Keratin 8 Phosphorylation by Protein Kinase C δ Regulates Shear Stress-mediated Disassembly of Keratin Intermediate Filaments in Alveolar Epithelial Cells*

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Phosphorylation of keratin intermediate filaments (IF) is known to affect their assembly state and organization; however, little is known about the mechanisms regulating keratin phosphorylation. In this study, we demonstrate that stress, but not stretch, causes disassembly of keratin IF in lung alveolar epithelial cells (AEC) and that this disassembly is regulated by protein kinase C δ-mediated phosphorylation of keratin 8 (K8) Ser-73. Specifically, in AEC subjected to shear stress, keratin IF are disassembled, as reflected by their increased solubility. In contrast, AEC subjected to stretch showed no changes in the state of assembly of IF. Pretreatment with the protein kinase C (PKC) inhibitor, bisindolylmaleimide, prevents the increase in solubility of either K8 or its assembly partner K18 in shear-stressed AEC. Phosphoserine-specific antibodies demonstrate that K8 Ser-73 is phosphorylated in a time-dependent manner in shear-stressed AEC. Furthermore, we showed that shear stress activates PKC δ and that the PKC δ peptide antagonist, δ V1-1, significantly attenuates the shear stress-induced increase in keratin phosphorylation and solubility. These data suggested that shear stress mediates the phosphorylation of scrine residues in K8, leading to the disassembly of IF in alveolar epithelial cells. Importantly, these data provided clues regarding a molecular link between mechanically induced signal transduction and alterations in cytoskeletal IF.

Keratin intermediate filaments (IF) are the major cytoskeletal components of epithelial cells. They are assembled as obligate heteropolymers of type I (K9–K20) and type II (K1–K8) IF proteins (1–3). In simple epithelia, such as those found in lung, liver, intestine, and pancreas, combinations of K7, K8, K18, K19, and K20 are expressed in a type I/type II stoichiometric ratio of 1:1 (1). For example, lung alveolar epithelial cells (AEC) express primarily K8 and K18 with variable levels of K7 and K19 (4, 5). The prototype structure of all IF proteins, including keratins, consists of a structurally conserved central coiled-coil α-helix termed the “rod” that is flanked by non-α-helical N-terminal “head” and C-terminal “tail” domains (2). Most of the structural heterogeneity of the different keratins resides in their head and tail domains, which also contain all of the known phosphorylation sites.

Phosphorylation of keratins at specific sites affects their assembly state, and it has been suggested that these modifications play a role in cell signaling (6). For example, phosphorylation of the N-terminal Ser-33 on K18 enables a cell cycle-dependent interaction between K8 and K18 IFs with members of the 14-3-3 protein family, promotes depolymerization of K8 and K18 in vitro (7), and plays a role in the intracellular distribution of K8 and K18 IF polymers (8). K8 and K18 are also phosphorylated in response to cell stress (9–12). In cultured HT-29 cells, hyperphosphorylation of K8 at Ser-73 has been associated with apoptosis induced by anisomycin or etoposide. More recently, it has been shown that soluble K8 is phosphorylated by p38 kinase upon FasR stimulation (11). Phosphorylation of K8 involves three major sites in vivo identified as: Ser-23, Ser-431, and Ser-73. Ser-23 is a highly conserved site among all type II keratins, suggesting a common function for this modification, whereas phosphorylation at Ser-431 increases during mitosis and upon exposure to epidermal growth factor in association with filament reorganization (13). During a variety of cellular stresses, including heat and drug exposure, Ser-73 is phosphorylated, whereas under normal conditions, it remains dephosphorylated.

Mechanical stimuli are important modulators of cellular function in tissues, especially in the mechanically ventilated lungs of patients with acute lung injury. Shear stress is a key physical force experienced by AEC in lungs that are fluid-filled and atelectatic. Shear stress is created by the cyclic opening and closing of the edematous, surfactant-depleted, collapsed alveoli. Another physical force experienced by alveolar epithelial cells is stretch, which occurs in the uninjured regions of the lung that are subjected to mechanical ventilator-induced overdistension. Mechanical stimuli, such as shear stress and stretch, are known to activate kinases, such as protein kinase C (14, 15). Shear stress has also been shown to activate phospholipase C (16), resulting in the cleavage of phosphatidylinositol bisphosphate into inositol 1,4,5-trisphosphate, a calcium-

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mobilizing second messenger, and diacylglycerol, an activator of PKC. Indeed, recent studies have implicated PKC in shear stress-mediated release of nitric oxide (17), shear stress-mediated ERK1/2 activation (18), and cytoskeletal reorganization. With respect to the latter, external forces such as shear stress can induce rapid and global changes in the patterns of IF in various cell types (19–21).

The importance of IF in maintaining the structural integrity of cells such as AEC derives in part from the unique viscoelastic properties of IF that are not shared by microfilaments and microtubules in vitro (22). In support of this, it has been shown in situ that fluid shear stress across the apical surface of cultured cells induces a rapid deformation/displacement of vimentin and keratin IF networks in endothelial and epithelial cells, respectively (19–21). The rapid responses of IF networks to external forces is consistent with the suggestion that the IF cytoskeleton transmits mechanical signals from the cell surface to all regions of the cytoplasm.

In this report, we have examined the effects of shear stress and stretch on the assembly state of keratin IF in AEC. We demonstrated that shear stress, but not stretch, results in the disassembly of keratin IF. This disassembly is mediated by the activation of protein kinase C, specifically PKC δ, which selectively phosphorylates Ser-73 on K8. Inhibition of PKC δ prevents the disassembly of keratin IF in shear-stressed AEC. These results showed that PKC is involved in the regulation of the assembly of K8/K18 IF in AEC in response to a mechanical stimulus.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phorbol 12-myristate 13-acetate (PMA; Sigma) and GF109203x (bisindolylmaleimide; Calbiochem) were used in some experiments. γ-[32P]ATP (Amersham Biosciences) was used for kinase activity assays. PKC antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and BD Transduction Laboratories. All other reagents were commercial products of the highest grade available.

**Isolation and Culture of Alveolar Epithelial Cells**—Human and rat AEC were used. Human A549 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 50 μg/ml gentamicin, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated in a humidified atmosphere of 5% CO2, 95% air at 37 °C. Rat alveolar epithelial type II cells were isolated from pathogen-free male Sprague-Dawley rats (200–225 g), as described previously (23). Cells were cultured on 25-mm glass slides and subjected to continuous laminar flow to generate shear stress (7.5, 15, and 30 dynes/cm2) in vitro (24). In support of this, it has been shown in situ that fluid shear stress across the apical surface of cultured cells induces a rapid deformation/displacement of vimentin and keratin IF networks in endothelial and epithelial cells, respectively (19–21). The rapid responses of IF networks to external forces is consistent with the suggestion that the IF cytoskeleton transmits mechanical signals from the cell surface to all regions of the cytoplasm.

**Immunofluorescence**—AEC grown on glass slides or Silastic membranes were rinsed 3× in phosphate-buffered saline (PBS) and fixed in either methanol (−20 °C) for 4 min or 3.5% formaldehyde at room temperature for 7 min. Following formaldehyde fixation, cells were permeabilized with 0.05% Tween 20 for 5 min. Cells were then washed 3× with PBS and processed for indirect immunofluorescence as described previously (28, 29). Following staining, the glass slides or Silastic membranes were washed in PBS and mounted in gelvatol containing 100 mg/ml 1,4-diazabicyclo (2.2.2) octane (Aldrich (28)). Images of fixed, stained preparations were taken with a Zeiss LSM 510 microscope (Carl Zeiss) (28).

**Protein Isolation, Immunoblotting, and Immunoprecipitation Analysis**—Total cell lysates were obtained from AEC after solubilization in Laemmli sample buffer. Keratin-enriched cytoskeletal preparations were made according to previously published procedures (28, 29). Briefly, the resulting Triton X-100-soluble or Triton X-100-insoluble fractions were prepared by solubilizing cells for 10 min at 4 °C with buffer containing 1% Triton X-100, 5 mM EDTA, and a protease inhibitor mixture. Following formaldehyde fixation, cells were solubilized in PBS (pH 7.4) followed by centrifugation (16,000 × g, 10 min). The supernatant was collected as the soluble fraction. The pellet was homogenized in 1 ml of 10 mM Tris-HCl, pH 7.6, 140 mM NaCl, 1.5 mM KCl, 5 mM EDTA, 0.5% Triton X-100, and the protease inhibitor mix. After 30 min (4 °C), the homogenate was pelleted (16,000 × g, 10 min), and the pellet (insoluble fraction) was washed twice with 5 mM EDTA in PBS (pH 7.4), 1% Triton X-100 (insoluble fraction), and dissolved in Laemmli sample buffer containing 1% β-mercaptoethanol, sonicated, and boiled for 5 min (30). The samples were separated on 7.5 or 10% polyacrylamide gels according to the method of Ref. 30. Equal amounts of proteins were loaded on 10% SDS-PAGE, transferred to nitrocellulose membranes, and blotted with the primary antibodies as follows: 1) LJ4-anti-pK8Ser-73 epitope (1:1000 in TBS; 2) anti-K8/18 monoclonal antibody (Research Diagnostics, Inc.; 1:500 in TBS); 3) anti-PKC (1:1000 in TBS); 4) anti-phospho-K8Ser-73 (Upstate; 1:500 in TBS); and 5) 5B3-anti-pK8Ser-431 (1:500 in TBS). Membranes were washed three times with PBS containing 0.1% of Tween for 30 min and then incubated with secondary antibodies coupled to horseradish peroxidase (in dilutions recommended by the supplier) and visualized using enhanced chemiluminescence (Amer sham). For immunoprecipitation studies, cells were solubilized using modified RIPA buffer containing (16,000 × g; 15 min), keratin was immunoprecipitated from the supernatant using an anti-K8 antibody coupled to protein A/G-Sepharose. The beads were washed once with RIPA buffer and twice with PBS (3 mM EDTA). Proteins were solubilized in 3× Laemmli sample buffer and then immunoblotted as described above with the relevant antibodies.

**PKC Translocation Assay**—Following the exposure of AEC cells to shear stress, cells were solubilized into a lysis buffer containing 0.5% Triton X-100, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml trypsin inhibitor, 20 μM leupeptin, 100 mM microcystin, and homogenized for 2 min. Lysates were then centrifuged at 100,000 × g for 10 min to obtain P1: nuclear and supernatant fractions. The supernatant fraction was further centrifuged at 100,000 × g for 60 min to obtain the P2: membrane fraction and the S: cytosol fraction. The P2 fraction was suspended in lysis buffer containing 0.1% Triton X-100 for 20 min and centrifuged (16,000 × g, 20 min, 4 °C) to separate the detergent-insoluble and -soluble material. Twenty to 50 μg of cytosolic and membrane fractions were then subjected to immunoblotting using isozyme-specific anti-PKC antibodies. Specificity of membrane fractionation was determined by histone H1, a nuclear protein that mainly localizes in the P1: nuclear fraction, and MEK-1, a marker of cytosolic protein, present in the S: cytosol fraction but not in the P1 or P2 fractions (23, 31).

**Miscellaneous**—ATP levels were measured by the luciferin/luciferase method using an ATP bioluminescence assay kit HIS II (Roche Applied Science). Lactate dehydrogenase release was measured using a commercially available assay (cytotoxicity detection kit, Roche Pharmaceuticals). Protein kinase C activity was measured using commercially available assay (SigmaTect, Promega, Madison, WI). Protein content was determined according to Bradford (34) using a commercial dye reagent (Bio-Rad Laboratories) and analyzed by SDS-PAGE and Western blot using a Western Lightning chemiluminescence reagent Plus kit (PerkinElmer Life Sciences). Quantification of phosphorylation by PKC was carried out with a phosphorimaging device (Bio-Rad).

**Statistical Analysis**—Comparisons were performed using the unpaired Student’s t test. One-way analysis of variance with Tukey’s test was used to analyze the data. p < 0.05 values were considered significant.
RESULTS

Effect of Stretch and Shear Stress on the Assembly State of Keratin IF—No differences in cell viability (Fig. 1A) or ATP levels (Fig. 1B) were observed in AEC exposed to shear stress (30 dynes/cm², 24 h) or in AEC exposed to cyclic stretch at strain conditions equivalent to 20% deformation for 24 h. AEC exposed to shear stress (30 dynes/cm²) for 0, 4, and 24 h exhibited a time-dependent decrease in the amount of pelletable (i.e. filamentous) K8 and K18, which coincided with significant increases in the soluble (disassembled) keratin protein detected in the supernatant (Fig. 2A and see “Experimental Procedures”). Keratin assembly depends on the degree of shear stress because in AEC exposed to 0, 7.5, 15, and 30 dynes/cm² for 24 h, there was a shear stress-dependent decrease in insoluble K8 and K18 protein (Fig. 2B). In contrast, AEC exposed to cyclic stretch at strain conditions equivalent to 20% deformation for 24, 48, and 72 h did not have any detectable effects on the assembly state of keratin IF as monitored by changes in their overall solubility. In control and stretched cells, >95% of the keratin IF protein was found in the insoluble pellet fraction (filamentous), and only a small amount of keratin was detected in the supernatant (Fig. 2C).

Activation of Protein Kinase C by Stretch and Shear Stress—Physical forces, such as cyclic stretch and shear stress, are known to activate protein kinases, such as protein kinase C (14, 15). Therefore, we assayed PKC activity in control and mechanically stimulated AEC. As shown in Fig. 3, AEC that were exposed to shear stress, cyclic stretch, or the phorbol ester PMA (used as a positive control) showed increased PKC activity as compared with control AEC. It is known that AEC express several isozymes of PKC, including PKCα, βI, βII, δ, ε, θ, and ζ (23). PKC isoforms become activated by translocating from the cytosol to new subcellular sites, including the plasma membrane (32), nucleus (33), and cytoskeletal elements (34). To determine which PKC isoforms were activated, AEC were exposed to either shear stress or cyclic stretch and then subfractionated into cytosol, membrane, and cytoskeletal fractions. The classical PKCs, PKCα and β, were not activated in response to either shear stress or cyclic stretch at any time point (data not shown). The novel and atypical PKCs, PKCδ, PKCε, and PKCζ, were activated in shear-stressed AEC, but only PKCζ was translocated to the cytoskeletal fraction following 4, 12, and 24 h of shear stress (data not shown).

Effects of Mechanical Stimulation on the Phosphorylation of K8—The state of phosphorylation is known to regulate keratin IF assembly and their dynamic properties (10, 35, 36). Thus, the effects of mechanical stimuli on keratin phosphorylation were determined. Changes in phosphorylation were assessed by immunoprecipitating K8 from AEC exposed to shear stress or cyclic stretch followed by immunoblotting with a phosphoserine antibody. As shown in Fig. 4, AEC exposed to shear stress resulted in a time-dependent increase in phosphorylation of Ser K8, as compared with control AEC (Fig. 4A). These changes in phosphorylation corresponded to the shear stress-mediated increase in keratin IF solubility (Fig. 1). Pretreatment with bisindolylmaleimide, a PKC inhibitor, prevented the shear stress-mediated increase in K8 phosphorylation. Additionally, treatment with a PKC activator, PMA (1 μM, 15 min), increased K8 phosphorylation (Fig. 4A). In contrast, AEC exposed to cyclic stretch for 24 h exhibited increased levels of keratin phosphorylation, which was not affected by the pretreatment with bisindolylmaleimide (Fig. 4A). To determine whether PKC mediated the shear stress-dependent increase in keratin IF solubility, AEC were treated with bisindolylmaleimide, a general PKC inhibitor. As shown in Fig. 4B, bisindolylmaleimide prevented the shear stress-induced increase in keratin IF solubility (i.e. disassembly).

Protein Kinase C-mediated Phosphorylation of K8 on Ser-73 in Shear-stressed AEC—To determine which PKC isoforms mediate keratin IF disassembly, AEC were pretreated with the PKC peptide inhibitors (PKCδ V1-1 and PKCε V1-2 peptide; gifts from Daria Mochly-Rosen) and then exposed to shear stress for 24 h. The PKCδ peptide antagonist attenuated the shear stress-mediated increase in keratin IF solubility as shown by Western blot analysis (Fig. 5A). There was no effect on the basal expression of keratin protein in cells treated with...
the PKC peptide inhibitors alone (data not shown). Pretreatment with the PKC ε peptide antagonist or scrambled peptide (used as a negative control) did not prevent the shear stress-mediated increase in keratin IF solubility (data not shown).

Three major phosphorylation sites were identified in K8: Ser-23, Ser-73, and Ser-431. To determine whether these sites were phosphorylated in response to shear stress and activation of PKC δ, AEC were exposed to shear stress for 4 and 24 h. K8 immunoprecipitates were immunoblotted with phospho-specific monoclonal antibodies against K8 pSer-431 and K8 pSer-73. As shown in Fig. 5B, shear stress induced the phosphorylation of K8 pSer-73 at 4 and 24 h but not of K8 pSer-431. Finally, pretreatment with PKC δ peptide inhibitor (PKC δ V1-1) prevented the shear stress-mediated phosphorylation of K8 pSer-73 (Fig. 5C).

Effect of Mechanical Stimuli on the Keratin IF Network in AEC—Since shear stress induces changes in phosphorylation/solubility in the keratin IF, we assessed whether the changes in phosphorylation were associated with keratin IF reorganization as studied by indirect immunofluorescence. The keratin IF network in control, unstressed cells was organized into arrays of relatively straight, thin keratin fibrils (Fig. 6A). In AEC exposed to shear stress (30 dynes/cm²) for 24 h, the keratin IF network was disorganized as shown by the formation of large particles and squiggles (Fig. 6B). These latter forms of IF are known to be alternate forms of IF which are typical of disassembled IF networks or involved in assembly of IF (28, 29). In contrast to shear-stressed cells, stretched cells showed no obvious signs of disorganization or disassembly of keratin IF network.

DISCUSSION

Mechanical ventilation is often required to manage patients with acute respiratory failure of different origins. However, there is considerable experimental evidence that mechanical ventilation itself can also cause or exacerbate lung injury, resulting in ventilator-induced lung injury (37, 38). The mechanisms associated with ventilator-induced lung injury have not been fully elucidated yet. Two mechanisms have received the most attention. One is related to the excessive overdistension or stretching of alveolar structures, termed volutrauma (39). Another potential mechanism is the repeated recruitment and derecruitment of atelectatic lung units (38). In principle, opening a fluid-filled, collapsed airway requires very high pressure (40). During the process of reopening the airway, high shear stresses and pressure gradients are generated across the epithelium (41). The shear stress gradient generated under these conditions has been estimated to be between 25 and 325 dynes/cm² (41). In this report, we examined which mechanical stimuli, shear stress or stretch, effects the keratin IF network in AEC.

Several physiological roles for IF have been proposed (42,
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43), including the determination and maintenance of cell shape, transmission of mechanical stress, and targeting of molecules between the nucleus and cytoplasm. Traditionally, the IF network has been viewed as a static architecture, primarily due to the low solubility and detergent extractability of IF proteins. Several reports have now demonstrated that IF are highly dynamic cellular structures as reflected by their assembly/disassembly states throughout the cell cycle (13, 21, 36). Although details regarding the mechanisms regulating the assembly dynamics of keratin IF are not known, there are a number of reports that demonstrate the significance of phosphorylation in regulating the assembly dynamics of keratin IF (6, 13, 21, 36, 45). Unlike previous studies that utilized pharmacologic approaches to effect changes in phosphorylation, we have established in vitro models of mechanical stimulation to assess the effects of both stretch and shear stress on the activation of protein kinases and the assembly dynamics of keratin IF.

Our studies demonstrated that shear stress, but not stretch, affects the assembly state of keratin IF. As shown in Fig. 2, when AEC are stretched for up to 72 h at 20% deformation (the maximum level of biaxial deformation), there is no change in the assembly state of keratin IF, suggesting that stretch does not activate signal transduction pathways involved in their disassembly. In contrast, when AEC are exposed to shear stress for as little as 4 h, there is an increase in the soluble fraction keratin, indicating that shear stress promotes the disassembly of IF. The rate of keratin IF disassembly is dependent upon the degree of shear stress; increased levels of shear stress accelerate the rate of disassembly of keratin (Fig. 2B).

It has been shown that both the assembly and organization of IF are regulated by post-translational modifications, especially phosphorylation. Phosphorylation is known to occur within the head and tail domains, which are responsible for most of the structural heterogeneity and presumed tissue-specific functions of IF (45). In the case of K8, a number of in vivo phosphorylation sites have been mapped, and these play essential roles in regulating filament assembly and disassembly in vivo (45). IF can be phosphorylated by a variety of kinases, including cell cycle-dependent kinases, mitogen-activated kinase (MAPK), and protein kinase C. Previous studies have also demonstrated that mechanical forces activate PKC (15, 46).

We have previously demonstrated that AEC express PKC α, β1, β2, δ, ε, θ, but not PKC γ of the classical and novel PKC isozymes (23, 27). The present study showed that shear stress, but not stretch, activates PKC δ and phosphorolysis K8 Ser-73, which then mediates the disassembly/reorganization of keratin IF in AEC. Several lines of evidence supported this notion. First, shear stress increases PKC activity in AEC (Fig. 3), and there is a transmigration of PKC δ from the cytosol to the cytoskeleton fraction (data not shown). Second, bisindolylmaleimide, a PKC inhibitor, prevents the shear stress-mediated increase in solubility (i.e. disassembly) of keratin IF (Fig. 4). Third, AEC pretreated with the PKC δ peptide antagonist attenuates the shear stress-mediated increase in keratin IF solubility (Fig. 5). These data suggested that PKC δ may be involved in the phosphorylation and assembly dynamics of keratin IF. AEC treated with the PKC δ peptide antagonist (V1-1) and exposed to shear stress (24 h) showed no indication of disassembly or cellular injury (i.e. Mallory bodies/aggresomes) but appeared to have increased tonofibril formation as compared with non-treated, shear-stressed AEC (Fig. 6D). This suggested that another kinase (and perhaps different pSer residue) may regulate tonofibril formation in AEC. Fourth, K8 Ser-73, but not Ser-431, is phosphorylated in response to mechanical stimuli (Fig. 5B), and this is mediated by PKC δ (Fig. 5C).

One of the most commonly described functions of IF is to provide mechanical integrity to cells. Disease-causing mutations have been associated with hyperphosphorylation of keratin with subsequent abnormal keratin filament reorganization (44). As shown in Fig. 6, AEC exposed to shear stress, which was associated with increased phosphorylation of K8 Ser-73, resulted in abnormal keratin filament reorganization (Fig. 6B). In contrast, AEC exposed to stretch, which was not associated with extensive phosphorylation, had thickened tono-
fibril formation (Fig. 6D), consistent with function of providing mechanical integrity to the cell. We postulated that the degree and specific site of phosphorylation of keratin may be critical in defining keratin IF reorganization.

In summary, we showed that PKC is a key kinase for the regulation of IF assembly and organization in response to mechanical stimuli, particularly shear stress, in AEC. Although the mechanisms underlying the activation of PKC isozymes during the cellular responses to mechanical forces remains unclear, it is evident that shear stress, and not stretch, results in the reorganization and changes in the assembly state of keratin IF in AEC via PKC. These changes in the assembly states of the IF networks in AEC could have a significant impact on signaling networks, and in this fashion, alter the cellular response to changes in its environment.

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