Mechanical Strain Induces pp60-src Activation and Translocation to Cytoskeleton in Fetal Rat Lung Cells*

(Received for publication, October 16, 1995, and in revised form, December 15, 1995)

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We have previously shown that mechanical strain-induced fetal rat lung cell proliferation is transduced via the phospholipase C-γ-protein kinase C pathway. In the present study, we found that protein-tyrosine kinase activity of fetal lung cells increased after a short period of strain, which was accompanied by tyrosine phosphorylation of proteins of ~110–130 kDa. Several components of this complex were identified as pp60-src substrates. Strain increased pp60-src activity in the cytoskeletal fraction, which coincided with a shift in subcellular distribution of pp60-src from the Triton-soluble to the cytoskeletal fraction. Strain-induced pp60-src translocation did not appear to be mediated via the focal adhesion kinase-paxillin pathway. In contrast, strain increased the association between pp60-src and the actin filament-associated protein of 110 kDa. Preincubation of cells with herbimycin A, a tyrosine kinase inhibitor, abolished strain-induced phospholipase C-γ pathway. In the present study, we found that protein-tyrosine kinase activation of strains was accompanied by tyrosine phosphorylation of phospholipase C-γ1 and protein kinase C (PKC) activity. Strain-induced PKC activation and DNA synthesis were blocked by PLC and PKC inhibitors (9). These results suggest that mechanical strain-induced fetal lung cell proliferation is mediated through the PLC-γ1-PKC pathway. Herein, we report that mechanical strain of fetal rat lung cells induced a rapid activation and translocation of pp60-src from the Triton-soluble to the cytoskeletal fraction. Strain-induced pp60-src translocation appears to be mediated via the actin filament-associated protein of 110 kDa (AFAP-110). Preincubation of cells with a protein-tyrosine kinase (PTK) inhibitor, herbimycin A, blocked strain-induced PLC-γ1 tyrosine phosphorylation and its association with pp60-src as well as strain-induced DNA synthesis. These results suggest that strain-induced PTK activation is an upstream event of the PLC-γ1-PKC pathway.

EXPERIMENTAL PROCEDURES

Materials—Pregnant Wistar rats (200–250 g) were obtained from Charles River (St. Constant, Quebec, Canada). Eagle’s minimal essential medium and antibiotics were purchased from Life Technologies, Inc. Fetal bovine serum, PTK (Src family) assay kit, and monoclonal anti-pp60-csrc antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal anti-AFAP-110 and monoclonal anti-pp130 antibodies were generous gifts of Dr. J. T. Parsons (University of Virginia). Monoclonal anti-paxillin antibody was from Transduction Laboratories (Lexington, KY). PTK substrate peptide (RR-SRC) was from Life Technologies, Inc. (Burlington, Ontario, Canada). Gelfoam® sponges were from Upjohn (Toronto, Ontario).

†This work was supported by a grant group (to M. P. and A. K. T.) and Operating Grant MT-13270 (to M. L.) from the Medical Research Council of Canada, by Grant R01 HL 43416 from the National Institutes of Health (to M. P.), by an operating grant (to M. L.) and equipment grants (to M. P., A. K. T., and M. L.) from the Ontario Thoracic Society, and by the Dean’s Fund from the Faculty of Medicine, University of Toronto (to M. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PLC-γ1, phospholipase C-γ1; PKC, protein kinase C; AFAP-110, actin filament-associated protein of 110 kDa; PTK, protein-tyrosine kinase; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; PDGF, platelet-derived growth factor.
Protein assays were performed using a kit from Bio-Rad. All other chemicals were from Sigma.

Strain of Fetal Lung Cell Organotypic Cultures—Pregnant rats were killed by an excess of diethyl ether on day 19 of gestation (term = 22 days). Organotypic cultures of fetal lung cells were established as described previously (7, 10). The mechanical strain apparatus used in these experiments has been described in detail elsewhere (7, 11). After inoculation, fetal rat lung cells were cultured on Gelfoam sponges in Eagle's minimal essential medium + 10% (v/v) fetal bovine serum. After 24 h, cells were rinsed three times with Eagle's minimal essential medium and cultured in serum-free Eagle's minimal essential medium for another 24 h. Sponges were then subjected to a 5% elongation from the cell length, at 60 cycles/min for 15 min, which optimally enhanced DNA synthesis and cell division without cell injury (7). In some experiments, cells were incubated for 3 h with cytochalasin B (1 μM) or herbimycin A (1 μg/ml) prior to the onset of strain.

Cell Lysate Preparation—For most experiments, cells were lysed by placing sponges in radioimmune precipitation assay buffer (1% (v/v) Triton X-100, 1% (v/v) sodium deoxycholate, 0.1% (v/v) SDS, 150 mM NaCl, 10 mM Tris, 1 mM EDTA, 250 μg/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, 1.0 mM benzamidine, 10 mM Na3VO4, and 100 mM kallikrein inactivator units/ml aprotinin, pH 7.2) for 1 h. Sponges and cells were homogenized using a tissue homogenizer and centrifuged at 12,000 × g for 10 min at 4°C to remove insoluble debris. Supernatants were stored as aliquots at −70°C until analyzed.

The Triton-soluble and cytoskeletal fractions were prepared according to a modified method of Clark and Brugge (12). Briefly, sponges were immersed into Triton buffer (2% (v/v) Triton X-100, 2% EDTA, 100 mM Tris, 500 μg/ml leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, 2.0 mM benzamidine, 2.0 mM Na3VO4, and 200 kallikrein inactivator units/ml aprotinin, pH 7.2) and incubated overnight at 4°C. The Triton-soluble fraction was separated by centrifugation of sponges over glass-wool filters. The supernatant was then washed several times with Triton buffer and resuspended in dilution buffer containing 10% (v/v) fetal bovine serum. After washing, sponges were lysed in radioimmune precipitation buffer for 5 min. The supernatant (25 μl) was spotted on phosphocellulose filters. The filters were washed four times with 0.75% (v/v) phosphoric acid and resuspended in dilution buffer containing 10% (v/v) fetal bovine serum. After washing, sponges were lysed in radioimmune precipitation buffer for 5 min. The supernatant (25 μl) was spotted on phosphocellulose filters. The filters were washed four times with 0.75% (v/v) phosphoric acid and resuspended in dilution buffer containing 10% (v/v) fetal bovine serum.

Immunoprecipitation and Western Blotting—For immunoprecipitation, cell lysates were adjusted to an equal amount of protein (400–1000 μg) and equal volume, designated antibody (IgG) was added, and samples were incubated at 4°C overnight. Immune complexes were recovered by the addition of 50 μl of Zysorbin (10% (v/v) Formalin-fixed Staphylococcus aureus Cowan stain A in phosphate-buffered saline) for polyclonal antibodies or 100 μl of protein G-Sepharose (10% (v/v) for monoclonal antibodies. Samples were incubated for 1 h with agitation at 4°C. The immunoprecipitates were washed twice with TBS buffer. The immunoprecipitated proteins were eluted by boiling in sample buffer (60 mM Tris-HCl, 20% (v/v) glycerol, and 0.1% (v/v) Nonidet P-40, pH 7.0) and resuspended in dilution buffer, and an aliquot (5 μl) of 5% (v/v) sodium dodecyl sulfate-PAGE sample buffer (60 mM Tris-HCl, 20% (v/v) glycerol, and 0.1% (v/v) Nonidet P-40, pH 7.0) was then used for the activity assay.

RESULTS

Strain-induced pp60src Activation and Protein Tyrosine Phosphorylation—We have previously shown that mechanical strain-induced fetal lung cell proliferation is primarily mediated through the PLC-γ/PKC pathway (9). The observed strain-induced PLC-γ1 tyrosine phosphorylation implies activation of PTKs by mechanical strain. To confirm strain-induced PTK activity, we first measured PTK activity in cell lysates after a short period of strain, using the RR-SRC peptide as a substrate (14). Total PTK activity increased 10-fold within 15 min of strain, declined slightly during a 45-min resting period, and then increased again during the second period of strain (Fig. 1). We then examined protein tyrosine phosphorylation. Cell ly-
Strain-induced pp60src Activation

Mechanical strain induces protein tyrosine phosphorylation. Fetal rat lung cells were subjected to strain for 5–15 min and then lysed, subjected to SDS-PAGE, and analyzed with antibodies to phosphotyrosine. An illustrative blot is shown in A, and results (mean \( \pm S.E. \)) of densitometric analyses of four separate experiments are shown in B. Statistical analysis was by one-way analysis of variance followed by Duncan’s multiple range test. *, \( p < 0.05 \) compared with static control.

Mechanical Strain Selectively Increases Tyrosine Phosphorylation of pp60src Substrates—The apparent molecular masses of several characterized pp60src substrates are in the range of 110–130 kDa, e.g. AFAP-110, pp120, pp125FAK, and pp130. To identify the strain-induced tyrosine-phosphorylated proteins, fetal lung cells were subjected to 5 min of strain and lysed, and lysates were immunoprecipitated with polyclonal anti-phosphotyrosine antibody. The immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting with specific antibodies against individual proteins. Alternatively, cell lysates were immunoprecipitated with monoclonal anti-pp125FAK antibody, which has been shown to immunoprecipitate pp125FAK from rat embryo fibroblasts (18), and immunoblotted with anti-phosphotyrosine antibody. The tyrosine phosphorylation of these proteins was compared with that of static controls. Again, 5 min after the onset of strain, tyrosine phosphorylation of proteins corresponding to 110–130 kDa was increased (Fig. 3). The tyrosine phosphorylation of pp120, which was almost undetectable in static culture, was markedly increased by mechanical strain (Fig. 3). The tyrosine phosphorylation of AFAP-110 (2.5-fold increase; \( n = 3 \), \( p < 0.05 \)) and cortactin (pp80/85) was also increased by mechanical strain (Fig. 3). The latter has been identified together with AFAP-110, pp120, pp130, and pp125FAK to be a substrate for pp60src (19). In contrast, the tyrosine phosphorylation of pp130 and pp125FAK was not influenced by mechanical strain (Fig. 3). No tyrosine phosphorylation of RasGAP, a protein of 120 kDa implicated in growth factor receptor signaling, was observed in either strained or static cell cultures (data not shown).

Strain-induced pp60src Activation and Translocation—pp60src can bind to membrane by lipid anchors and is then well positioned to interact with the membrane-cytoskeletal complex (20). These features and the observed selective increase in tyrosine phosphorylation of pp60src substrates suggested that pp60src may be activated by mechanical strain. The activities of Src family PTKs in the Triton-soluble and cytoskeletal fractions were measured with a synthetic peptide, [Lys\(^{19}\)]Cdc2-(6–20)-NH\(_2\). Its relative phosphorylation rates with pp60src-related PTKs are up to 180-fold greater than those with non-Src-related PTKs (13). Total Src PTK activity increased from 8.9 \( \pm \) 3.2 to 70.4 \( \pm \) 17.2 pmol/min/mg of protein in the Triton-soluble fraction and from 58.9 \( \pm \) 17.7 to 60.4 pmol/min/mg of protein in the cytoskeletal fraction. No phosphorylation of two pseudosubstrates (see “Experimental Procedures”) was observed (data not shown). To test the specific activity of pp60src in the Triton-soluble and cytoskeletal fractions was immunoprecipitated with polyclonal anti-pp60src antibody. The PTK activities in the immunoprecipitates were then determined using the [Lys\(^{19}\)]Cdc2-(6–20)-NH\(_2\) peptide as substrate. The specific activity of pp60src in the Triton-soluble fraction did not change, while that in the cytoskeletal fraction increased 3.77 \( \pm \) 0.73-fold (\( p < 0.05 \)) after 5 min of mechanical strain (Fig. 4). The percentage of measurable pp60src activity in the Triton-soluble fraction decreased, whereas a corresponding increase of pp60src activity in the cytoskeletal fraction (from 11 to 35%) was noted. The subcellular distribution of pp60src was also determined by Western blotting using anti-pp60src mAb. In static culture, pp60src was mainly present in the Triton-soluble fraction (Fig. 5). The amount of pp60src in the cytoskeletal fraction increased after 5 min of strain (Fig. 5). When cells were preincubated with cytochalasin B (1 \( \mu \)M) to interrupt the dynamic polymerization of actin filaments, mechanical strain did not induce pp60src activation and translocation to the cytoskeletal fraction (Figs. 4 and 5). Preincubation of cells with herbimycin A (1 \( \mu \)g/ml) inhibited both basal and strain-induced pp60src activities (Fig. 4) and blocked strain-induced pp60src translocation to the cytoskeletal fraction (Fig. 5). These results suggest that pp60src activity in fetal lung cells depends on its association with the cytoskeletal matrix and that mechanical strain-induced translocation of pp60src requires the integrity of actin filaments as well as the existence of tyrosine-phosphorylated proteins prior to the onset of strain. The tyrosine phosphorylation of pp60src was determined by immunoprecipitation of Triton-soluble and cytoskeletal fractions with polyclonal anti-phosphotyrosine antibody followed by immunoblotting with anti-pp60src mAb. No significant difference between control and strained cells was observed (Fig. 6), which may be due to the simultaneous dephosphorylation of tyrosine-phosphorylated Tyr(5)-P-527 and phosphorylation of Tyr-416 (21).

Mechanical Strain-induced pp60src Translocation to Cytoskeleton Is Mediated through AFAP-110—We further examined the mechanism(s) by which pp60src is translocated to the cytoskeleton. Several pp60src substrates are either cytoskeletal...
pp60<sup>src</sup> proteins or associated with the cytoskeleton. These include pp125<sup>Fak</sup> (22); pp120, a tyrosine-phosphorylated protein that shares sequence similarity with cadherin-binding factors (23); AFAP-110 (24, 25); cortactin, an 80/85-kDa filamentous actin-binding protein that is enriched in the cell cortex (19); and paxillin, a focal adhesion protein associated with vinculin (26).

Both pp120 and cortactin were coimmunoprecipitable with pp60<sup>src</sup> and cytoskeletal (cyk) fractions were prepared, and aliquots, equalized for protein content, were immunoblotted with antibodies to pp60<sup>src</sup> (mAb 2G11). The position of pp60<sup>src</sup> is indicated. Similar results were obtained in two separate experiments. C, control static cultures; S, strained cultures.

PPK Activation Mediates PLC-γ1 Activation—To determine whether PTK activation is related to the strain-induced PLC-γ1-PKC activation, cells were preincubated with or without herbimycin A (1 μg/ml), a PTK inhibitor, and then subjected to strain or static culture. Consistent with our previous observation (9), tyrosine phosphorylation of PLC-γ1 was increased after 5 min of strain (Fig. 9, left panel). Both basal and strain-induced PLC-γ1 tyrosine phosphorylations were blocked by herbimycin A. When cell lysates were immunoprecipitated with anti-PPK mAb, electrophoresed, and immunoblotted with anti-PLC-γ1 mAb, an association between pp60<sup>src</sup> and PLC-γ1 was observed in fetal rat lung cells. The association was significantly increased by mechanical strain (p < 0.05) and attenuated by pretreatment with herbimycin A (Fig. 9, right panel). Herbimycin A also completely blocked the en-
Mechanical strain does not activate the pp125\textsuperscript{FAK}-paxillin pathway. Fetal rat lung cells were subjected to strain for 5 min and then lysed. Cell lysates, equalized for protein content, were immunoprecipitated (ptp.) with polyclonal anti-pp60\textsuperscript{src} or monoclonal anti-pp125\textsuperscript{FAK} antibody. Lysates (−) and immunoprecipitates (Src or FAK) were analyzed by SDS-PAGE. The blots were immunoblotted (blot) as indicated with antibodies to pp130, paxillin, and Src. Positions are indicated by arrowheads. Similar results were obtained in two separate experiments. C, control static cultures; S, strained cultures.

FIG. 8. Strain-induced PLC-1 tyrosine phosphorylation and PLC-1-pp60\textsuperscript{src} association are blocked by the PTK inhibitor herbinycin A. Fetal rat lung cells were preincubated with or without 1 \(\mu g/ml\) herbinycin A and then subjected to strain or static culture. DNA synthesis was measured. Data are mean ± S.E. from three sponges. Statistical analysis was by one-way analysis of variance followed by Duncan’s multiple range test. *, \(p < 0.05\) compared with the static control group.

PTK inhibitor herbinycin A blocked strain-induced PLC-1 tyrosine phosphorylation, PKC activation, and DNA synthesis, suggesting that the rapid activation of cytoplasmic PTKs is an upstream event of the PLC-1-PKC pathway. In vitro studies have shown that PLC-1 can be tyrosine-phosphorylated by pp60\textsuperscript{src} and other Src family PTKs (32). In platelets, electrotransfection of monoclonal pp60\textsuperscript{src} antibody inhibited activation of PLC-1 (33). Moreover, pp60\textsuperscript{src} has been coimmunoprecipitated with PLC-1 in platelets (33), and this association was increased after treatment of platelets with platelet-activating factor (33). Similarly, we observed coimmunoprecipitation of pp60\textsuperscript{src} and PLC-1 in fetal lung cells, and this association was increased by mechanical strain and inhibited by herbinycin A treatment. Thus, our present data are compatible with strain-induced PLC-1 tyrosine phosphorylation in fetal lung cells being, at least in part, mediated via pp60\textsuperscript{src}. Phosphorylated PLC-1 then activates the PKC pathway and downstream signal cascades.

Increased tyrosine phosphorylation of proteins ranging from 110 to 130 kDa is a common phenomenon in extracellular matrix-integrin-cytoskeleton-mediated signal transduction initiated by integrin clustering (15) or cell attachment (16). An integrin-related increase of tyrosine phosphorylation has been found for proteins such as pp130 (15) and pp125\textsuperscript{FAK} (16, 34). Although tyrosine phosphorylation of pp130 and pp125\textsuperscript{FAK} was observed in fetal lung cells, it was not affected by short periods of mechanical strain, and the association between these two proteins and pp60\textsuperscript{src} in fetal rat lung cells was not detected by coimmunoprecipitation. Paxillin has been suggested to be a direct substrate for pp125\textsuperscript{FAK} (35) and has been found to bind in vitro to the SH3 domain of c-Src (36). In this study, coimmunoprecipitation of pp60\textsuperscript{src} and paxillin was demonstrated in fetal rat lung cells. However, the tyrosine phosphorylation and association of paxillin with pp60\textsuperscript{src} were not altered by mechanical strain. The strain-induced pp60\textsuperscript{src} translocation therefore appears not to be mediated through the pp125\textsuperscript{FAK}-paxillin pathway. In addition, mechanical strain-induced cell proliferation was not affected when fetal lung cells were preincubated with an RGD peptide to block the extracellular matrix-integrin interaction.2 Taken together, these results suggest that the mechanical strain-induced signals are different from those initiated by integrins, although the cytoskeleton system appears to be involved in the transmission of both signals.

Tyrosine phosphorylation of pp130, pp125\textsuperscript{FAK}, pp120, and

2 M. Liu and M. Post, unpublished observation.
contactin has been observed upon epidermal growth factor, PDGF, and colony-stimulating factor-1 stimulation of several cell types (35, 37, 38). The effect of these growth factors is likely mediated through intermediate PTKs such as Src family members. PDGF stimulation of quiescent NIH 3T3 cells and human fibroblasts activates Src family tyrosine kinases (39). Treatment of A172 glioblastoma cells with PDGF or epidermal growth factor induces activation and translocation of c-Src to the cytoskeleton (40). We have recently demonstrated that strain-induced pp60$^{src}$ activation is a result of an exocytosis of PDGF. We found no measurable changes of PDGF in the culture medium within 4 h of mechanical strain (8). Tyrosine phosphorylation of RasGAP has been observed for various cell types upon activation of epidermal growth factor or PDGF receptors (35, 41, 42). We also observed tyrosine phosphorylation of RasGAP and PDGF $\beta$-receptors after PDGF-BB treatment of fetal rat lung cells. In contrast, we did not observe tyrosine phosphorylation of RasGAP or PDGF $\beta$-receptors after 5 min of mechanical strain (data not shown).

One of the regulatory mechanisms for pp60$^{src}$ activation is through its association with the cytoskeletal matrix (43). All oncogenic variants of pp60$^{src}$ have been shown to be tightly associated with the Triton-insoluble cytoskeletal matrix, and this association correlates with the elevated tyrosine kinase activity of pp60$^{src}$ (44-46). Normally, pp60$^{src}$ does not associate with the detergent-insoluble cellular matrix (44). However, redistribution of activated pp60$^{src}$ to the cytoskeletal matrix has been reported for thrombin-stimulated platelets (12) and growth-factor-stimulated A172 glioblastoma cells (40). The reason for Src activation following its translocation to the cytoskeleton is unknown. In the current model for Src regulation, the tyrosine-phosphorylated C-terminal Tyr(P)-S27 sequence binds by an intramolecular interaction to Src's own SH2 domain to maintain Src in an inactive state (47, 48). The tyrosine-phosphorylated Src C-terminal Tyr(P)-S27 sequence does, however, not resemble the consensus high affinity SH2-binding site and therefore binds poorly to the SH2 domain of Src. In contrast, AFAP-110, a distinctive cytoskeleton-associated protein (24, 25), contains four putative SH2-binding sites including a consensus high affinity tyrosine-phosphorylated SH2-binding site (25). Mechanical strain-induced cytoskeleton deformation may physically facilitate the approximation of these binding sites to pp60$^{src}$ and activate pp60$^{src}$ by competing with Src Tyr(P)-S27 for Src SH2 binding. In this study, we indeed observed an increased association between pp60$^{src}$ and AFAP-110. The increased association between AFAP-110 and pp60$^{src}$ may activate pp60$^{src}$, which results in an increased tyrosine phosphorylation of AFAP-110 and other proteins, such as PLC- $\gamma$1.

Acknowledgment—We thank Dr. J. T. Parsons for the gifts of anti-AFAP-110 and anti-pp130 antibodies.

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J. Biol. Chem. 1996, 271:7066-7071.
doi: 10.1074/jbc.271.12.7066

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