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Insulin-like Growth Factor-binding Protein-5-induced Laminin γ1 Transcription Requires Filamin A*

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Christine K. Abrass[1] **and Kim M. Hansen**

From the Department of Medicine, Allergy & Inflammation Program, University of Washington School of Medicine, Seattle, Washington 98109

Insulin-like growth factor-binding protein-5 (IGFBP-5) has IGF-1-independent intranuclear effects that are poorly defined. Treatment of cells with IGFBP-5 induces migration, prevents apoptosis, and leads to increased laminin subunit transcription. Similarly, filamin A (FLNa), an actin-binding protein that participates in cell attachment, plays important additional roles in signal transduction and modulation of transcriptional responses. In this report, we show that IGFBP-5 leads to dephosphorylation of FLNa with subsequent FLNa cleavage. Following cleavage, there is enhanced recruitment of Smad3/4 to a C-terminal FLNa fragment with nuclear translocation and subsequent binding to the promoter region of the laminin γ1 (*lamc1*) gene. FLNa knockdown prevents IGFBP-5-mediated increases in *lamc1* transcription. These data indicate that IGFBP-5 induces formation of a FLNa-based nuclear shuttle that recruits transcription factors and regulates transcription of IGFBP-5 target genes. These studies provide new insights into the mechanisms whereby IGFBP-5 and FLNa exert intranuclear effects.

Insulin-like growth factor-binding proteins (IGFBPs)[2] modulate IGF-1 action by preventing IGF-1 degradation and by modulating IGF-1 receptor binding (1, 2), yet among the six IGFBP that bind IGF-1, IGFBP-3 and IGFBP-5 exhibit IGF-independent effects (3–6). IGFBP-5 has important effects on cell proliferation, apoptosis, migration, and regulation of transcription (7–9), which have been shown to be relevant to understanding cell differentiation, senescence, and cancer (3). An N-terminal domain of IGFBP-5 contains the IGF-1-binding region and caveolin-binding sites (10). The C-terminal domain does not bind IGF-1, but it can influence the IGF-1 binding affinity (11). A highly basic domain in the C-terminal end of IGFBP-5 (amino acids 201–218) has a functional nuclear localization sequence (12), yet secretion and reuptake of protein and possibly proteolytic processing appears to be required for nuclear translocation (13). Cellular reuptake can occur by several mechanisms that are attributed to the C-terminal domain including uptake by a protein transduction domain (14), binding to transferrin with subsequent uptake by the transferrin receptor (15), a possible IGFBP-5 receptor (6), or interaction with the type V transforming growth factor β receptor (16). It is possible that each of these uptake mechanisms lead to different biological responses. Studies to understand the intranuclear effects of IGFBP-5 have revealed a cryptic domain in the N-terminal portion of the molecule that can act as a transcriptional transactivator (13). Amaar *et al.* (17) showed that IGFBP-5$^{201-218}$ binds to the second and third LIM domains of FHL2, and they proposed that IGFBP-5 participates in transcriptional regulation through binding to this transcriptional co-activator. IGFBP-5 also influences gene transcription through interaction with the retinoic acid receptor-rexinoid receptor system (18). Less is known about IGFBP-5 gene targets and other steps involved in transcriptional control by IGFBP-5.

Filamin A (FLNa) is a 280-kDa protein with an N-terminal actin-binding domain followed by 24 repeats that are interrupted by two hinge regions and a C terminus that is responsible for dimerization (19). The hinge regions allow FLNa to function as a molecular leaf spring, lending flexibility and stiffness to the actin filaments (19) when FLNa participates in connections between the intracellular domain of integrins and the cytoskeleton. The 24 Ig-like repeats serve as docking sites for a variety of proteins that regulate cellular responses to growth factors and perturbants of cell-matrix attachments. Thus far, more than 20 FLNa binding partners have been described, and more have been proposed (20). Sites that bind the intracellular domains of integrins (21), the potassium channel, androgen receptor (22), calcium sensing receptor (23), and prostate-specific antigen (24) are near the C terminus (repeats 16–24). Signal transduction molecules, such as the Rho GTPases (Rho/Rac/cdc42), RalA, and Smads, bind in repeats 17–23 (25–28). Other proteins can bind to FLNa when signal transduction cascades are activated (29). Proteins that primarily regulate cleavage of FLNa and actin assembly bind to repeats 10–13 and the first hinge region (*e.g.* furin, presenilins, and FILIP) (30). With this structural arrangement and the distribution of binding sites, FLNa brings together integrins, the submembrane actin network, and intracellular signaling components to allow participation in the regulation of many cellular processes.

In its traditional role as an actin-binding protein, FLNa binds to the intracellular domains of integrins and assembles actin to form stress fibers (31). In this configuration, the cells are adherent and nonmigratory (32). With inside-out signaling of integrins, the cells can attach to different matrix proteins, and FLNa and actin become assembled into new focal adhesions (33). In association with migration, FLNa and actin provide the rigidity

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[1] To whom correspondence should be addressed: UW Medicine South Lake Union, 815 Mercer St., Seattle, WA 98109. Fax: 206-897-1300; E-mail: cabrass@u.washington.edu.

[2] The abbreviations used are: IGFBP, insulin-like growth factor-binding protein; FLNa, filamin A; GFP, green fluorescent protein; MC, mesangial cell(s); FCS, fetal calf serum; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; siRNA, small interfering RNA; ChIP, chromatin immunoprecipitation; DAPI, 4′,6-diamidino-2-phenylindole.

necessary for propulsion of the cell (34, 35). Migration promoted by mitogens (*e.g.* insulin (36), IGF-1, and epidermal growth factor) is associated with RS6K-mediated phosphorylation of FLNa at Ser^{2152}, which inhibits FLNa cleavage, facilitates caveolin binding, and drives migration associated with Rac1 activation and lamellipodia formation (10, 37, 38). In response to IGF-1, the caveolin 1-FLNa complexes contain p-Akt, and the pI3K inhibitor wortmannin blocks FLNa-caveolin interaction and prevents migration (10). Although some migratory stimuli such as IGF-1 lead to formation of a Rac1-dependent, β-actin-rich leading lamella, other stimuli (*e.g.* IGFBP-5) induce migration characterized by cdc42-dependent filopodia formation (35). In its GTP-bound, activated form, RalA binds FLNa (29), which elicits filopodia formation and recruits FLNa to these structures. Blockade of FLNa-RalA interaction prevents cdc42-dependent filopodia formation. Thus, FLNa-actin interactions are required for both forms of cell migration.

FLNa is also associated with a variety of membrane receptors where the association can influence receptor retention in the membrane, recycling to the membrane, protection from or enhanced degradation, or receptor signaling. FLNa binds to FcγR1, which enhances retention in the cell membrane by preventing targeting to lysosomes (39). Upon receptor ligation, the two dissociate. With bradykinin activation in endothelial cells, FLNa plays a role in receptor internalization and recycling to the membrane (24). When calcium-sensing receptor binding to hinge 1 and repeats 15–17 of FLNa is disrupted, calcium-sensing receptor-mediated signaling to extracellular signal-regulated kinase (ERK) or c-Jun N-terminal kinase (JNK) are impaired, and Rho activation, which is required for inhibition of parathyroid hormone, does not occur (40–42). In these examples, activation of membrane receptors involves signal transduction cascades that lead to phosphorylation of FLNa, which inhibits its cleavage and facilitates membrane reorganization, thereby allowing receptor recycling.

In other instances, cellular activation leads to dephosphorylation of Ser^{2152} upon FLNa rendering the molecule susceptible to calpain cleavage (43). When FLNa is cleaved, the C-terminal fragment can bind activated factors such as phospho-Smad (25) and translocates to the nucleus enhancing transforming growth factor β-mediated responses. Binding of the C-terminal fragment of FLNa to the cytoplasmic androgen receptor facilitates nuclear translocation and modulates the responses of androgen target genes (22, 44, 45). FLNa binding is required for androgen-mediated stimulation of cell proliferation, and the loss of cleavable FLNa plays a role in the development of androgen independence in prostate cancer (46). Transcriptional activity of the transcription factor FOXC1 is also modulated by intranuclear binding to FLNa (47). These studies highlight the importance of FLNa cleavage and intranuclear effects of FLNa, yet the breadth of nuclear effects of FLNa remains to be examined.

Previously, we showed that IGFBP-5 induces migration in glomerular mesangial cells, a pericyte-like, vascular smooth muscle cell (7, 48). IGFBP-5-induced migration requires cdc42 activation and attachment to laminin 421 via a α6-containing integrin (48). Treatment with IGFBP-5 also induces transcription of the laminin subunits (α4β2γ1) that compose laminin 421 (49). Each of these effects can be induced by intact IGFBP-5 or the heparin-binding peptide $IGFBP\text{-}5^{201-218}$, but they cannot be duplicated with the N-terminal $IGFBP\text{-}5^{1-169}$, IGFBP-3, IGFBP-4, or IGF-1. The effects of IGFBP-5 on laminin 421 expression are of particular interest because of the role of this laminin isoform in control of angiogenesis and stabilization during microvessel maturation (50), functions that also influence metastatic potential of cancers (51). During studies of IGFBP-5-induced migration, we noted that FLNa reorganized with the cytoskeleton as expected, but IGFBP-5 also led to nuclear accumulation of FLNa. This report details the studies that were undertaken to investigate the role of IGFBP-5 in nuclear accumulation of FLNa and its role in mediating the increased transcription of laminin γ1.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The following reagents were obtained from the designated suppliers: intact IGFBP-5 (Abcam, Cambridge, MA); IGFBP-5 peptide (amino acids 201–218, RKGFYKRKQCPKSRGRKR) (Sigma); pcDNA3.1/NT-GFP-TOPO-TA and the control vector pcDNA3.1/NT-GFP (Invitrogen); DAPI and phalloidin (Molecular Probes, Eugene, OR); fluoromount (Southern Biotechnology, Birmingham, AL); antibodies to α-actinin, epidermal growth factor receptor, and β-actin (Sigma); TFIIB (Transduction Laboratories, Franklin Lakes, NJ); and goat anti-human phospho-Smad2/3 (Santa Cruz Biotechnology, Santa Cruz, CA). Antibodies to FLNa included goat anti-chicken gizzard FLNa (Sigma), polyclonal rabbit antibody to a COOH peptide fragment (Santa Cruz Biotechnology), NH3 peptide fragment (Santa Cruz Biotechnology), human FLNa, and phosphoserine 2113 and 2152 (Cell Signaling Technology, Beverly, MA). Polyclonal rabbit antibody to C-terminal repeat 24 was kindly provided by Dr. R. Tyler Miller (Case Western Reserve University, Cleveland, OH). Peroxidase-conjugated goat anti-rabbit IgG was from Pierce, and Alexa Fluor 488 and 568 antibodies to mouse, goat, and rabbit IgG were from Molecular Probes.

Cell Culture—Rat glomerular mesangial cells (MC) were prepared by modification (52, 53) of routine methods (54). In brief, minced rat kidney cortex was sieved. Isolated glomeruli were plated in medium containing a 1:1 mix of 20% FCS-RPMI 1640 and previously collected glomerular conditioned medium. MC outgrowths were harvested and passed in this medium for an additional week, after which the conditioned medium was omitted. MC were cloned and studied at passages 8–12. Supplemental insulin routinely added to MC cultures was omitted. MC are vascular smooth muscle-like cells analogous to microvascular pericytes or mural cells. For microscopic analysis cells (2×10^4) were plated in glass chamber slides (Nunc, Rochester NY), grown for 24 h in 20% FCS-RPMI medium, and growth-arrested in 2% FCS-RPMI for 48 h. The cells were treated with $IGFBP\text{-}5^{201-218}$ (30 μg/ml) or intact IGFBP-5 (100 nM) and examined at the times noted in the results. The cells were fixed and examined by light, standard immunofluorescence, and confocal microscopy.

Fluorescence Microscopy—Chamber slides were rinsed in PBS and fixed in 2% paraformaldehyde for 20 min. After rinsing in PBS, the cells were permeabilized with 0.05% Triton X-100

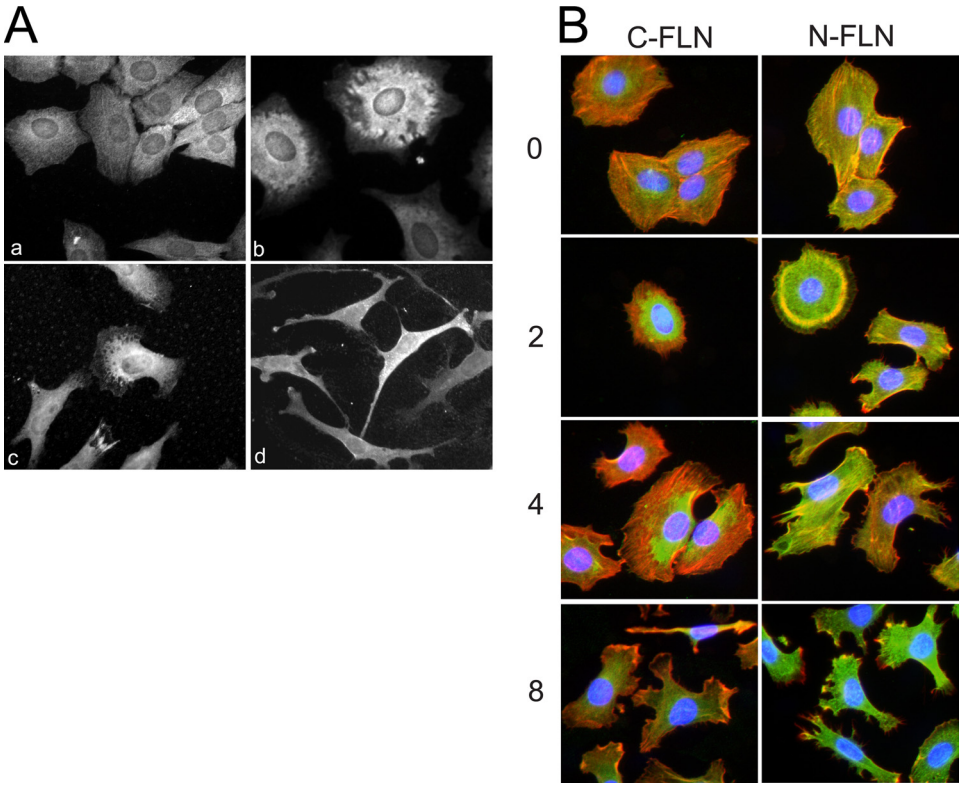

FIGURE 1. **IGFBP-5 induces FLNa reorganization.** *A*, MC were stained with Gt anti-FLNa before (*panel A*) and 4 (*panel B*), 8 (*panel C*), and 24 (*panel D*) h after the addition of IGFBP-$5^{201-218}$. FLNa reorganizes, leaving the periphery of the cell and then concentrating in attachment sites, as the filopodial phenotype that is associated with IGFBP-5-induced migration develops. *B*, MC were stained for F-actin (phalloidin, *red*), FLNa (*green*), and nuclei (DAPPI, *blue*) before and at 2, 4, and 8 h after the addition of IGFBP-$5^{201-218}$. As seen with the N-terminal anti-FLNa antibody, intact FLNa is diffusely present throughout the cytoplasm and co-localizes with F-actin (*yellow*) in a submembrane location at 2 h and in discrete focal adhesions as they develop after treatment with IGFBP-5. In contrast, staining with antibody specific for repeat 24 at the C terminus shows intense perinuclear staining followed by co-localization with DAPI, indicating an increase in nuclear staining. This pattern is less apparent by 8 h as the altered cellular phenotype develops. *C-FLN*, C-terminal FLNa; *N-FLN*, N-terminal FLNa.

for 3 min and rinsed three times in PBS. The slides were incubated with primary antibody for 20 min at room temperature, rinsed, and incubated with fluorescein-conjugated secondary antibody for an additional 20 min. Controls included the non-immune IgG and the secondary antibody alone. Two different antigens were visualized by using Alexafluor 488-conjugated as well as Alexafluor 568-conjugated secondary antibody. F-actin was visualized with phalloidin staining, and nuclei were visualized with DAPI. All of the slides were mounted in Fluoromount and observed with a Leitz microscope equipped for epi-illumination or a Leica TCS-SP confocal microscope. The scanned images collected in the sequential xy series were acquired at 400× magnification. The digital images were exported to Adobe Photoshop for further processing.

Preparation of Cellular Fractions—MC were plated in 75-cm^2 culture flasks, grown to near confluency, and growth-arrested in 2% FCS-RPMI medium for 48 h. Some flasks were treated with IGFBP-$5^{201-218}$(30 μg/ml) for the times indicated in the results, rinsed, and harvested with cell lysis buffer. The cell lysates were prepared by three different methods: 1) whole cell lysates were prepared by treating cells with PBS containing 0.1% SDS, 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, pepstatin A (20 μg/ml), aprotinin (10 μg/ml), and leupeptin (10 μg/ml); 2) nuclear and cytoplasmic fractions were prepared as described in Ref. 46 by first extracting with 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 10% Nonidet P-40, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each leupeptin, aprotinin, and pepstatin A) followed by extraction of the nuclear pellet with 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 10% glycerol containing the same protease inhibitors; and 3) differential detergent fractionation was performed as described (33). Untreated or IGFBP-$5^{201-218}$-treated cells were grown in 75-cm^2 flasks. At the designated times the cells were rinsed with PBS and extracted in ice-cold digitonin buffer (0.01% digitonin, 10 mM PIPES, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 10 μg/ml phalloidin, and protease inhibitors). Following incubation on ice for 10 min, the supernatant was collected (cytosolic fraction). The remaining cell components were sequentially incubated for 30 min at room temperature in a Triton solution (0.5% Triton X-100, 10 mM PIPES, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 3 mM EGTA, protease inhibitors, and 10 μg of phalloidin) to extract the membrane/organelle fraction. The residual cell material was extracted on ice for 10 min with a Tween 40/deoxycholate buffer (1% Tween 40, 0.5% deoxycholate, 10 mM PIPES, protease inhibitors), and the supernatant was collected (nuclear fraction). Hot (100 °C) SDS buffer (2.5% SDS in 10 mM Tris-HCl, 200 mM dithiothreitol, and protease inhibitors) was added to the dish and then incubated on ice for 5 min. The cytoskeletal fraction is collected and heated at 100 °C for 5 min. All of the cellular fractions were acetone-precipitated, dissolved in equal volumes of sample buffer, subjected to electrophoresis under reducing conditions in 5% SDS-polyacrylamide gel, transferred to nitrocellulose, and incubated with polyclonal goat anti-FLNa. The FLNa bands within each lane were analyzed on Kodak EDAS 290. Fraction purity was monitored by staining by Western blot using antibodies to α-actinin (cytoskeletal protein), epidermal growth factor receptor (membrane protein), β-actin (cytosolic protein), and TFIIB (nuclear protein).

Immunoprecipitation and Western Analysis—The protein amounts were determined by Bradford colorimetric assay. For immunoprecipitation, equal amounts of protein were incubated with antibodies overnight at 4 °C followed by incubation with protein A/G-Sepharose (GE Healthcare) for 2 h. The resulting complexes were washed with PBS containing 0.1% SDS and 0.5% Triton X-100. For Westerns blots, the proteins

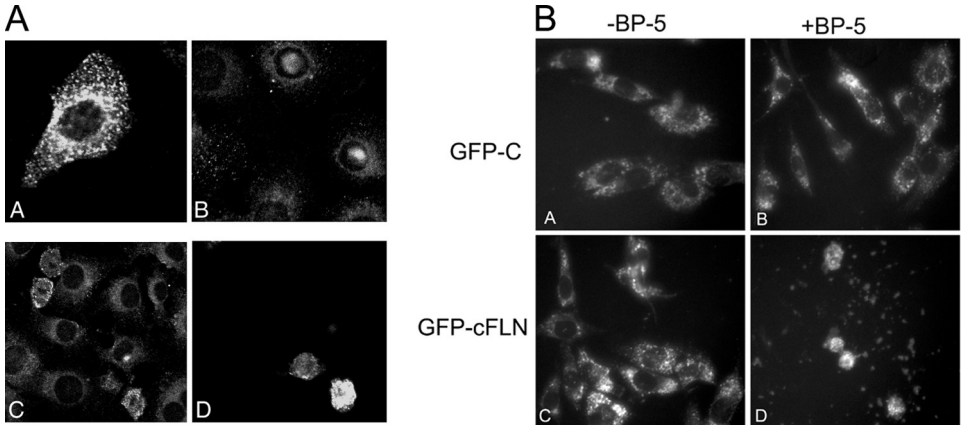

FIGURE 2. **Nuclear accumulation of C-FLNa.** *A*, mesangial cells were examined by confocal microscopy following treatment with IGFBP-5 and staining with antibody specific for C-FLNa (repeats 16–24). Diffuse granular staining of the cytoplasm is seen in untreated cells (*panel A*). By 2 h after the addition of IGFBP-$5^{201-218}$ (*panel B*) some cells show nuclear staining for FLNa and perinuclear clustering of FLNa (cut through center of cell). At 4 h after treatment, a cut through the bottom of the cells shows cytoplasmic staining (*panel C*), whereas the cut through the top of the cells shows predominantly nuclear staining (*panel D*). *B*, cells were transfected with empty vector (GFP-C) (*panels A* and *B*) or GFP-FLNa containing an insert for C-terminal repeats 16–24 (GFP-cFLNa) (*panels C* and *D*). The cells were untreated (*-BP-5*, *panels A* and *C*) or treated with IGFBP-$5^{201-218}$ (*+BP-5*, *panels B* and *D*) for 4 h. IGFBP-$5^{201-218}$ induced nuclear uptake of cFLNa but had no effect on the distribution of the control GFP-C.

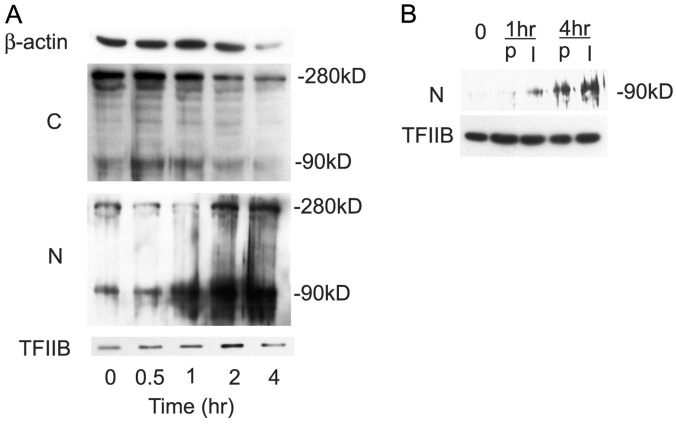

FIGURE 3. **IGFBP-5 induces fragmentation and nuclear translocation of FLNa.** *A*, cytosolic (*C*) and nuclear (*N*) fractions were isolated from cells prior to and at 0.5, 1, 2, and 4 h after the addition of IGFBP-$5^{201-218}$. Staining for FLNa (anti-COOH-FLNa) in Western blot shows the IGFBP5-induced loss of intact FLNa and appearance of fragments in the cytoplasm at 30 min and 1 h. Nuclear appearance of both intact FLNa and a 100-kDa fragment occur beginning at 1 h and are more pronounced at 2 and 4 h after the addition of IGFBP-$5^{201-218}$. β-Actin (a cytosolic protein) and TFIIB (a nuclear protein) serve as loading controls and purification markers for the subcellular fractions. *B*, the nuclear fraction was isolated at 1 and 4 h after treatment with intact IGFBP-5 (*I*) or IGFBP-$5^{201-218}$ (*p*) and subjected to Western blot using the anti-COOH-FLNa antibody. Both intact IGFBP-5 protein and peptide mediate nuclear uptake of a C-terminal fragment of FLNa. TFIIB serves as a loading control for the nuclear fraction.

were separated on 10% SDS-PAGE gels and immunoblotted onto nitrocellulose membranes. Processed membranes were incubated with primary antibodies, exposed to Super Signal chemiluminesence reagents (Pierce) and developed on Kodak X-Omat LS. Image analysis was done using a Kodak EDAS 290 system.

Reverse Transcription-PCR and Real Time PCR—0.5 μg of total RNA prepared using RNAqueous®-4PCR (Ambion, Austin, TX) was used per reverse transcription-PCR. An aliquot of the resulting cDNA generated for each condition was used for amplification of laminin γ1 (*lamc1*) and *gapdh*. Primers for *lamc1* included a sense primer (5′-GTTACGGGCAGAACCTCTCA) and an antisense primer (5′-GTTACGGGCAGAACCTCTCA), and the primers for *gapdh* were purchased (Qiagen). Real time PCR was preformed using SYBR® green SensiMix dT (Quantace, Norwood, MA) processed in an ABI 7900HT PCR machine.

Vector Construction—A GFP-tagged C-terminal FLNa (GFP-cFLNa) construct was prepared for transfection into MC. A C-terminal fragment (nucleic acids 5285–8122) of human FLNa (X53416) was made using pREP4 FLNa vector (a gift from Dr. T. Stossel, Brigham and Women's Hospital, Harvard Medical School, Boston, MA) and the following primers: CFilF (5′-GAC GGC ACT TTC GAC ATC TTC) and CFilR (5′-CCA GAC TCA GGG CAC CAC A). The inserts were prepared by PCR using AccuPrime Pfx DNA polymerase (Invitrogen) and gel-purified, and 3′A overhangs were added with *Taq* DNA polymerase. TA TOPO cloning of inserts into pcDNA3.1/NT-GFP vector (Invitrogen) was performed according to the manufacturer's instructions. The cloning reaction was transformed into One Shot TOP10 *Escherichia coli* (Invitrogen). Multiple clones were screened by PCR using GFP vector sequencing primers provided with the vector. Clones containing inserts of the correct size were sequenced. MC were transfected with GFP-cFLNa or an empty GFP vector using Lipofectamine 2000 (Invitrogen) and growth-arrested in 2% FCS-RPMI medium for 48 h. The cells were treated with IGFBP-$5^{201-218}$(30 μg/ml) for the times indicated in the results.

siRNA Inhibition of FLNa Expression—Stealth™ siRNA sequences derived from human FLNa sequence NM_001456 were as follows: F2 duplex, 5′-AAUACUCGAAGCCAUACACGCCAUC and 3′-UUAUGAGCUUCGGUAUGUGCGGUAG; F3 duplex, 5′-UAUCCAUGCAGGACAUCUUGGCCUC and 3′-AUAGGUACGUCCUGUAGAACCGGAG; and F4 duplex, 5′-UUAGGUGGGACAUACGCAUGGAGUC and 3′-AAUCCACCCUGUAUGCGUACCUCAG (Invitrogen). MC were transfected with Lipofectamine 2000 and siRNA at final concentrations of 0.5–0.1 μM. RNA and protein measurements were made 24–48 h post-transfection. The cells treated with IGFBP-$5^{201-218}$ (30 μg/ml) were growth-arrested in 2% FCS-RPMI medium for 48 h prior to treatment.

Chromatin Immunoprecipitation (ChIP)—ChIP assay was performed as described (55). 3×10^6 cells were treated with or without IGFBP-$5^{201-218}$ for 4 h, formaldehyde-cross-linked, sonicated, and immunoprecipitated with antibody to FLNa, and phenol/chloroform-purified DNA was subjected to PCR. PCR analysis included primers for *lamc1* (sense primer, 5′-AGGACATCAAGAAGACACTACCCAAC, and antisense primer, 5′-CTACCAACGTCTAATGACCAATACC) and Hba (a gene not expressed in MC; sense primer, 5′-AGG-

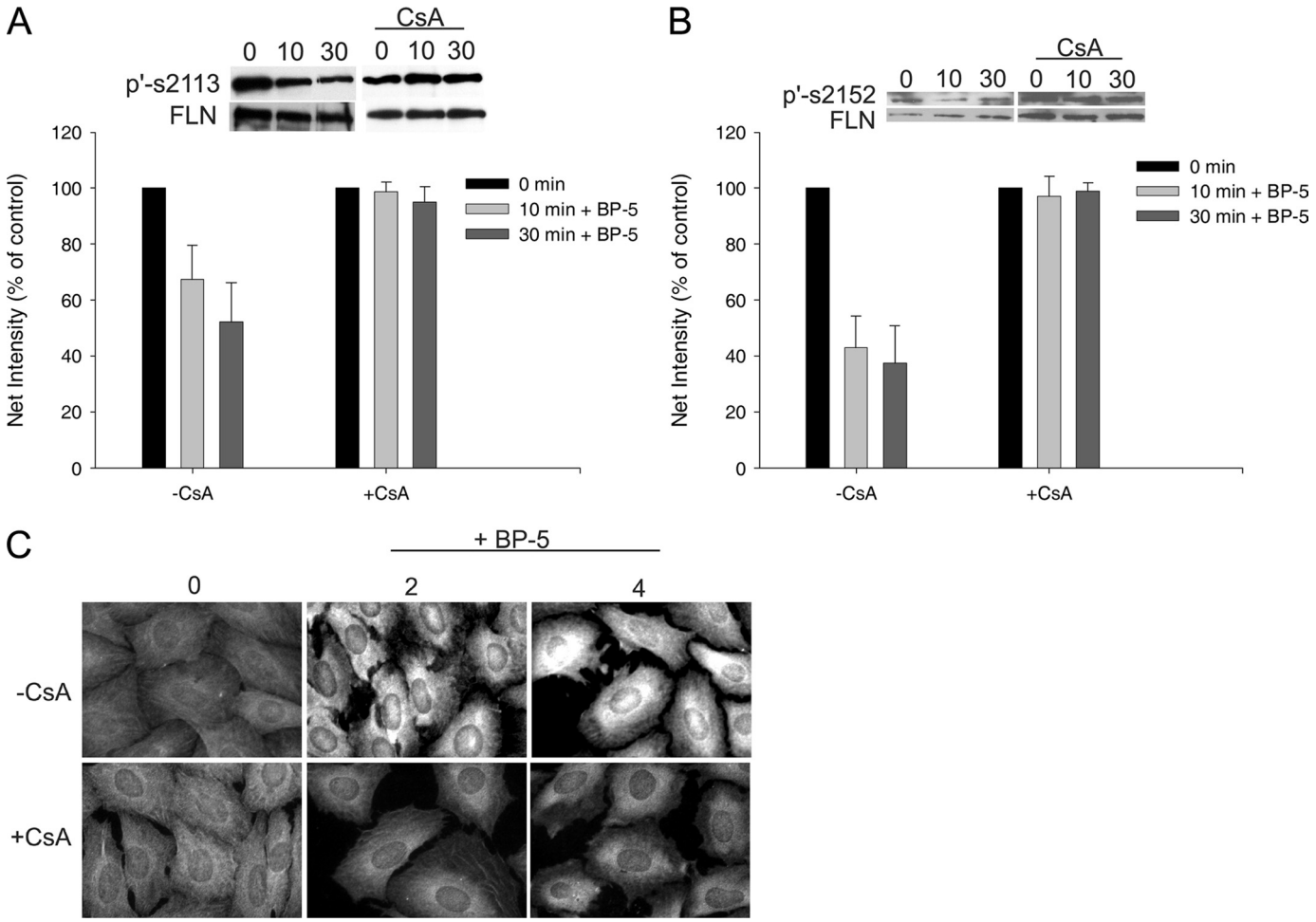

FIGURE 4. **IGFBP-5-induced FLNa dephosphorylation.** Cytosolic fractions isolated at the times indicated following the addition of IGFBP5$^{201-218}$ in control and cells pretreated with cyclosporine A were stained for intact FLNa and with antibodies to phospho-FLNa at serines 2113 (*A*) and 2152 (*B*). *Bar graphs* of the scanning densitometry results for p′FLN/total FLNa are shown. Although the total amount of FLNa detected did not significantly change within the first 30 min, the addition of IGFBP-5$^{201-218}$ led to a significant reduction in the amount of p′FLNa detected at both serine residues. To examine the role of phosphatases in mediating the loss of p′FLNa, cells were treated with cyclosporine A (*CsA*) for 30 min prior to the addition of IGFBP-5$^{201-218}$ and then stained for p′FLNa. Pretreatment with CsA prevented the dephosphorylation of FLNa at both Ser^{2113} (*A*) and Ser^{2152} (*B*). *C*, furthermore, the cells were stained with anti-FLNa before and at 2 and 4 h following the addition of IGFBP-5$^{201-218}$. Pretreatment with CsA prevented the increase in nuclear staining that followed treatment with IGFBP-5$^{201-218}$, which suggests that prevention of dephosphorylation prevents nuclear uptake of FLNa.

CCAATCTTGAGTTCATTC, and antisense primer, 5′-GTCTCAGTGCCAATGGGCTC). Controls included non-immune IgG.

Statistical Analysis—Group means were compared by one-way analysis of variance with subgroup testing by contrasts. $p < 0.05$ was considered significant.

RESULTS

IGFBP-5 Induces FLNa Reorganization—Previously we showed that IGFBP-5 induces MC to change phenotype and migrate (48). In association with these changes, the cytoskeleton reorganizes, and filopodial extensions occur. In the process of evaluating cytoskeletal reorganization with migration, we noted that FLNa reorganizes independently of actin in some subcellular locations. The present studies were undertaken to further understand the role of FLNa in mediating IGFBP-5 responses. MC were stained with polyclonal and region-specific anti-FLNa antibodies and analyzed by standard and confocal fluorescence microscopy. In quiescent MC, FLNa shows a diffuse cytoplasmic staining pattern (Fig. 1*A*, *panel A*). Following treatment with IGFBP-5, peripheral cytoplasmic staining becomes less distinct, and FLNa clusters around the nucleus (Fig. 1*A*, *panel B*), and in some cases, nuclei appear to have positive staining. As filopodial extensions begin to form, distinct coalesced areas of FLNa become detectable along the filopodial extensions coinciding with unique focal attachments (48). Eventually, FLNa is detected along the length of the filopodia (Fig. 1*A*, *panel D*).

Using antibodies specific for the C-terminal and N-terminal portions of FLNa, we noted that the intense perinuclear staining for FLNa was detected only with antibodies to C-terminal repeat 24 or to the C-terminal fragment containing repeats 16–24 (Fig. 1*B*). In contrast, polyclonal antibodies to full-length FLNa and to the N-terminal fragment showed association with actin and rearrangement into filopodia following treatment with IGFBP-5 (Fig. 1*B*). Nuclear staining was not observed with antibodies specific to the N terminus. These findings suggest that intact FLNa participates in cytoskeletal reorganization associated with IGFBP-5-mediated migration, and a C-termi-

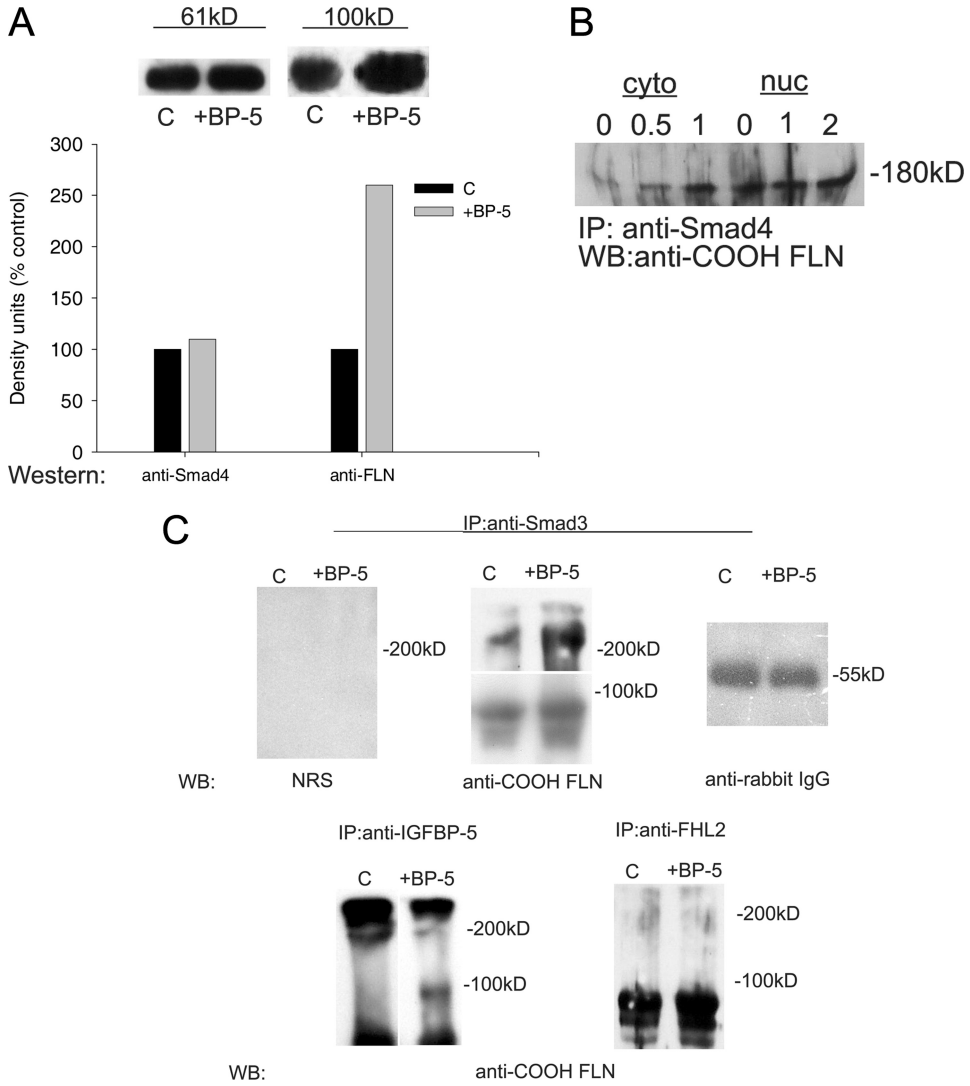

FIGURE 5. **IGFBP-5$^{201-218}$ induces increased binding of transcription factors to FLNa.** *A*, lysates collected from control and IGFBP-5$^{201-218}$-treated cells (after 4 h) were subjected to immunoprecipitation (*IP*) with either anti-Smad4 or anti-FLNa followed by Western blotting (*WB*) with anti-Smad4. The amount of Smad4 was equal; however, FLNa-bound Smad4 increased with IGFBP-5$^{201-218}$ treatment. *B*, cytoplasmic (*cyto*) and nuclear (*nuc*) extracts were subjected to immunoprecipitation with anti-Smad4 at the times shown, followed by Western blotting for FLNa. The amount of Smad4 bound to FLNa increased within 30 min of treatment with IGFBP-5$^{201-218}$, and an increase was detected in the nucleus by 1 h. *C*, immunoprecipitation of similar cell lysates showed that co-precipitation of FLNa with Smad3 and IGFBP-5 were also increased by treatment with IGFBP-5, whereas the content of FHL2 was unchanged.

nal fragment of FLNa translocates to the nucleus, which might participate in IGFBP-5-mediated activation of target gene transcription. Because we had previously shown that IGFBP-5-mediated migration required cell binding to laminin 421 and that IGFBP-5 is associated with increased mRNA expression for these laminin chains, we examined this relationship further (49).

Compartmental reorganization of FLNa has been examined following stimulation with various agents using differential detergent solubilization (33, 56). Using this approach, membrane, cytoskeletal, cytosolic, and nuclear fractions of control and IGFBP-5-treated cells were monitored for purity by staining for epidermal growth factor receptor (membrane), TFIIB (nuclear), α-actinin (cytoskeleton), and β-actin (cytosol) (33). Western blot of isolated fractions showed that in untreated cells intact FLNa is abundant in the membrane and cytoskeletal fractions, with less detected in the cytosolic fraction. Following treatment with IGFBP-5, intact FLNa was lost from the membrane, and fragments appeared in the cytosol (data not shown). Because these findings differed from previous reports in which FLNa shifts from the membrane to the cytosolic compartment and recycles back to the membrane following treatment of endothelial cells with bradykinin or H2O2 (33, 56) and because of the evidence described above that FLNa was translocated to the nucleus, additional studies were undertaken to define this unique response to IGFBP-5.

IGFBP-5 Induces Nuclear Translocation of FLNa—To further assess IGFBP-5-induced nuclear translocation of FLNa, the cells were stained with anti-C-FLNa repeats 16–24 and examined by confocal microscopy. Nuclear uptake of endogenous protein was confirmed by this method (Fig. 2*A*). Furthermore, the cells were transfected with GFP-C-FLNa$^{16-24}$ or an empty vector. IGFBP-5 induced nuclear accumulation of GFP-C-FLNa, but had no effect on nuclear accumulation of the empty vector (Fig. 2*B*). These data support the conclusion that IGFBP-5 induces nuclear uptake of FLNa. Although these studies confirm that the C-terminal fragment is sufficient for nuclear uptake, they do not exclude the possibility that intact FLNa is also present in the nucleus.

IGFBP-5 Induces Fragmentation and Nuclear Uptake of FLNa—To further confirm the development of FLNa fragmentation and establish the form of FLNa that translocates to the nucleus, nuclear and cytoplasmic fractions were processed and analyzed by Western blot. As seen in Fig. 3*A*, within 30 min of adding IGFBP-5, the amount of intact FLNa decreased in the cytoplasm, and fragments appeared. As time progressed, intact FLNa and fragments decreased in the cytoplasm, and the 100-kDa, C-terminal FLNa fragment increased in the nucleus. Previously we had confirmed that both intact IGFBP-5 and the amino acid 201–218 peptide that corresponds to the heparin-binding domain equally induce migration (48) and FLNa reorganization. Studies were undertaken here to establish that both also lead to degradation and nuclear uptake of FLNa. As shown in Fig. 3*B*,

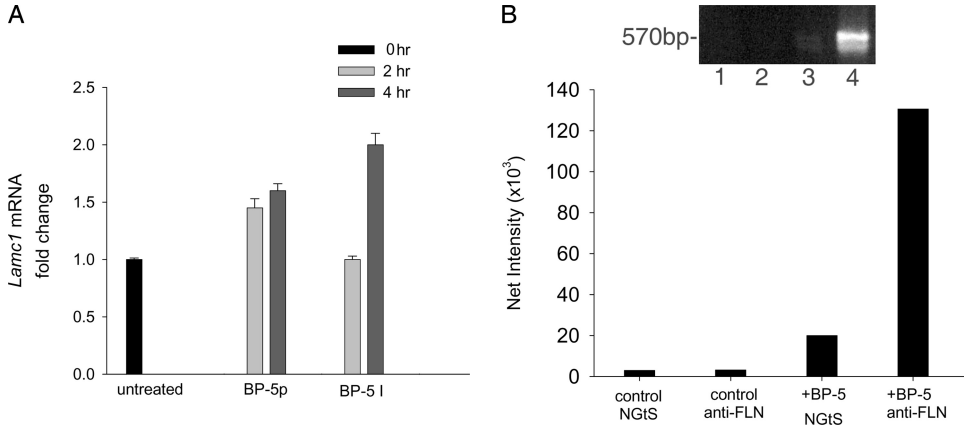

FIGURE 6. **Intact IGFBP-5 and IGFBP-5$^{201-218}$-mediated increase in *lamc1* mRNA is associated with FLN recruitment to the *lamc1* promoter.** *A*, effect of intact IGFBP-5 (*BP-5 I*) and IGFBP-5$^{201-218}$ (*BP-5p*) on *lamc1* mRNA. The results are expressed as fold change from the untreated control. The delayed and more robust response from intact IGFBP-5 suggests that cleavage may be required before it is taken up by the cell. *B*, ChIP assay shows that treatment with IGFBP-5$^{201-218}$ induces recruitment of FLNa to the *lamc1* gene. *lamc1* DNA is not detected in precipitates from normal goat serum (*NGtS*) (*lanes 2* and *4*), nor in the absence of treatment with IGFBP-5 (*lanes 1* and *2*). The *bar graph* reflects net intensity of the reverse transcription-PCR products shown above.

both intact and IGFBP-5$^{201-218}$ induced FLNa fragmentation and nuclear translocation.

IGFBP-5 Induces FLNa Dephosphorylation, Which Is Required for Nuclear Translocation—Phosphorylation of Ser^{2152} inhibits calpain-mediated cleavage of FLNa, whereas dephosphorylation of this residue leads to enhanced cleavage (57). To investigate the relationship of FLNa phosphorylation to cleavage and the response to IGFBP-5, cytosolic extracts of cells without and following treatment with IGFBP-5$^{201-218}$ were isolated, and Western blots were stained for FLNa and FLNa phosphorylated at Ser^{2152} or Ser^{2113}, both of which are adjacent to hinge 1 where cleavage occurs. FLNa is phosphorylated at both residues in quiescent MC, but the amount is markedly reduced following treatment with IGFBP-5 (Fig. 4, *A* and *B*). Changes are more apparent at Ser^{2113} than Ser^{2152}, which might reflect variations in antibody affinity for rat FLNa, or phosphorylation at Ser^{2113} may be more relevant to IGFBP-5-mediated effects. Treatment with cyclosporine A, a Ser/Thr phosphatase inhibitor, prevented the IGFBP-5-induced dephosphorylation at both serine residues (Fig. 4, *A* and *B*). Dephosphorylation of FLNa is consistent with enhanced sensitivity to calpain cleavage and the appearance of fragments as described above. Blockade of dephosphorylation significantly reduced nuclear translocation of FLNa in response to IGFBP-5 (Fig. 4*C*), which indicates that this effect is an important step in generation of a FLNa fragment with enhanced nuclear uptake. In summary, these data show that treatment with IGFBP-5 leads to dephosphorylation of FLNa, loss from the membrane, increased fragmentation, and increased nuclear translocation of a 100-kDa C-terminal fragment. For the most part, intact FLNa is reorganized along with the cytoskeleton as the phenotypes of cells change and they begin to migrate under stimulation with IGFBP-5.

IGFBP-5 Induces FLNa Binding and Nuclear Translocation of Transcription Factors—In prior studies of IGFBP-5-mediated migration, we reported that IGFBP-5 induces transcription of the laminin subunits ($\alpha4\beta2\gamma1$) contained in laminin 421, the laminin isoform that is required for IGFBP-5-mediated migration (49). Thus, we wondered whether nuclear translocation of FLNa participated in activation of laminin gene transcription through recruitment of transcription factors that regulate laminin gene transcription. Sasaki *et al.* (25) reported that FLNa binds Smads 2 and 5 and is required for transforming growth factor β-mediated Smad phosphorylation and transcriptional effects of transforming growth factor β. Previously we showed that *lamc1* (laminin $\gamma1$) transcription is activated by Smad3/4 (58). Based on these observations, we posited that these transcription factors would be recruited to the newly generated FLNa fragment following treatment with IGFBP-5. To examine this, cell lysates were subjected to immunoprecipitation with anti-Smad4 antibody followed by Western blotting for FLNa or vice versa. As shown in Fig. 5*A*, IGFBP-5 had no affect on the total cellular content of Smad4 but increased the amount of FLNa that was bound to Smad4. When similar experiments were conducted using both cytosolic and nuclear fractions, Smad4 was found bound to FLNa in the nuclear fraction (Fig. 5*B*), and the nuclear content increased with time following treatment with IGFBP-5. In similar experiments, the amount of Smad3 bound to FLNa was increased by IGFBP-5 (Fig. 5*C*). These studies do not establish whether or not Smad3 binds directly to FLNa or is recruited to the complex by binding to Smad4 (59). FLNa does not contain a putative nuclear localization sequence; however, the nuclear localization sequence in IGFBP-5 mediates its nuclear uptake (12), raising the possibility that IGFBP-5 participates in the nuclear import of the FLNa-based complex. As seen in Fig. 5*C*, treatment of cells with IGFBP-5 was associated with increased binding of IGFBP-5 to FLNa. FHL2 binds to IGFBP-5 (17), where it has been postulated to serve as a transcriptional co-activator; thus, we examined FHL2 binding as a possible partner in the FLNa complex generated by treatment with IGFBP-5. Although FHL2 was detected bound to the 100-kDa FLNa fragment in samples from untreated and IGFBP-5$^{201-218}$-treated cells; there was no significant change in amount (Fig. 5*C*). Because FHL2 was present, it may still function as a transcriptional modulator in the presence of activated transcription factors that are recruited to FLNa for transport to the nucleus following treatment with IGFBP5. These data demonstrate that transcription factors are recruited to the C-terminal fragment of FLNa and increase in the nucleus bound to FLNa. This is the first demonstration of the interaction of IGFBP-5 with FLNa both in the cytosol and nucleus of IGFBP-5-treated cells. Additional studies are required to determine whether IGFBP-5 is directly mediating FLNa import into the

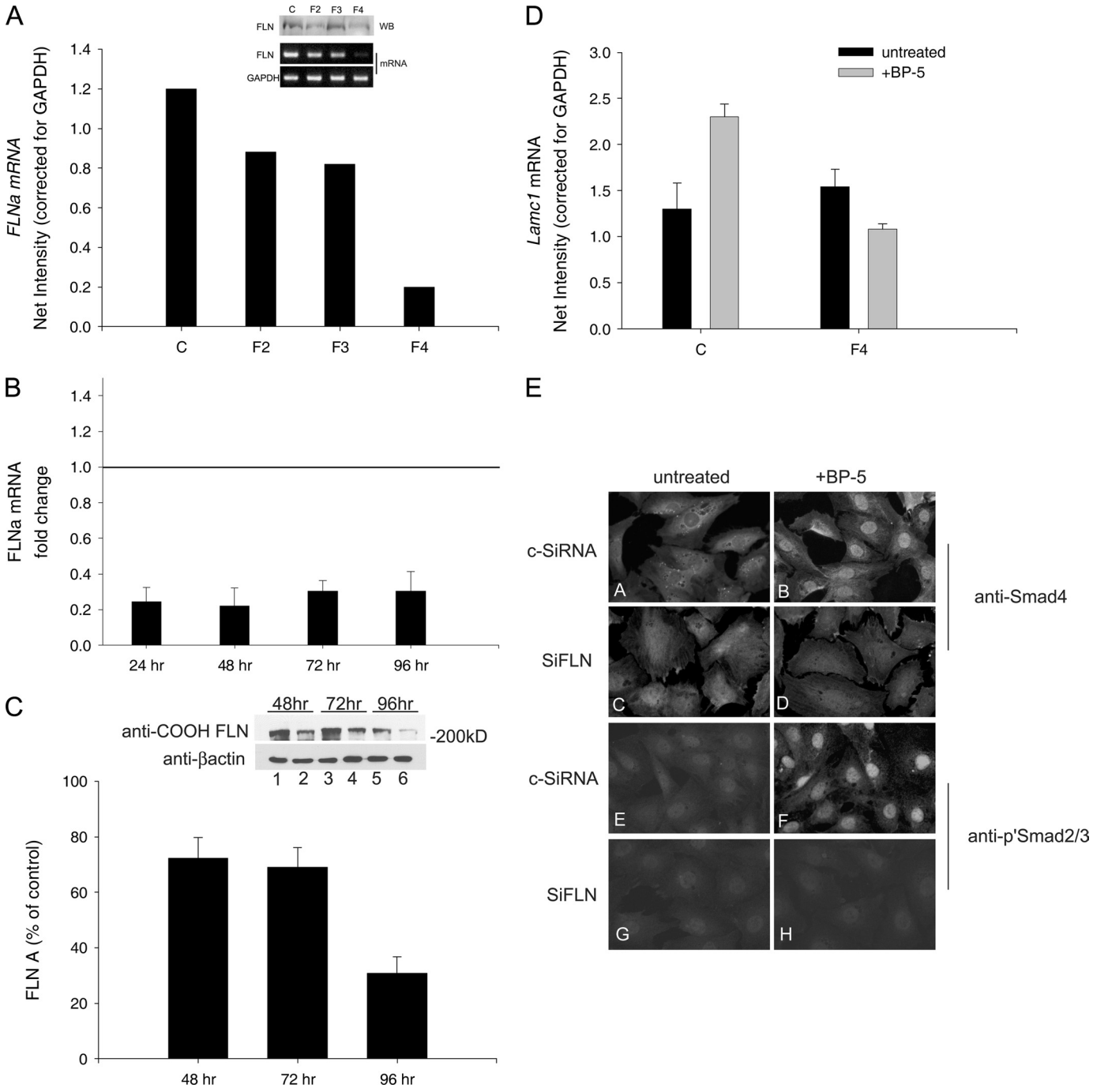

FIGURE 7. **siRNA-mediated knockdown of FLNa blocks IGFBP-5-induced *lamc1* transcription and nuclear translocation of Smad.** *A*, three separate FLNa siRNA sequences (F2, F3, and F4) were examined. Because F4 was the most effective, it was used in subsequent experiments. *B*, knockdown of FLNa mRNA with F4 persisted for 96 h ($n = 3$). *C*, the reduction in FLNa protein was maximal at 96 h ($n = 3$). *D*, in separate experiments using the time course defined above, FLNa knockdown with F4 blocks IGFBP-$5^{201-218}$-induced increases in *lamc1* mRNA as compared with the control siRNA ($n = 3$, $p < 0.05$, analysis of variance). *E*, as compared with cells transfected with a control siRNA (*c-SiRNA*), FLNa knockdown (*SiFLN*) was associated with loss of nuclear translocation of Smad4 and phospho-Smad2/3 that normally occurs following treatment with IGFBP-5.

nucleus or whether it has accessory effects on transcription (9, 13).

IGFBP-5 Induces lamc1 Transcription and FLNa Binding to the lamc1 Gene—Treatment of cells with intact IGFBP-5 or IGFBP-$5^{201-218}$ increases *lamc1* mRNA expression (Fig. 6*A*). As further evidence that FLNa is important to the IGFBP-5-mediated effects on *lamc1* transcription, ChIP assay revealed that IGFBP-5 treatment was associated with increased binding of FLNa to the *lamc1* gene (Fig. 6*B*). IGFBP-5 treatment was not associated with FLNa binding to genes not transcribed in MC (hemoglobin) or those unaffected by IGFBP-5 (*gapdh*) (data not shown). FLNa is not known to have a DNA-binding domain; thus, it is inferred that FLNa-bound transcription factors and possibly other molecules bound into the complex serve the direct DNA-binding function. To our knowledge, FLNa has not previously been reported to associate with promoters of expressed genes.

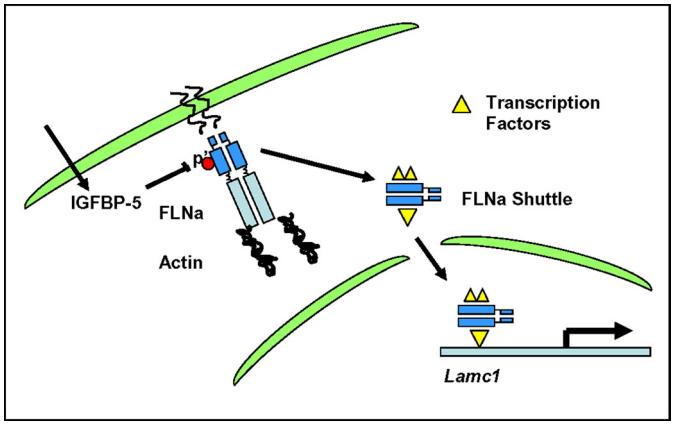

FIGURE 8. **Proposed schema.** IGFBP-5 induces dephosphorylation of FLNa, rendering it susceptible to calpain cleavage. The C-terminal FLNa fragment recruits transcription factors and other transcriptional modulators, and the FLNa-based shuttle is translocated to the nucleus. Within the nucleus, the shuttle modulates transcription of IGFBP-5 target genes.

FLNa Knockdown Prevents Nuclear Translocation of Smad3/4 and IGFBP-5-induced lamc1 Transcription—To confirm that FLNa was required for the effects of IGFBP-5 on *lamc1* transcription, several siRNA sequences were each tested a single time (Fig. 7*A*), and the most effective one (F4) was selected for use. siFLN (F4) markedly reduced FLNa mRNA levels for 96 h (Fig. 7*B*). Because the existing protein must be degraded before a reduction in the protein levels occurs, the fall in mRNA precedes the loss of protein as shown in Fig. 7*C*. Based on this time course, the effect of FLN knockdown on IGFBP-5-induced *lamc1* transcription was examined at 96 h after introduction of the siFLNa. IGFBP-5-mediated increases in *lamc1* mRNA occurred as expected in cells transfected with control siRNAs, but these effects were blocked in cells with FLNa knockdown (Fig. 7*D*). FLNa knockdown also prevented the nuclear uptake and accumulation of Smad4 and phospho-Smad2/3 (Fig. 7*E*), which indicates that recruitment of these transcription factors to the FLNa shuttle was important to their role in mediating IGFBP-5 effects on transcription.

DISCUSSION

Based on the above findings, IGFBP-5 leads to dephosphorylation and cleavage of FLNa. The C-FLNa fragment serves as a FLNa-based nuclear shuttle that recruits transcription factors, translocates to the nucleus, and activates transcription of IGFBP-5 target genes (Fig. 8). Several recent reviews have implicated intranuclear FLNa in mediating the link between signal transduction and target gene effects (60–62), yet few of these systems have been studied in detail. Berry *et al.* (47) showed in A7 cells that nuclear FLNa blocks FOXC1-mediated transcription. In the A7 melanoma cell line with stable transfection of FLNa, FLNa accumulates in the nucleus and is sequestered in untranscribed regions of chromatin. Through interaction with PBX1a and FLNa, FOXC1 was partitioned to a heterochromatic portion of the nucleus, thereby preventing its interaction with target genes. A role for FLNa-FOXC1 interaction in the expression of target genes in normal cells under physiological stimulation has not been established. The best studied example of the intranuclear effects of FLNa involves FLNa interaction with the androgen receptor (46). This interaction does not require androgen, but it is increased by androgens (63). Nuclear translocation of the androgen receptor requires FLNa (63), and it is thought to associate only with the C-terminal FLNa fragment, because full-length FLNa remains cytoplasmic (22). Fragmentation of FLNa and androgen receptor binding to the C-terminal FLNa fragment are required for proliferative responses to androgens in prostate cells (64). In some cases, association between FLNa and the androgen receptor has repressed the response of androgen target genes (22), yet the molecular configuration or possible association with other factors that is responsible for this effect has not been defined.

In conclusion, these studies demonstrate that IGFBP-5 induces laminin gene transcription through formation of a FLNa-based nuclear shuttle that binds IGFBP-5, recruits transcription factors, translocates to the nucleus, and binds to IGFBP-5 target genes. Loss of FLNa is associated with a loss of IGFBP-5-mediated induction of *lamc1* transcription, thereby establishing the functional significance of these findings. Previous studies have indicated that IGF-1-independent effects of IGFBP-5 are mediated through intranuclear effects on transcription (9, 13, 18), yet details of those effects have not been elucidated. Similarly, intranuclear effects of FLNa that modulate effects of transcription factors on target genes have not been thoroughly defined. This report enhances our understanding of the means by which IGFBP-5 changes gene expression and the role that FLNa plays in this response. There is growing interest in the role of IGFBPs in control of the response to insulin/IGF-1, independent effects to preserve Rb function (65), and its role as a tumor suppressor, thereby modifying the progression of cancer (66, 67). Although the significance is not known, the loss of FLNa has been associated with some cancers (46, 68). This study raises the possibility that the loss of FLNa would be expected to impair IGFBP-5 effects on target genes and thus might be associated with cancer progression. These data add to the growing literature demonstrating intranuclear effects of both IGFBP-5 and FLNa. Additional studies are needed to understand the breadth of stimuli that lead to formation of a FLNa-based shuttle, the types and mechanisms for recruitment of transcription factors, and the families of genes that are regulated by this process. Additional studies are also needed to understand the breadth of IGFBP-5 target genes, the role IGFBP-5 plays in nuclear import of the shuttle, and potential intranuclear transactivator/repressor functions. These studies also expand our knowledge of the critical roles that FLNa specifically and members of the cytoskeleton in general play in controlling cellular responses to perturbants of cell-matrix and cell-cell contacts, as well as soluble ligands such as growth factors and hormones.

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