Regulation of serum-induced fibronectin expression by protein kinases, cytoskeletal integrity, and CREB

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Received 6 December 2000; accepted in final form 17 October 2001

Michaelson, Jeffrey E., Jeffrey D. Ritzenthaler, and Jesse Roman. Regulation of serum-induced fibronectin expression by protein kinases, cytoskeletal integrity, and CREB. Am J Physiol Lung Cell Mol Physiol 282: L291–L301, 2002; 10.1152/ajplung.00445.2000.—Lung injury, characterized by the flooding of interstitial and alveolar spaces with serum proteins, induces the expression of fibronectin (FN). This cell-adhesive extracellular matrix (ECM) glycoprotein is believed to modulate inflammation and wound repair. Murine NIH/3T3 fibroblasts transfected with a 1.2-kb human FN promoter-reporter gene were studied to gain insight into the mechanisms involved in the induction of FN by serum. Transcription of the FN gene, followed by FN protein production, was enhanced by 10% fetal bovine serum. This effect was blocked by inhibitors of protein kinase C and mitogen-activated protein kinases. ECMs typically found in injured tissues (i.e., type I collagen, fibrin, and FN) had no effect. Conversely, disruption of actin microfilaments inhibited, whereas disruption of microtubular assembly enhanced, the serum-induced FN response. The stimulatory effects of serum and microtubular disruption on FN gene transcription were related to increased DNA binding of the transcription factor cAMP response element binding protein. The data suggest that regulation of serum-induced FN expression in fibroblasts is dependent on protein kinases and on cytoskeletal integrity.

The biological response to lung injury is characterized by a complex set of events that has not yet been entirely elucidated. In many instances of injury, it is unpredictable if the response will be adaptive, perfectly restoring function and form, vs. maladaptive, leading to the replacement of injured tissue with mesenchymal profibrotic components, a process known as the fibroproliferative response (7, 8). Numerous cellular signals are involved in promoting the fibroproliferative response, of which only a fraction has been determined. They include soluble signals found in serum, lung alveolar lining fluid, and tissue such as growth factors and cytokines (13, 26, 35, 67), and solid phase factors such as the extracellular matrix (ECM) components collagens and proteoglycans (reviewed in Refs. 54, 55, and 58).

One ECM component implicated in the pathogenesis of fibroproliferative disorders is fibronectin (FN) (10, 37, 55, 58). This multifunctional cell adhesive glycoprotein can be found in soluble form in plasma and in insoluble form as an integral part of tissues (42, 58). After injury, fibroblasts (among other cell types) residing in or recruited to a wound site synthesize and secrete FN in a soluble monomeric form. The secreted FN is then assembled into an insoluble matrix by poorly understood mechanisms that require the activation of integrins and possibly other cell surface receptors (45, 71, 72). This newly deposited matrix is further supplied with serum FN that, after extravasation from injured vascular structures, gets incorporated into the growing matrix.

In addition to contributing to coagulation (44) and participating in the architectural changes that result from fibroproliferation after injury, FN might play other roles in injured tissues (54). Accurately defining the roles of FN under these circumstances, however, has proven to be a daunting task, mainly because there exist no in vivo models by which to study it. The generation of knockout mice deficient in either FN or one of its cellular receptors (i.e., the integrin α5β1) results in death during early embryogenesis (27, 74). Consequently, what is known about FN function comes from in vitro models with cultured cells and organs. These studies suggest that during the fibroproliferative response to tissue injury, FN might exert its influence by providing a provisional substrate on which cells can organize and become activated (16, 28). Once in contact with cells, FN binds integrin surface receptors capable of signal transduction (15, 28). The binding of these receptors results in the activation of intracellular secondary messengers capable of inducing potent transcription factors which can, in turn, affect the expression of many genes (15, 28). In this fashion, FN can modulate many cellular processes ranging from adhesion and migration to differentiation and proliferation, all considered necessary for tissue repair (2, 3, 7, 9, 24, 30, 47, 51). More recently, it has been demon-
strated that FN can stimulate the production of proinflammatory cytokines such as interleukin (IL)-1β (29, 46, 52). In view of this, it is speculated that increased FN expression and deposition not only may alter the structure of the lung, with obvious consequences to lung function, but might modulate (and even amplify) inflammatory and repair responses elicited in injured tissues.

Because of the many postulated effects on organ structure and function during fibroproliferation, our work is directed to exploring the intracellular signaling and transcriptional mechanisms involved in the control of FN expression in fibroblasts. One factor considered key for induction of FN expression in injured tissues is the extravasation of serum proteins with FN-inducing activity (e.g., transforming growth factor-β, platelet-derived growth factor, interleukins) (5, 6, 18, 19, 53). Accordingly, this report explores the intracellular mechanisms responsible for regulation of FN gene transcription in fibroblasts exposed to serum and presents data indicating a role for protein kinases and the state of cytoskeletal organization in modulation of FN expression.

MATERIALS AND METHODS

Experimental reagents. Colchicine, cytochalasin B, taxol, and calphostin C were purchased from Sigma Chemical (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). The MEK1 inhibitor PD-98059 was purchased from New England BioLabs (Beverly, MA). Rat tail type I collagen was purchased from Boehringer Mannheim (Indianapolis, IN) or Upstate Biotechnology (Lake Placid, NY). Fibrin was purchased from American Diagnostica (Greenwich, CT). FN was isolated from bovine plasma by affinity chromatography as previously described (46, 52). In view of this, it is speculated that increased FN expression and deposition not only may alter the structure of the lung, with obvious consequences to lung function, but might modulate (and even amplify) inflammatory and repair responses elicited in injured tissues.

The pFN (1.2 kb) LUC promoter construct was donated to Dr. Thomas Birkenmeier (Dept. of Medicine, Washington Univ. School of Medicine, St. Louis, MO). The FN promoter construct contains ∼1,200 base pairs (bp) of the 5′-flanking region of the human FN gene isolated from the human fibrosarcoma cell line HT-1080 (20). This construct includes 69 bp of exon 1, a CAAT site located at −150 bp, and the sequence ATATAA at −25 bp from the transcription start site. The FN promoter also contains several previously identified regulatory elements, such as three cAMP response elements located at −415 bp, −260 bp, and −170 bp, and an SP-1 site at −102 bp from the transcription start site. The FN promoter was subcloned into the SmaI site of the pGL3 basic luciferase reporter vector (Promega, Madison, WI).

To prepare deletion mutants of the promoter, pFN (1.2 kb) LUC was digested with the restriction endonucleases PvuII and HindIII, and the fragments were separated on a 1% agarose gel. The resulting FN promoter fragment extended to −510 bp and was religated into the pGL3 basic luciferase reporter vector to produce the construct pFN (0.5 kb) LUC. This construct lacks much of the 5′ sequences present in the FN promoter without affecting the cAMP response elements (CREs). To create the pFN (0.2 kb) LUC construct, pFN (0.5 kb) LUC was digested with SacI and AatII restriction endonucleases, blunt ends were produced using T4 DNA polymerase, and the linear DNA was religated using T4 DNA ligase. All deletion construct sequences were verified using an Applied Biosystems 373 automated DNA sequencer. The constructs pFN (1.2 kb) LUC and pFN (0.5 kb) LUC contain all three CREs, whereas pFN (0.2 kb) LUC contains no CREs.

Electrophoretic mobility shift assay. NIH/3T3 cells (3 × 10⁵) were seeded onto 150-mm² tissue culture flasks and incubated in either 0.4% or 10% FBS for 24 h with and without concurrent treatment with either colchicine or cytochalasin B at the doses described in Cell culture and treatment. Cells were washed with ice-cold PBS, and nuclear binding proteins were extracted by a published method (22). Protein concentration was determined by the Bradford method (12) using Bio-Rad protein assay reagent. Double-stranded cAMP response element binding (CREB) protein consensus oligonucleotide (5′-AGAGATTGCTGACGTCA-32P]ATP using T4 polynucleotide kinase enzyme. Nuclear protein (50 μM), taxol (20 μM), cytochalasin B (10 μM), calphostin C (1 × 10⁻⁷ M), or mitogen-activated protein kinase (MAPK) inhibitor (50 μM). The doses of experimental agents were chosen based on optimal doses reported in the literature. Cells were harvested at 24 h.

Electrophoretic mobility shift assay. NIH/3T3 cells (3 × 10⁵) were seeded onto 150-mm² tissue culture flasks and incubated in either 0.4% or 10% FBS for 24 h with and without concurrent treatment with either colchicine or cytochalasin B at the doses described in Cell culture and treatment. Cells were washed with ice-cold PBS, and nuclear binding proteins were extracted by a published method (22). Protein concentration was determined by the Bradford method (12) using Bio-Rad protein assay reagent. Double-stranded cAMP response element binding (CREB) protein consensus oligonucleotide (5′-AGAGATTGCTGACGTCA-32P]ATP using T4 polynucleotide kinase enzyme. Nuclear protein (5 μg) was incubated with radiolabeled CREB protein consensus oligonucleotide (5′-AGAGATTGCTGACGTCA-32P]ATP using T4 polynucleotide kinase enzyme. Nuclear protein (5 μg) was incubated with radiolabeled CREB for 30 min at room temperature as described previously (52). For competition reactions, nonradiolabeled consensus and mutated CREB double-stranded oligonucleotides (5′-AGAGATTGCTGACGTCA-32P]ATP using T4 polynucleotide kinase enzyme. Nuclear protein (5 μg) was incubated with radiolabeled CREB for 30 min at room temperature as described previously (52). For competition reactions, nonradiolabeled consensus and mutated CREB double-stranded oligonucleotides (5′-AGAGATTGCTGACGTCA-32P]ATP using T4 polynucleotide kinase enzyme. Nuclear protein (5 μg) was incubated with radiolabeled CREB for 30 min at room temperature as described previously (52). For competition reactions, nonradiolabeled consensus and mutated CREB double-stranded oligonucleotides (5′-AGAGATTGCTGACGTCA-32P]ATP using T4 polynucleotide kinase enzyme. Nuclear protein (5 μg) was incubated with radiolabeled CREB for 30 min at room temperature as described previously (52).
in low ionic strength buffer (22.25 mM Tris borate, 22.25 mM boric acid, and 500 mM EDTA) for 2–3 h at 4°C at 10 V/cm. Gels were fixed in a 10% acetic acid/10% methanol solution for 10 min, dried under vacuum, and exposed to X-ray film. Radiolabeled DNA-protein complexes were extracted from gels and quantified by scintillation counter.

Western blot. NIH/3T3 cells were treated with 0.4% FBS or 10% FBS for 24, 48, or 72 h, washed with ice-cold PBS, and lysed in 1 ml of homogenization buffer (50 mM NaCl, 50 mM NaF, 50 mM NaPO₄·12 H₂O, 5 mM EDTA, 5 mM EGTA, 2 mM Na₂VO₃, 0.5 mM phenylmethylsulfonlfyl fluoride, 0.01% Triton X-100, 10 μg/ml leupeptin, and 10 mM HEPES, pH 7.4) by repeated passages through a 26-gauge needle. The resulting homogenate was centrifuged at 14,000 rpm for 5 min at 4°C. Protein concentration was determined by the Bradford method (12). The protein (100 μg) was mixed with an equal volume of 2× sample buffer (125 mM Tris·HCl, pH 6.8, 4% SDS, 20% glycerol, 5–10% 2-mercaptoethanol, and 0.004% bromphenol blue), boiled for 5 min, loaded onto a 10% SDS-7.4, 4% SDS, 20% glycerol, 5 mM NaF, 50 mM NaPO₄·12 H₂O, 5 mM EDTA, 5 mM EGTA, 2

MECHANISMS OF SERUM-INDUCED FIBRONECTIN EXPRESSION

RESULTS

Serum enhances FN gene transcription and protein production. To characterize what effect serum has on FN expression, NIH/3T3 cells (clone P1d) permanently transfected with the human FN promoter connected to the luciferase reporter gene, pFN (1.2 kb) LUC were cultured in either 0.4% or 10% FBS for 24 h, harvested, and processed to determine luciferase activity. Figure 1A shows that exposure of fibroblasts to 10% FBS resulted in a significant increase in FN expression compared with fibroblasts cultured in 0.4% FBS (P = 0.0001). This effect was time dependent with the response being noticeable by 6.5 h and achieving a plateau after 18–24 h (Fig. 1B). Cells for all subsequent experiments were, therefore, harvested between 21 and 24 h. Cells cultured in 0.4% FBS also demonstrated a slight increase in FN expression with time, but this was not significant. The serum induction of FN gene transcription was associated with an increase in FN protein production as determined by Western blotting (Fig. 1C). Note that FN protein expression was increased at 24 h, with a peak detected at 48 h, followed by a decrease in FN protein production by 72 h. The variance in the intensity of the serum-induced FN response appeared dependent on the use of different lots of serum. However, all serum samples always induced FN expression significantly.

Role of ECMs in serum induction of FN gene expression. Injured tissues are characterized by alterations in ECM expression and composition and show increased deposition of FN and type I collagen and fibrin, among other ECMs (54, 55). Because of their strategic location and their ability to affect intracellular signaling and gene expression (see DISCUSSION), these ECM molecules could potentially modulate FN expression in injured tissues. To test this possibility, cells cultured in both 0.4% and 10% FBS were treated with monomeric soluble ECM components or insoluble ECMs coated onto culture dishes followed by harvesting at 24 h. Control cells were grown in serum without additions. FN and type I collagen were tested at 100 μg/ml, whereas fibrin was tested at 500 μg/ml. We previously demonstrated that these doses maximally stimulate another gene (i.e., IL-1β) in human monocytic cells (29, 49, 50, 52, 59). The soluble monomeric ECM components tested individually did not significantly affect FN gene expression compared with controls (not shown). The results were consistent for both concentrations of serum. Similar results were obtained when the soluble ECMs were added simultaneously (Fig. 2A).

In other experiments, cells were exposed to dishes coated with insoluble polymeric ECMs at the concentrations described above (see Fig. 2B). Cells were subsequently added in both 0.4% and 10% FBS, allowed to adhere, and harvested after 24 h. Here again, FN and type I collagen did not have a major effect on the serum-induced FN response, although the intensity of the response to serum appeared diminished. Fibrin, however, had a small inductive effect compared with the controls, but this effect did not reach statistical significance (P = 0.083).

Role of protein kinases in the serum induction of FN expression. We next focused on the signal transduction mechanisms affecting FN expression induced by serum. First, we examined the possibility that protein kinase C (PKC) could be involved. This kinase is increased in cells exposed to serum and has been implicated in the resulting induction of FN and other genes (38). Consistent with this, we found that exposure of transfected cells with phorbol ester (25 nM, 24 h), an inducer of PKC, consistently caused an increase in FN gene transcription in our system (not shown). We then tested the effects of a potent inhibitor of PKC activation, calphostin C, on FN expression (36). As demonstrated in Fig. 3A, light-activated calphostin C not only blocked the serum response (P = 0.00001) but also ablated the constitutive expression of FN, whereas...
inactive calphostin C had no effect. We also tested the effects of another kinase inhibitor, PD-98059. This MEK1 inhibitor blocks the activation of MAPK (4). Like the PKC inhibitor, PD-98059 also caused a reduction in FN expression, but to a lesser extent than that seen with calphostin C ($P < 0.005$; Fig. 3B). It did not affect the constitutive expression of the gene.

**Role of cytoskeletal organization in serum-induced FN expression.** The role of cytoskeletal integrity on FN expression was also tested. To this end, microtubule and actin microfilament structures were separately disrupted with colchicine or cytochalasin B, respectively, in cells cultured in both 0.4% and 10% FBS. As seen in Fig. 4, A–C, opposite effects occurred. Microtu-
bule disruption with colchicine resulted in enhanced gene expression, particularly when the cells were cultured in 10% FBS ($P = 0.0001$; Fig. 4A). Interestingly, when the microtubule stabilizing chemotherapy drug taxol was used, the serum induction of FN gene expression was prevented (Fig. 4B). The effects of these agents were dose dependent (see inset). Disruption of actin microfilament structures with cytochalasin B produced a marked decrease in expression, to the extent of constitutive inhibition ($P = 0.0001$; Fig. 4C). Results were consistent for both concentrations of serum. Cells remained 99% viable, even with concentrations of cytochalasin B of 30 μM (not shown).
Fig. 4. Effects of cytoskeletal-disrupting agents on FN gene transcription. 

A: effects of colchicine (Colch). NIH/3T3 cells (1.5 × 10⁵ cells/ml) permanently transfected with pFN (1.2 kb) LUC were cultured for 24 h in DMEM supplemented with either 0.4% or 10% heat-inactivated FBS with or without 50 μM colchicine. Gene expression was quantified as before, recorded as normalized luciferase values, and standardized according to total protein measurements. Note that microtubule disruption (Colch, 10% FBS) enhanced the serum stimulation of the FN gene (P < 0.0001).

B: effects of taxol. Transfected NIH/3T3 cells cultured as described above were treated with 10 μM taxol, a microtubule stabilization agent. Taxol diminished the serum stimulation of the FN gene (control vs. taxol, P = 0.185). Inset: effects of taxol were dose dependent.

C: effects of cytochalasin B (Cyto B). NIH/3T3 cells permanently transfected with pFN (1.2 kb) luciferase were cultured for 24 h in DMEM supplemented with either 0.4% or 10% heat-inactivated FBS with or without 6.25 μM cytochalasin B and incubated in 5% CO₂ at 37°C. Note that disruption of microfilaments with cytochalasin B (Cyto B, 10% FBS) abrogated the serum stimulation of the FN gene (10% FBS, Control; P = 0.0001).

D: role of PKC. NIH/3T3 cells (1.5 × 10⁵ cells/ml) permanently transfected with the human FN promoter were cultured for 24 h in DMEM supplemented with either 0.4% or 10% heat-inactivated FBS with or without 50 μM colchicine, 6.25 μM cytochalasin B, and active (CC⁺) and inactive (CC⁻) calphostin C and incubated in 5% CO₂ at 37°C. Note that active calphostin C inhibited the stimulation of the FN gene by colchicine. The inhibition of the serum-induced response observed with cytochalasin was retained in the presence of calphostin C.
We then examined the role of PKC in the effects of colchicine and cytochalasin on FN expression. As demonstrated in Fig. 4D, we found that the PKC inhibitor calphostin C prevented the induction of FN produced by colchicine but did not affect the inhibition of FN expression induced by cytochalasin. This suggests that PKC mediates the effects of microtubular disruption by colchicine on FN but plays no role in the ability of actin depolymerization by cytochalasin to inhibit FN expression.

Role of CRE in FN gene transcription. We found the aforementioned observations regarding the role of cytoskeletal integrity on FN expression intriguing and decided to examine them in more detail. The work by Dean and colleagues (20, 21) identified the CRE as the transcription factor predominantly responsible for serum-induced FN gene expression, and, therefore, we attempted to determine whether the effects of the cytoskeletal-disrupting drugs were related to alterations in DNA binding of CREs contained in the FN promoter by CREB. Indeed, we found this to be the case. Using electrophoretic mobility shift assay, we demonstrated that serum enhanced DNA binding of CREB (Fig. 5, compare lane 2 vs. lane 5). Colchicine did not inhibit this induction (compare lanes 2 with 3 and 5 with 6). Conversely, cytochalasin B was found to inhibit DNA binding of CREB, suggesting that its inhibitory effects on FN induction by serum are mediated by inhibition of this transcription factor (compare lanes 2 with 4 and 5 with 7). To show the specificity of the probe-DNA complexes, nonradiolabeled CREB or mutated CREB oligonucleotides were added to the mixtures at a 50-fold molar excess concentration. As seen in lanes 8 and 9, binding was blocked with the nonradiolabeled probe but was not inhibited by the mutated nonradiolabeled oligonucleotide (lanes 10 and 11).

To confirm the role of CREs in the serum induction of FN, the following experiments were conducted. First, we tested fibroblasts transfected with deletion constructs of the FN gene promoter (Fig. 6). All previous experiments were performed using pFN (1.2 kb) LUC. In the experiments described in Fig. 6, we used cells transfected with pFN (0.5 kb) LUC, which lacks most of the 5’ sequences present in pFN (1.2 kb) LUC but contains the three CRE, and pFN (0.2 kb) LUC, with no CREs. As demonstrated in Fig. 6, 10% FBS stimulated
the transcription of pFN (1.2 kb) LUC over control as before. The stimulatory effect of serum and colchicine was diminished when the deletion construct pFN (0.5 kb) LUC was tested, indicating that 5' sequences proximal to the CREs are needed for optimal stimulation. As before, cytochalasin inhibited the effect. No stimulation was noted for either reagent when we used pFN (0.2 kb) LUC, a construct lacking all three CREs.

The studies performed using deletion constructs of pFN (1.2 kb) LUC further support a role for CREs in the serum induction of FN. To strengthen this association, we measured FN expression in cells exposed to 10% FBS after transfection with a competing CRE oligonucleotide (Fig. 7). As previously reported by others, the CRE oligonucleotide greatly diminished the serum-induced FN response.

DISCUSSION

In this report, we confirmed that serum induces FN gene transcription in a dose- and time-dependent manner using stably transfected murine fibroblasts. As expected, this stimulatory effect was followed by increased production of FN protein. These observations are consistent with those of other investigations (5, 18, 20). The intracellular signals mediating the serum response are not well understood, but the activation of protein kinases such as PKC is thought to be important (38, 39). Herein, we show that serum induction of FN expression in our system does require PKC activation because the inhibition of this protein kinase prevented the serum-induced response. Additionally, we found that serum induction of FN gene transcription is decreased by a potent inhibitor of MEK1 activation, an intermediary step in the MAPK pathway (4).

A role for intracellular protein kinases in the stimulation of genes by serum is not surprising. Serum contains a number of soluble factors capable of stimulating signal transduction and FN gene transcription such as vitamin D, transforming growth factor-β, platelet-derived growth factors, interleukins, and corticosteroids (18–20). In general, these agents have been shown to induce PKC and MAPK activation, among other effects on signal transduction (25). Both pathways appear to intersect at the level of Raf-1, which can be activated by PKC (25). Also, members of the ribosomal S6 kinase (RSK) protein family (RSK2), which can be activated by PKC, are substrates of extracellular regulated kinases generated through the MAPK pathway, can phosphorylate CREB at Ser133, a critical residue for activation of CREB and for expression of c-fos (73). The latter pathway might be partially responsible for the induction of FN by serum.

Another important finding of this report relates to the observation that the serum-induced FN gene transcription appears to be dependent on cytoskeletal integrity. This phenomenon is known as mechanotransduction (31, 32) and has been demonstrated for a variety of genes, including the ECM molecule osteopontin (11, 48, 50, 62, 68). This process is dependent on the physical and functional links established between the cytoplasmic domains of ligand-bound integrin receptors, intracellular cytoskeletal proteins, and secondary signaling molecules (e.g., kinases) at the cell membrane in structures termed focal adhesion complexes or FACs (69). This work begins to define the intracellular signals stimulated during mechanotransduction that lead to FN expression. First, we found that microtubule destabilization with colchicine results in further enhancement of serum-stimulated FN expression, whereas “stabilization” of microtubules with taxol inhibited the response. These results suggest that the influence of microtubule integrity on FN expression runs along a spectrum of stability; the more stable the microtubule system, the less effect serum has on FN expression. The observation that a PKC inhibitor could block both the serum- and colchicine-induced FN expression suggests a role for this kinase in both processes. Other genes appear to be affected in a similar fashion (11). Other kinases might contribute to the effects of colchicine as well (43, 63). Taxol, on the other hand, was found to inhibit the serum induction of FN, and this effect might be related to its ability to act on specific signaling and transcriptional pathways. For example, taxol has been shown to inhibit microtubule-dependent NF-κB activation by phorbol esters (66), whereas others have shown taxol able to induce c-jun NH2-terminal kinase signaling pathways (41).
Together, these studies suggest that, similar to serum treatment, disruption of microtubules results in the activation of specific kinase-dependent pathways including PKC, cAMP/protein kinase A, and MAPK.

This is not surprising in view of the reported physical association between certain protein kinases and microtubules for which some investigators have postulated that the microtubule network might act as a "retention receptor for kinases or kinase activators" (43). In this scheme, microtubules serve to restrain kinases that could become available for the transduction of signals elicited at the cell surface under the right conditions. Colchicine might facilitate the "release" of these kinases by unfolding the COOH-terminal region of β-tubulin (61).

Interestingly, disruption of the cytoskeleton with cytochalasin also resulted in alterations in FN expression; however, in contrast to our observations with colchicine, cytochalasin B inhibited FN expression in response to serum without affecting viability. This suggests a role for the actin microfilament system in this process. As described for the microtubular system, actin microfilament disruption may also affect signaling pathways potentially relevant to FN expression (33). PKC does not appear to mediate the inhibitory role of cytochalasin in our system, but other, less well-defined kinase systems might be involved (1).

The transcriptional mechanisms by which serum induces FN expression were also examined. The studies performed with cells transfected with deletion mutation constructs of pFN (1.2 kb) LUC suggest that the signals induced by serum act directly on the FN gene to promote its expression. In particular, they point to CREs present within the human FN promoter as necessary to generate the serum-induced response. A significant response was observed when all three CREs were present, whereas the response was prevented in the absence of CREs. A maximal stimulation required sequences present 5' of the CREs. This association was further strengthened by the CRE oligonucleotide cotransfection experiments. This information, together with studies showing that dibutyryl adenosine 3',5'-cyclic monophosphate (an analog of cAMP) and forskolin (an inducer of adenyl cyclase) stimulate FN gene transcription in our system (unpublished observations), implicate CREB in the serum-induced FN response. This is consistent with our findings showing that DNA binding by CREB was indeed stimulated by both serum and colchicine, but inhibited by cytochalasin. Therefore, it appears that the signals elicited at the cell membrane by serum ultimately result in the induction of CREB, which, in turn, is responsible for the observed effects on FN gene transcription. Furthermore, the work suggests that microtubular disruption might act by stimulating common downstream signals that lead to CREB induction. Similarly, others have demonstrated alterations in transcription factor production in response to cytoskeletal disruption, including activator protein-1 and NF-κB (40, 48, 60, 64). More relevant to our work, colchicine has been shown to increase cAMP in S49 lymphoma cells (33). Interestingly, taxol has been shown to antagonize the increase in cAMP induced by colchicine, and this might explain its inhibitory effect in our system (70).

Finally, we tested the effects of ECMs on FN gene expression. Injured tissues are characterized by increased deposition of fibrin, FN, type I collagen, and other ECMs. These ECM components can trigger PKC-dependent signals as well as induce transcription factors involved in the regulation of gene expression. Moreover, intracellular events elicited by ECMs also appear to be modulated by cytoskeletal organization (15, 30, 34, 46, 49, 50, 52, 54, 59). Because of the possibility that a change in ECM composition might feed back to either stimulate or inhibit FN production, we tested the effects of some ECMs in our in vitro system. Our results suggest that, at least in vitro, FN and type I collagen display little, if any, feedback control on the constitutive expression of FN or on its response to serum. Fibrin, however, tended to stimulate gene transcription above control, but only when presented polymerized onto a substrate. Although this effect did not reach statistical significance in our system, it might be relevant in vivo because fibrinogen is present in serum and can be incorporated into newly formed insoluble matrices in injured tissues (37, 50, 65). Also, we have shown previously that fibrinogen can stimulate DNA binding by CREB in U-937 cells (49).

Implications to lung injury. In view of the above, we postulate that FN expression in injured lungs is stimulated via at least two mechanisms. The first relates to the stimulation induced by serum and the injurious agent. Acute lung injury is characterized by increased vascular permeability and the flooding of alveoli with proteinaceous material from serum (14). Under these circumstances, FN expression might be stimulated by the injurious agent directly, as demonstrated for paraquat and other agents (54, 55), or indirectly by the serum that floods the alveoli, which contains FN-stimulating factors. These factors stimulate PKC- and MAPK-dependent signals in interstitial and alveolar fibroblasts, resulting in increased FN expression. This process could be amplified by the release of chemotactic growth factors that promote the recruitment and proliferation of fibroblasts into the affected site.

Mechanical ventilation is yet another mechanism likely to be involved in the processes that lead to increased FN expression in acute lung injury. During mechanical ventilation, regional overinflation can result in cellular stretch and cytoarchitectural distortion that compromises cytoskeletal integrity (23). On the basis of our observations with agents capable of cytoskeletal disruption, it is conceivable that such an effect could also result in enhanced FN expression. If one of the consequences of ventilator-induced lung injury is microtubule destabilization, this would enhance the stimulatory effect of serum and, consequently, FN expression and deposition. Consistent with this, Berg and colleagues (6) found that inflation of rabbit lungs with high levels of positive end-expiratory pressure (PEEP) increased the expression of FN mRNA twofold compared with low PEEP ventilation.
In summary, we have demonstrated that serum induces the transcription of the FN gene in cultured fibroblasts and that this effect is mediated via the activation of specific protein kinases and the induction of the transcription factor CREB. Serum-induced FN expression was altered by disruption of the cytoskeleton with microtubule destabilization, resulting in further induction of FN, and microfilament depolymerization, resulting in inhibition of the response. Further work will be required to determine the true relevance of these mechanisms in the ability of the lung to heal after injury.

The authors thank William Schuyler and Susanne Roser for excellent technical assistance.

This work was supported by an Established Investigator Award from the American Heart Association (to J. Roman).

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