**d**) represent mean and standard deviation ($n = 5$)

NO-induced apoptosis (Figure 1c and d). NO-induced apoptosis was also blocked by ectopic expression of wild-type PKCζ via adenovirus infection (Figure 1c and d). The above results collectively indicate that NO production induces chondrocyte apoptosis through p38 kinase activation and subsequent inhibition of PKCζ.

Direct interaction of p38 kinase with PKCζ via its regulatory domain

To better understand p38 kinase-mediated inhibition of PKCζ, we investigated a possible interaction between PKCζ and p38 kinase. PKCζ was overexpressed in chondrocytes using adenoviruses coding for wild-type PKCζ. Immunoprecipitation of p38 kinase from lysates prepared from these cells resulted in co-precipitation of PKCζ (Figure 2a), suggesting an interaction between PKCζ and p38 kinase. In addition, PKCζ coimmunoprecipitated with both p38 kinase and phosphorylated p38 kinase after addition of SNP to chondrocytes (Figure 2b). The complex formation between p38 kinase and PKCζ was blocked by the inhibition of p38 kinase activation with SB203580 (Figure 2c), indicating that p38 kinase activation is required for its association with PKCζ.

We next examined whether p38 kinase interacted with PKCζ directly or indirectly via an intermediate protein. This was explored by *in vitro* assays using recombinant GST-p38 kinase fusion protein and recombinant PKCζ protein. A GST pull-down from the mixture of GST-p38 kinase and recombinant PKCζ showed co-precipitation of PKCζ (Figure 3a, left panel). Inversely, immunoprecipitation of PKCζ from the mixture showed co-precipitation of p38 kinase (Figure 3a, right panel). These *in vitro* data indicate a direct interaction between p38 kinase and PKCζ. The interaction of p38 kinase with PKC appears to be specific for the ζ isoform among the PKC isoforms expressed in chondrocytes (i.e., α, ε, and ζ), since overexpressed PKCα or PKCε did not associate with p38 kinase (Figure 3b).

We investigated which PKCζ domain(s) was involved in the interaction with p38 kinase. Chondrocytes were engineered to overexpress myc-tagged wild-type PKCζ, or myc-tagged PKCζ regulatory or catalytic domains. Cell lysates were prepared, to which recombinant GST-p38 kinase was added, and the mixture was then analyzed in GST pull-down assays. Western blotting for the myc-tag revealed that both wild-type PKCζ and the PKCζ regulatory domain co-precipitated with GST-p38 kinase, while the PKCζ kinase domain did not (Figure 4). These data indicate that the PKCζ regulatory domain is responsible for the interaction with p38 kinase.

p38 kinase inhibits PKCζ by blocking autophosphorylation

We examined whether the interaction of p38 kinase with PKCζ was responsible for inhibition of PKCζ activity. Recombinant

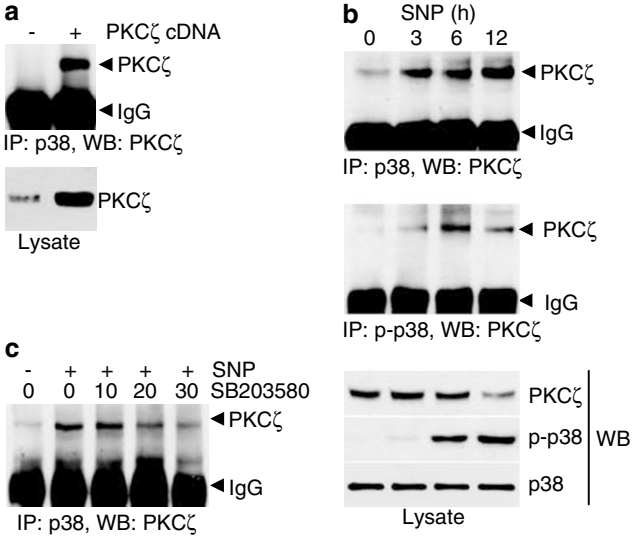

Figure 2 NO causes physical association of p38 kinase with PKCζ in chondrocytes. Articular chondrocytes were infected with empty adenovirus or virus containing PKCζ cDNA (**a**). Chondrocytes were treated with 1 mM SNP for the indicated time periods in the absence (**b**) or presence of indicated concentrations (μM) of SB203580 to inhibit p38 kinase (**c**). Total p38 kinase (p38) or phosphorylated p38 (p-p38) kinase was immunoprecipitated, and co-precipitation of PKCζ was detected by Western blot analysis. Levels of PKCζ, phosphorylated p38 kinase and p38 kinase were determined by Western blot analysis (**b**, lower panel). The data represent a typical result from at least five independent experiments

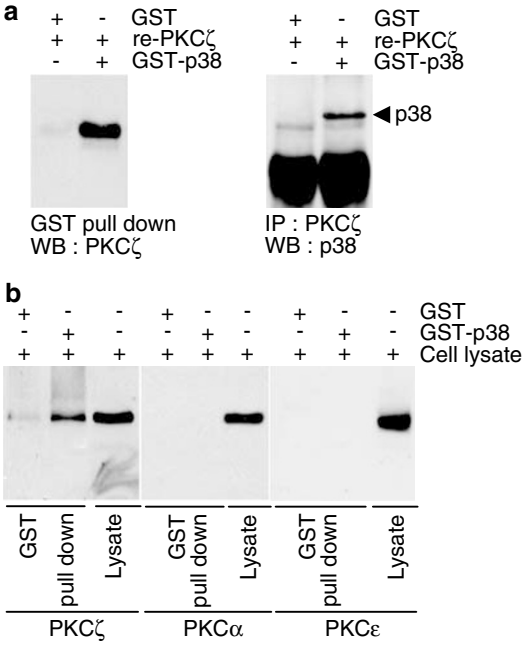

Figure 3 *In vitro* interaction of PKCζ and p38 kinase. (**a**) GST or GST-p38 kinase (1 μg) were incubated with recombinant PKCζ (1 μg) for 4 h in NP-40 buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40). Following pull-down of GST or GST-p38 kinase with GST beads, or immunoprecipitation of PKCζ with antibody, bound PKCζ or p38 kinase was detected by Western blot analysis. (**b**) Chondrocytes were infected with adenovirus carrying PKCζ, α or ε cDNA, and infected cells were cultured in complete medium for 24 h. Total cell lysates prepared from infected cells were incubated with recombinant GST or GST-p38 kinase (1 μg) for 4 h. Following GST pull-down, PKCζ, α or ε were detected by Western blot analysis. Total cell lysates were used as a positive control for Western blot analysis. The data represent a typical result from at least five independent experiments

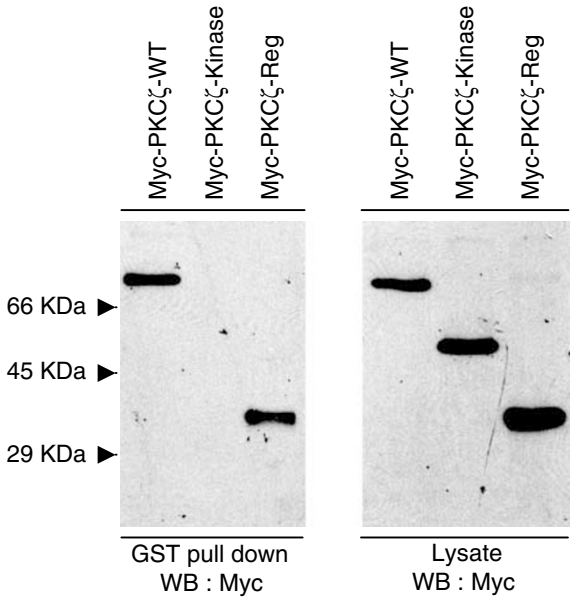

Figure 4 Regulatory domain of PKCζ interacts with p38 kinase. Articular chondrocytes were transfected with myc-tagged wild-type (WT) PKCζ, or myc-tagged PKCζ regulatory (Reg) or kinase (Kinase) domain. Total cell lysates were prepared after 24 h culture in complete medium, and 500 μg lysate was incubated with recombinant GST-p38 kinase (1 μg) for 4 h at 4°C. Following GST pull-down, co-precipitation of ectopically expressed PKCζ was determined by Western blotting using anti-myc antibody. Expression of PKCζ was determined by Western blotting of total cell lysates. The data represent a typical result from at least four independent experiments

proteins or immunoprecipitated cellular proteins were used in these studies. We found that the *in vitro* activity of immunoprecipitated cellular PKCζ was reduced to 62% of control level by addition of recombinant GST-p38 kinase (Figure 5a). As expected, cellular PKCζ activity was dramatically blocked by pseudosubstrate (PS)-PKCζ, and GST-p38 kinase itself did not phosphorylate the PKCζ substrate (Figure 5a). Recombinant PKCζ activity was also reduced to 33 and 45% of control level by the addition of immunoprecipitated cellular p38 kinase or phosphorylated p38 kinase, respectively (Figure 5b). The effect of p38 kinase interaction on PKCζ activity was further investigated by overexpressing p38 kinase and/or PKCζ in chondrocytes. Ectopically expressed myc-tagged PKCζ activity was determined by immune complex kinase assay using anti-myc antibody. As shown in Figure 5c and d, the activity of ectopically expressed PKCζ was significantly reduced by the coexpression of p38 kinase, indicating the inhibition of PKCζ activity by p38 kinase. While the association of PKCζ with p38 kinase resulted in lower PKCζ activity, it did not affect p38 kinase activity (Figure 5e). Taken together, the above results suggest that binding of p38 kinase to PKCζ results in the inhibition of PKCζ activity.

We next examined the role of PKCζ phosphorylation in p38 kinase-mediated inhibition of PKCζ activity. It has been shown that human PKCζ is phosphorylated on Thr-410 and Thr-560. PDK-1 phosphorylates the Thr-410 site,[6,7] whereas Thr-560 is reported to be an autophosphorylation site.[8] We found that recombinant PKCζ was phosphorylated following the addition of [γ-^{32}P] ATP alone, and that [γ-^{32}P] ATP incorporation was blocked by addition of PS-PKCζ or 'cold' ATP, suggesting autophosphorylation of PKCζ (Figure 6a). This PKCζ autophosphorylation was dramatically blocked by addition of recombinant GST-p38 kinase (Figure 6a), or by addition of cellular p38 kinase or phosphorylated p38 kinase immunoprecipitated from chondrocytes (Figure 6b). The effect was specific to p38 kinase since bead alone or the related ERK-1 did not affect PKCζ phosphorylation (Figure 6b). In contrast to this inhibition of autophosphorylation, phosphorylation on Thr-410 of recombinant PKCζ was not affected by addition of recombinant GST-p38 kinase, as determined by Western blot analysis (Figure 6a). Furthermore, addition of SNP to articular chondrocytes also did not affect PKCζ Thr-410 phosphorylation (Figure 6c). These data indicate that neither NO nor p38 kinase affect PKCζ Thr-410 phosphorylation. Taken together, these results suggest p38 kinase inhibits PKCζ activity via direct interaction and subsequent blocking of PKCζ autophosphorylation.

Micro LC–MS/MS analysis of PKCζ autophosphorylation sites

MicroLC–MS/MS experiments were performed to identify autophosphorylation sites on PKCζ. Control and autophosphorylated recombinant PKCζ protein were reduced and alkylated with iodoacetamide, digested with proteolytic enzymes (chymotrypsin, proteinase K, endoproteinase Lys-C or trypsin), and analyzed by microLC–MS/MS. Digested peptides from the four different proteolytic enzymes were combined to cover all phosphorylation sites present in PKCζ and to confidently identify closely situated modification sites in a single peptide. A total of six modification sites were identified from control and autophosphorylated PKCζ proteins, which comprised four newly identified phosphorylation sites (i.e., Ser-113, Ser-186, Ser-217/Ser-218 and Ser-520) and two known phosphorylation sites (i.e., Thr-410 and Thr-560) (Figure 7). Since MS/MS spectra from ion trap mass spectrometry often fail to provide sufficient information on very small fragment ions (e.g., b_1, y_1, b_2 and y_2), it was not easy to confidently assign phosphorylation sites among three consecutive serine residues, -^{216}SerSerSer^{218}-. However, Ser-216 can be ruled out because ^{217}SS*RKHDSIK DDSEDLKPVIDGM DGIKIS^{244} obtained from proteinase K digestion was identified as a phosphorylated peptide.

Selected ion chromatograms of phosphorylated peptides were compared between control and autophosphorylated recombinant PKCζ to determine autophosphorylation sites. Phosphorylated peptides containing Ser-113 and Ser-186 were dramatically increased in autophosphorylated PKCζ (Figure 8a and b). Control PKCζ contained phosphorylated Thr-560, which is a known autophosphorylation site. Thr-560 phosphorylation was increased in autophosphorylated PKCζ protein, although the degree of increase (1.9-fold) was less than that of Ser-113 or Ser-186 (Figure 8c). In contrast, the relative abundance of phosphorylated peptides containing Ser-217/218 and Ser-520 was not significantly different between control and autophosphorylated PKCζ (data not shown). Therefore, ion chromatogram analysis indicates that Ser-113, Ser-186 and Thr-560 can be autophosphorylated in PKCζ protein.

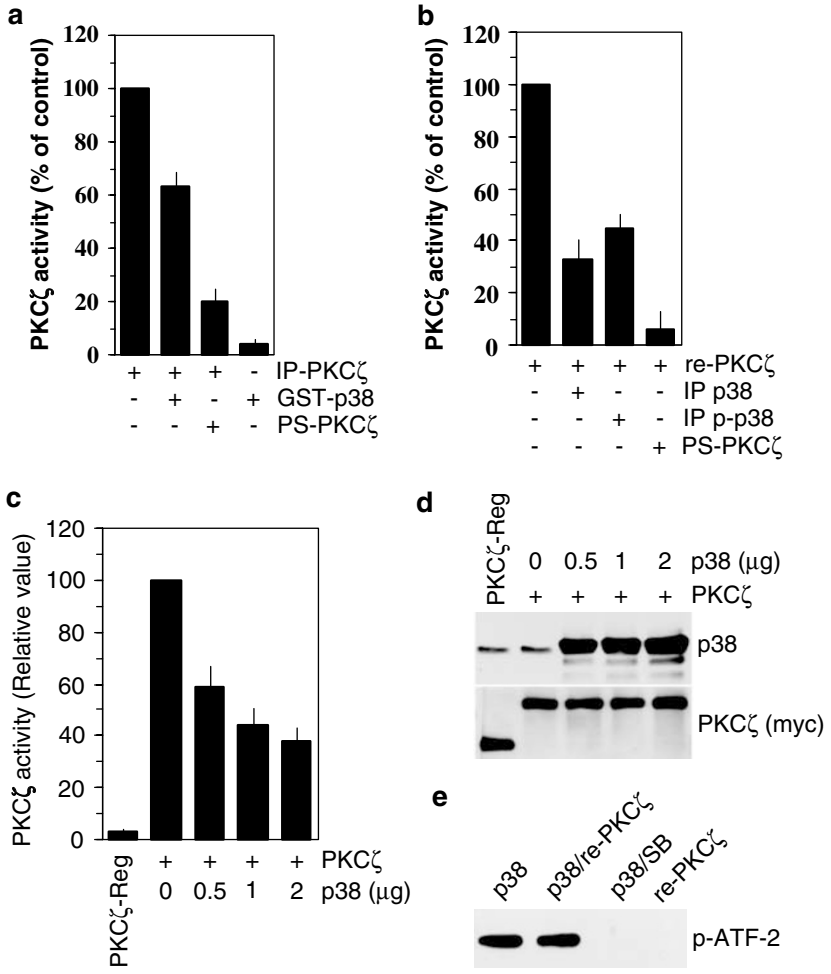

Figure 5 p38 kinase inhibits PKCζ activity. (**a**) Cellular PKCζ was immunoprecipitated from control chondrocyte cultures, and the immune complex (IP-PKCζ) was incubated with recombinant GST-p38 kinase (0.3 μg) or PS-PKCζ (30 μM). PKCζ activity was determined using a kinase assay kit. (**b**) Recombinant PKCζ (re-PKCζ, 0.1 μg) was incubated for 4 h with p38 kinase (IP p38) or phosphorylated p38 kinase (IP p-p38) (both immunoprecipitated from control chondrocyte cultures), or PS-PKCζ (30 μM). PKCζ activity was determined using an assay kit. (**c**, **d**) Articular chondrocytes were transfected with 1 μg of myc-tagged PKCζ regulatory domain (PKCζ-Reg) or wild type (PKCζ) and the indicated amount of p38 kinase (p38). Cells were cultured for 48 h. Following immunoprecipitation of PKCζ using anti-myc antibody, PKCζ activity was determined using an assay kit (**c**). Expression of p38 kinase and PKCζ (using anti-myc antibody) was determined by Western blot analysis (**d**). (**e**) Recombinant GST-p38 kinase (1 μg) was incubated alone (p38) or with recombinant PKCζ (1 μg) or 20 μM SB203580 (SB) for 1 h. p38 kinase activity was determined by *in vitro* kinase assay using ATF-2 as a substrate. Phosphorylation of ATF-2 was detected by Western blot analysis. The data in (**a**–**c**) represent mean and standard deviation, and in (**d**) and (**e**) represent a typical result from at least four independent experiments

To determine the significance of the inhibition of autophosphorylation by p38 kinase binding, myc-tagged PKCζ mutants (S113A, S186A, and T560A) were ectopically expressed and kinase activity was monitored by immune complex kinase assy. As shown in Figure 9, point mutation on Ser-113 or Ser-186 or double mutation on both sites did not affect kinase activity, whereas mutation of Thr-560 to alanine dramatically blocked kinase activity. The above results collectively indicate that inhibition of autophosphorylation on Thr-560, but not Ser-113 and Ser-186, is responsible for the inhibition of PKCζ activity by the binding of p38 kinase.

Discussion

This study investigated the mechanisms underlying p38 kinase-mediated inhibition of PKCζ following the addition of an NO donor to chondrocytes. As depicted in Figure 10, our data indicate that PKCζ activity was inhibited following p38 kinase activation due to a direct physical interaction between the two molecules. Furthermore, our data indicate the reason this interaction inhibited PKCζ activity was because the association reduced PKCζ autophosphorylation on Thr-560. Thus, our results provide evidence of a novel mechanism for PKCζ inhibition by p38 kinase. In relation to other known PKCζ inhibitors, Par-4 is known to bind to the regulatory domain of PKCζ and inhibit its activity.[4] It is unlikely that Par-4 is involved in p38 kinase-mediated PKCζ inhibition since we could not detect any changes in Par-4 levels as determined by Western blot analysis (data not shown).

p38 kinase and PKCζ play opposing roles in NO-induced apoptosis of primary culture articular chondrocytes. Activation of p38 kinase by NO functions as a proapoptotic signal,[13,14]

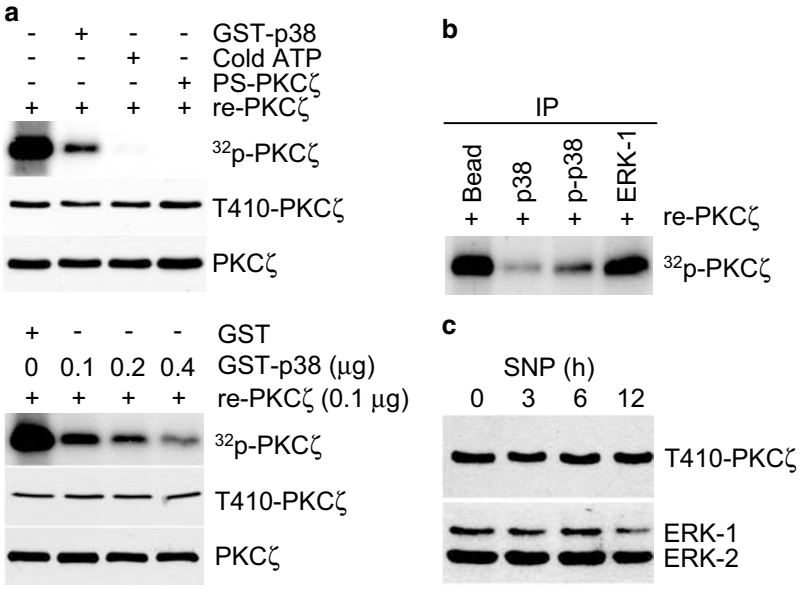

Figure 6 p38 kinase inhibits autophosphorylation of PKCζ. (**a**) Recombinant PKCζ (0.1 μg) was preincubated alone or with GST-p38 kinase (0.1 μg), PS-PKCζ (30 μM), or 'cold' ATP (200 μM) for 4 h (upper panel). Alternatively, recombinant PKCζ (0.1 μg) was incubated with the indicated amounts of GST-p38 kinase (lower panel). GST protein (0.1 μg) was used as a negative control for GST-p38 kinase. The extent of PKCζ autophosphorylation was measured by determining incorporation of [γ-^{32}P] ATP using autoradiography. Levels of total PKCζ and Thr-410-phosphorylated PKCζ (T410-PKCζ) were determined by Western blot analysis. (**b**) Articular chondrocytes were treated with 1 mM SNP for 12 h. p38 kinase, phosphorylated p38 kinase, or ERK-1 was immunoprecipitated from total cell lysates and mixed with recombinant PKCζ (0.1 μg). Following incubation for 4 h, autophosphorylation of PKCζ was determined by autoradiography. (**c**) Chondrocytes were treated with 1 mM SNP for the indicated time period. Levels of Thr-410-phosphorylated PKCζ and ERK were determined by Western blotting. The data represent a typical result from at least four independent experiments

with NO-induced apoptosis requiring downregulation of PKCζ.[15–19] The chondrocyte pro- and antiapoptotic functions of p38 kinase and PKCζ, respectively, are also observed in other cell types.[21–24] Evidence that PKCζ provides survival signals in chondrocytes is supported by the observations that ectopic expression of wild-type or constitutively active PKCζ inhibited NO-induced apoptosis of articular chondrocytes, whereas inhibition of PKCζ activity by PS-PKCζ potentiated NO-induced apoptosis.[15] Currently, underlying molecular mechanism leading to the inhibition of p38 kinase-induced apoptosis of chondrocyte by PKCζ is not known. Our previous study indicated that p38 kinase causes chondrocyte apoptosis by accumulating p53 protein in two different ways, transcriptional activation by NF-κB and post-translational stabilization.[13] We have also shown that accumulation of p53 leads to the induction of Bax,[14] translocation of Bax to the mitochondria (data not shown), and subsequent activation of caspase-3.[14] Ectopic expression of PKCζ did not affect NO-induced p38 kinase activation but blocked downstream signaling such as NF-κB activation, accumulation of p53, induction of Bax, and activation of caspase-3.[15] We also observed that NO production in chondrocyte causes nuclear translocation of phosphorylated p38 kinase, p53, and PKCζ (data not shown). Nuclear translocation of PKCζ has also been observed in other cell type that coincides with the inhibition of apoptosis.[25] Therefore, it is our hypothesis that PKCζ may function as an antiapoptotic signal by interrupting nuclear signaling such as NF-κB activation, p53 accumulation, and Bax expression.

The current results indicate that physical formation of a complex between p38 kinase and PKCζ is a critical step for the inhibition of PKCζ activity. While the principles underlying this interaction are yet to be fully identified, the present data show PKCζ and p38 kinase interact directly *in vitro*, suggesting the interaction does not require other proteins. It has been shown that p38 kinase can interact directly with numerous proteins with high specificity.[26–28] This specific interaction is mediated by a binding domain on p38 kinase, known as the CD domain, and a specific interacting motif on the target molecule, known as the D domain.[29] The D domain is found to form a modular structure, which comprises a cluster of positively charged amino-acid residues (LXL motif) surrounded by a hydrophobic region. Sequence analysis indicates PKCζ has a potential D domain as it contains an LXL motif (^{56}LTL^{58}) and a hydrophobic pocket in its regulatory domain.[30,31] That this potential D domain exists in the PKCζ regulatory domain is consistent with our present findings that p38 kinase interacts directly with PKCζ through its regulatory domain. In terms of identifying other factors involved in p38 kinase and PKCζ binding, the present study showed activation of p38 kinase was necessary for binding to PKCζ, suggesting that phosphorylation of p38 kinase is required for its interaction with PKCζ.

p38 kinase is a known Pro-directed Ser/Thr kinase. We initially hypothesized that p38 kinase inhibits PKCζ by phosphorylating PKCζ. There are five possible (Ser/Thr)-Pro p38 kinase phosphorylation sites on PKCζ, namely Ser-98, Thr-99, Thr-413, Ser-448 and Thr-559. The possibility that these sites are phosphorylated by p38 kinase was tested using recombinant GST-p38 kinase and synthetic peptides containing each site. MALDI-TOF analysis failed to detect phosphorylation of any of these peptides (data not shown), suggesting that PKCζ is not phosphorylated by p38 kinase.

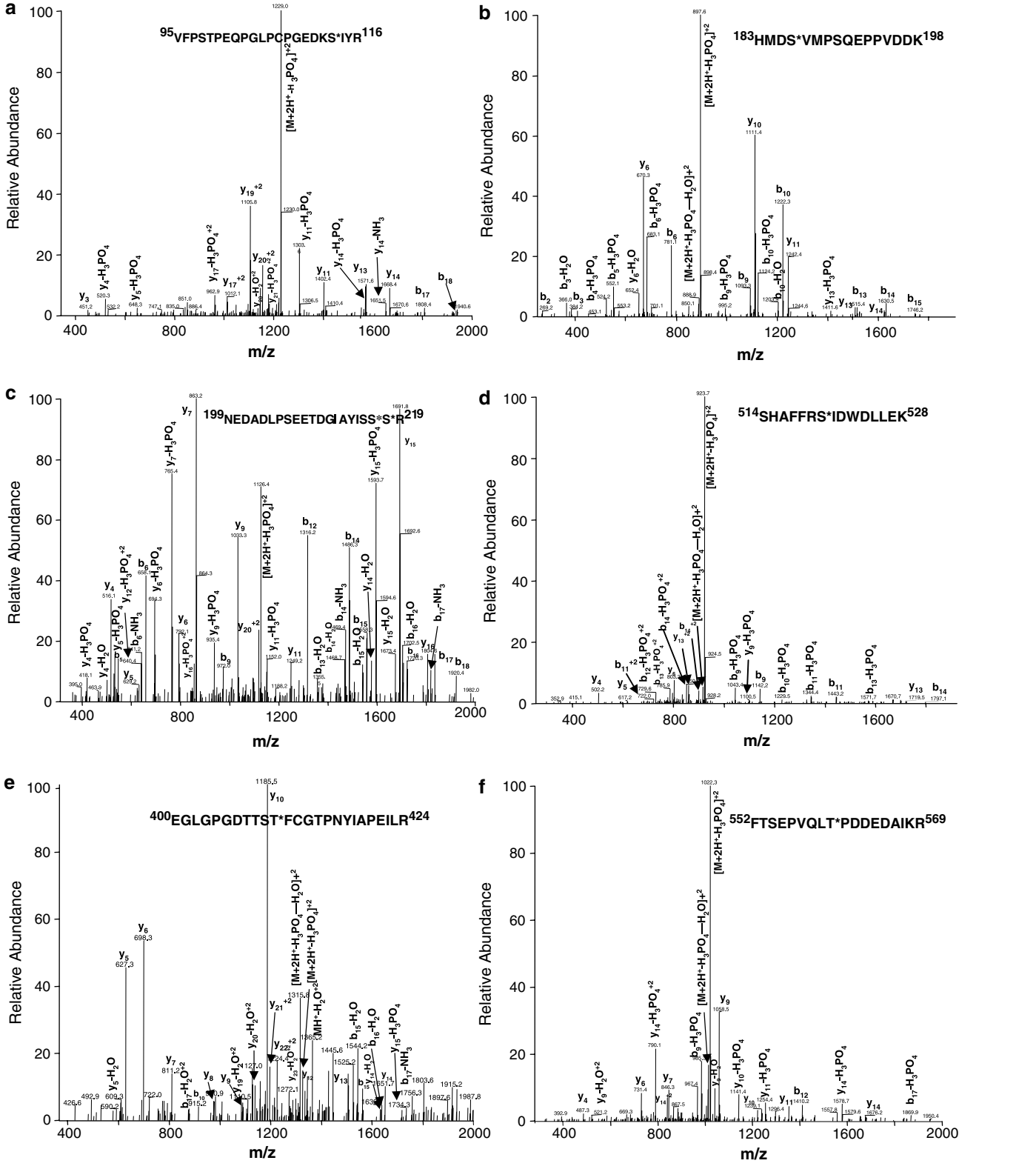

Figure 7 Mass spectrometry analysis of PKCζ phosphorylation sites. Peptides were generated by trypsin digestion of autophosphorylated recombinant PKCζ. MS/MS spectra of tryptic peptides were acquired using LCQ Deca XP Plus ion trap mass spectrometry. Peptide sequences and phosphorylation sites were identified using TurboSequest and Bioworks. The MS/MS spectra of six phosphorylated tryptic peptides are shown, indicating phosphorylation of Ser-113 (**a**), Ser-186 (**b**), Ser-216/217 (**c**), Ser-520 (**d**), Thr-410 (**e**) and Thr-560 (**f**). Fragment ions, especially b and y type ions, were assigned in each MS/MS spectra. Phosphorylation sites in each peptide are denoted with *. The data represent a typical result from at least three independent experiments

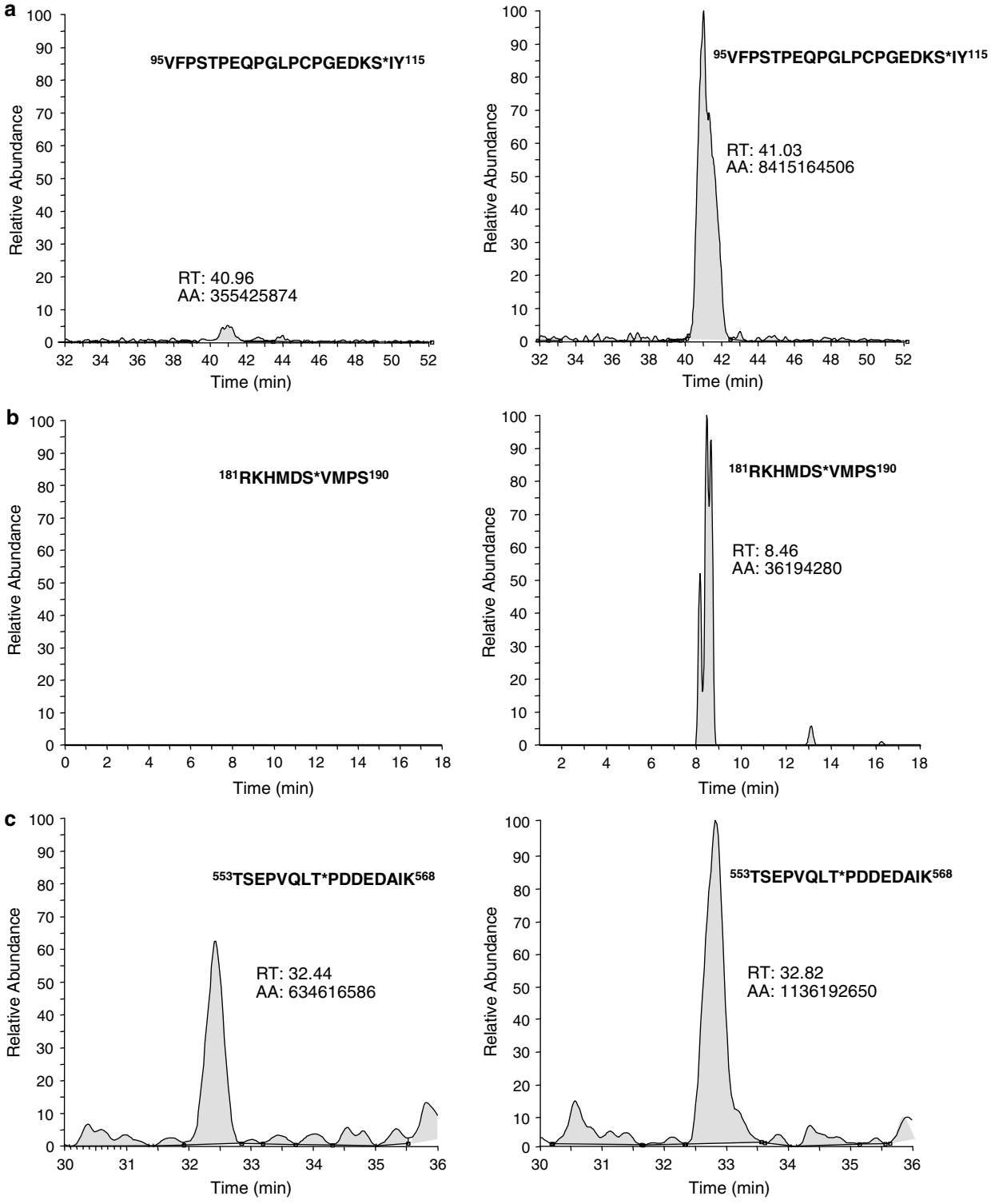

Figure 8 Quantitation of PKCζ autophosphorylation. Control (left panel) and autophosphorylated (right panel) PKCζ was digested with chymotrypsin (**a** and **c**) or proteinase K (**b**). Ion chromatograms of peptides containing Ser-114 (**a**), Ser-186 (**b**) and Thr-560 (**c**) are shown. AA denotes the integrated peak area. The data represent a typical result from at least three independent experiments

Instead, our data indicate that p38 kinase inhibits PKCζ activity by inhibiting PKCζ autophosphorylation.

MicroLC/MS/MS analysis identified two known and four novel phosphorylation sites in PKCζ. The known phosphorylation sites include Thr-560 in the Turn motif and Thr-410 in the activation loop. Thr-560 has been previously reported as an autophosphorylation site by several research groups.[1,5,8] It has also been demonstrated that inhibition of

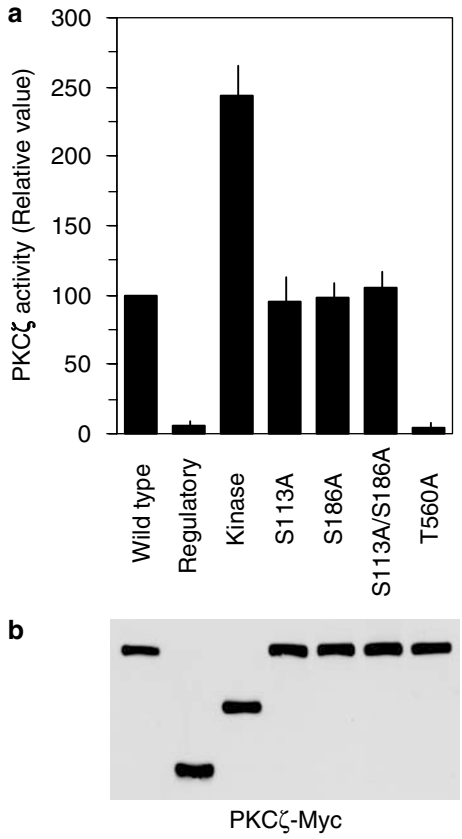

Figure 9 Inhibition of autophosphorylation on Thr-560 but not Ser-113 and Ser-186 blocks PKCζ activity. Chondrocytes were transfected with myc-tagged PKCζ constructs: wild-type, kinase domain (Kinase), regulatory domain (Regulatory), S113A, S186A, S113A/S1686A, and Thr-560. Following immunoprecipitation using anti-myc antibody, PKCζ activity was monitored by using an assay kit (**a**). Expression levels of individual PKC constructs was determined by Western blotting using anti-myc antibody (**b**). The data represent mean and standard deviation (**a**) and a typical result (**b**) from three independent experiments

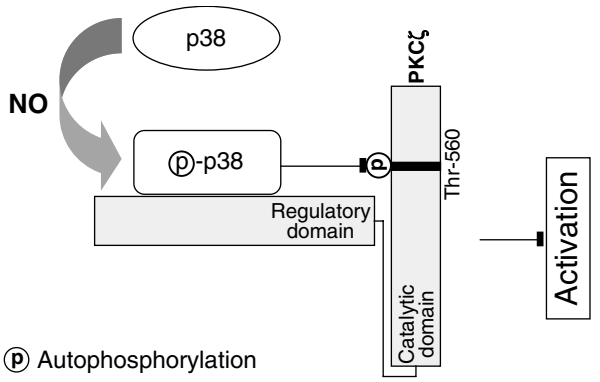

Figure 10 Schematic summary of PKCζ inhibition by p38 kinase. PKCζ activity is inhibited following p38 kinase activation due to a direct physical interaction between the two molecules, and this interaction inhibited PKCζ activity by blocking PKCζ autophosphorylation on Thr-560

Thr-560 autophosphorylation blocks PKCζ activity.[8] Phosphorylation status of Thr-410, which is phosphorylated by PDK-1,[6–8] was not affected by p38 kinase binding, indicating that p38 kinase does not interfere with Thr-410 phosphorylation by PDK-1. Among the novel phosphorylation sites identified by micro LC/MS/MS analysis, Ser-113 is located in the pseudosubstrate region, whereas Ser-186, Ser-217/218, and Ser-520 are not located in the specific motif or domain.

To identify autophosphorylation sites in PKCζ, the degree of autophosphorylation was measured by comparing peak areas of corresponding peptide ion chromatogram of control and autophosphorylated samples. Although this may not be a widely accepted way to monitor changes in the degree of modification, it appears sufficient for relative quantification of an autophosphorylation in simple protein systems for the following reasons. First, ion chromatograms of nonmodified peptides were very similar for control and autophosphorylated protein samples. Second, autophosphorylation did not appear to alter the digestion patterns of PKCζ, mostly yielding the same digested peptides. Using this technique, we identified Ser-113, Ser-186 and Thr-560 as autophosphorylation sites in PKCζ. Recombinant PKCζ contains both unphosphorylated and phosphorylated Thr-560, and autophosphorylation increased the relative abundance of phosphorylated Thr-560. In contrast, Ser-113 and Ser-186 were not significantly phosphorylated in recombinant PKCζ protein, and autophosphorylation dramatically increased phosphorylation of these sites. Ser-113 and Ser-186 appear to be novel autophosphorylation sites, as we could not find any other reports regarding their autophosphorylation. However, inhibition of autophosphorylation in these sites by mutation did not affect PKCζ activity, whereas mutation in Thr-560 blocked PKCζ activation. While the principles underlying the inhibition of autophosphorylation are yet to be fully identified, it appears to be clear that inhibition of Thr-560 phosphorylation by p38 kinase binding is critical for the inhibition of PKCζ activation, and the functional significance of the autophosphorylation on Ser-113 and Ser-186 remains to be elucidated.

In summary, we demonstrated in this study that PKCζ activity is inhibited following p38 kinase activation due to a direct physical interaction between the two molecules, and this interaction inhibited PKCζ activity by blocking PKCζ autophosphorylation on Thr-560. Thus, our results provide evidence of a novel mechanism for PKCζ inhibition by p38 kinase.

Materials and Methods

Cell culture

Articular chondrocytes were isolated from knee joint cartilage slices of 2-week-old New Zealand white rabbits by enzymatic digestion as described previously. [32] Briefly, cartilage slices were dissociated enzymatically for 6 h in 0.2% collagenase type II (381 U/mg solid, Sigma, St. Louis, MO, USA) in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Rockville, MD, USA). The cells were plated on culture dishes at a density of 5×10^4 cells/cm^2 in DMEM containing 10% bovine serum. Cells at day 3.5 cultures were treated with various pharmacological reagents, as specified in each experiment.

Assay of apoptosis

SNP-induced death of chondrocytes is due to apoptosis, as demonstrated by DNA fragmentation and terminal deoxynucleotidyl transfer-mediated nick end labeling (TUNEL).[14] In this study, apoptotic cells were identified using TUNEL assays and quantified by analyzing 1×10^4–2×10^4 cells using a FACSort flow cytometer.

Construction of PKC mutants

Constructs of myc-tagged wild-type PKCζ or myc-tagged PKCζ regulatory domain or catalytic domain were kindly provided by Dr. Peter J Parker (Cancer Research, UK). Point mutation to alanine of PKCζ on Ser-113 (S113A), Ser-186 (S186A), Thr-560 (T560A), and double mutation on Ser-113 and Ser-186 (S113A/S186A) was prepared by site-directed mutagenesis. Following mutagenizing oligonucleotides were used: 5′-CCTGGAGAAGACAAGGCCATCTACCGCCGTGG-3′ for S113A mutant that substitutes a serine TCC codon to an alanine GCC codon, 5′-GCATATGGATGCTGTCATGCCTTCCCAAG-3′ for S186A that substitutes a serine TCT codon to an alanine GCT codon, and 5′-CGAGCCCGTACAGCTGGCCCCAGATGATGAGG-3′ for T560A that substitutes a threonine ACC codon to an alanine GCC codon. Each construct with myc tag was ligated into pcDNA3 mammalian expression vector. Mutations were confirmed by sequence analysis.

Ectopic expression of PKCζ and p38 kinase

Chondrocytes from day 3 cultures were infected with either control adenovirus or adenovirus containing wild-type mouse PKCζ cDNA, as previously described,[18] and infected cells were cultured in complete medium for 24 h. In some experiments, infected cells were treated with 1 mM SNP for an additional 12 h. Alternatively, chondrocytes were transfected with plasmids coding for wild-type p38 kinase, myc-tagged PKCζ wild-type, regulatory domain, catalytic domain, or mutants (S113A, S186A, T560A, and S113A/S186A) using LipofectAMINE PLUS (Invitrogen, Carlsbad, CA, USA) as previously described.[19,33] The cells were cultured in complete medium for 24 h prior to further use.

Immunoprecipitation and kinase assays

The activity of cellular PKCζ and p38 kinase was determined by immune complex kinase assay. Briefly, cell lysates were prepared in a lysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate) containing protease inhibitors (10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride) and phosphatase inhibitors (1 mM NaF and 1 mM Na_3VO_4). The cell lysates were precipitated with polyclonal antibody against p38 kinase, PKCζ, or myc tag (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Immune complexes were collected using protein A sepharose beads and resuspended in 20 μl kinase reaction buffer (25 mM Tris-HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$, [γ-^{32}P] ATP and 1 μg substrate). Activating transcription factor-2 (ATF-2) (Santa Cruz Biotechnology Inc.) and myelin basic protein (MBP) (Sigma) were used as substrates for p38 kinase and PKCζ, respectively.

Activities of human recombinant PKCζ protein (produced in Sf-9 cells) and recombinant GST-p38 kinase (Calbiochem, La Jolla, CA, USA) were determined by *in vitro* kinase assay. Recombinant PKCζ activity was determined using a SignaTECT PKC assay kit (Promega, Madison, WI, USA). Briefly, recombinant PKCζ (0.1 μg) was added to the kinase reaction buffer described above, supplemented with phosphatidylserine and PKC-biotinylated peptide substrate. The kinase reaction was performed for 5 min at 30°C, and [γ-^{32}P] ATP incorporation into peptide substrate was measured by scintillation counting. Recombinant GST-p38 kinase fusion protein (0.1 μg) was added to 20 μl kinase reaction buffer, and the kinase reaction was performed for 30 min at 30°C using ATF-2 as a substrate. Phosphorylated ATF-2 was detected by Western blotting using an antiphosphorylated ATF-2 antibody (New England Biolabs, Beverly, MA, USA).

Autophosphorylation of PKCζ

Recombinant PKCζ (0.1 μg) was incubated for 30 min at 30°C in the kinase reaction buffer described above, in the absence or presence of various reagents, as specified in each experiment. The autophosphorylation reaction was stopped by adding 2× SDS sample buffer. Following size-fractionation of PKCζ by electrophoresis on SDS-polyacrylamide gels, PKCζ phosphorylation was detected using autoradiography.

GST pull-down assay

GST or GST-p38 fusion protein (1 μg) was incubated for 4 h on a rotator in a cold room with 1 μg recombinant PKCζ or 1 mg total cell lysate prepared in NP-40 buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40). GST or GST-p38 kinase complexes were collected using GST-beads and washed three times with NP-40 buffer. The bound proteins were size-fractionated by electrophoresis and detected using Western blotting.

Western blot analysis

Whole cell lysates were prepared by extracting proteins using a buffer (50 mM Tris-HCl, pH 7.4,. 150 mM NaCl, 1% Nonidet P-40 and 0.1% SDS) supplemented with protease inhibitors and phosphatase inhibitors, as described above. The proteins were size-fractionated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and detected using specific antibodies. Antibodies against PKCα, PKCε, myc-tag, PKCζ phosphorylated at Thr-410, phosphorylated ATF-2 and phosphorylated p38 kinase were purchased from New England Biolabs. Anti-PKCζ and anti-p38 kinase were from Santa Cruz Biotechnology Inc. Blots were developed using a peroxidase-conjugated secondary antibody and enhanced chemiluminescence kit.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated by a single-step guanidinium thiocyanate–phenol chloroform method, using RNA STAT-60 (Tel-Test B, Inc., Friendswood, TX, USA) according to the manufacturer's protocol. Total RNA (3 μg) was reverse-transcribed with AMV reverse transcriptase for 60 min at 42°C, followed by PCR. PCR conditions were 94°C for 40 s, 50°C for 40 s and 72°C for 40 s for a total of 33 cycles. A pair of PCR primers was designed to amplify PKCζ mRNA isolated from rabbit articular chondrocytes. PCR primers used were as follows: PKCζ sense 5′-ACTACGGCATGTG CAAGG-3′ and antisense 5′-GAACTGCGTGTCGGAGTTG-3′, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sense 5′-TCAC CATCTTCCAGGAGCGA-3′ and antisense 5′-CACAAT GCCGAAGTG GTCGT-3′. PCR products were analyzed on 1.5% agarose gels and visualized by ethidium bromide staining. Sequencing of rabbit PKCζ PCR product showed 92 and 88% homology to human and mouse PKCζ, respectively.

Enzymatic digestion for mass spectrometry analysis of PKCζ

A measure of 1 μg of control recombinant PKCζ or autophosphorylated PKCζ was reduced and alkylated by addition of dithiothreitol and iodoacetamide. To generate peptides covering different sequences, the samples were digested in a digestion buffer (100 mM Tris-HCl, pH 7.8, 10 mM $CaCl_2$) with one of four different proteolytic enzymes (chymotrypsin, proteinase K, endoproteinase Lys-C and trypsin). Digestion conditions were 14 h at 25°C for chymotrypsin, 1 h at 37°C for proteinase K, 14 h at 25°C for endoproteinase Lys-C and 14 h at 24°C for trypsin, with an enzyme to substrate ratio of 1 : 100. Protein digestion was quenched by adding 90% formic acid (final concentration of 5%).

Micro LC–MS/MS analysis of PKCζ

Digested proteins were loaded onto fused silica capillary columns (100 μm i.d. , 360 μm o.d.,) containing 8 cm of 5 μm particle size Polaris C-18 column material (Metachem, Ventura, CA, USA). The column was placed inline with an Agilent HP1100 quaternary LC pump and a splitter system was used to achieve a flow rate of 250 nl/min. Buffer A (5% acetonitrile and 0.1% formic acid) and buffer B (80% acetonitrile and 0.1% formic acid) were used to make a 110 min gradient. The gradient profile started with 5 min of 100% buffer A, followed by a 70 min gradient from 0 to 55% buffer B, a 30 min gradient from 55 to 100% buffer B, and a 5 min gradient of 100% buffer B. Eluted peptides were directly electrosprayed into an LCQ Deca XP Plus mass spectrometer (ThermoFinnigan, Palo Alto, CA, USA) by applying 2.3 kV of DC voltage. A data-dependent scan consisting of one full MS scan (400–1400 *m/z*) and three data-dependent MS/MS scans was used to generate MS/MS spectra of eluted peptides. Normalized collision energy of 35% was used throughout the data acquisition. MS/MS spectra were searched against an in-house protein database containing the PKCζ sequence using TurboSequest and phosphorylation modification (+ 80 on Ser, Thr, Tyr) was considered in the differential modification search. Bioworks Ver 3.1 was used to filter the search results and the following Xcorr values were applied to different charge states of peptides: 1.8 for singly charged peptides, 2.5 for doubly charged peptides and 3.5 for triply charged peptides. MS/MS spectra for phosphorylated peptides were generated using Xcalibur Ver 3.1 and manual assignment of fragment ions was performed to confirm the search results. Changes in the level of phosphorylation before and after autophosphorylation reaction were monitored by selected ion monitoring of each phosphorylated peptide. Peak area was integrated using a built-in integration function of Xcalibur.

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