Molecular cloning of actin filament-associated protein: a putative adaptor in stretch-induced Src activation

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The lung is exposed to physical forces derived from breathing, blood flow, and surface tension, which contribute to the regulation of lung structure, metabolism, and function (36). Fetal breathing movements, for example, are essential for lung growth, maturation, and development (10, 11, 15, 16). Using a mechanical strain device for organotypic cell cultures, we have demonstrated that a stretch regimen, which simulates the frequency, amplitude, and periodicity of normal fetal breathing movements (10), enhanced DNA synthesis and cell division of fetal rat lung cells (22). Mechanical stretch-induced mitogenic activity is related to the increased expression of growth factors and their receptors (18, 27, 45). Mechanical stretch also stimulated production of extracellular matrix molecules (44), such as glycosaminoglycans and proteoglycans (43) and fibronectin (29), as well as gene expression of surfactant proteins (30), a marker of fetal lung maturation. In addition, we have shown that a continuous mechanical stretch regimen, which simulates injurious mechanical ventilation in premature lungs, induced cell damage that is associated with increased cytokine production from fetal rat lung cells (28). These effects of mechanical stretch are mediated through specific signal transduction pathways (20, 23).

The phospholipase C-γ (PLC-γ)-protein kinase C (PKC) pathway is one of the major routes for transmission of stretch-induced signals for fetal lung cell proliferation (21, 24–26). A rapid activation of protein tyrosine kinases (PTKs) is an early response of cells to mechanical stretch and an upstream event of the PLC-γ-PKC pathway (21). One of the activated PTKs, identified as c-Src, is translocated from the cytosolic to the cytoskeletal fraction (21). This process appears to be mediated through actin filament-associated protein (AFAP) of 110 kDa (AFAP-110) (21).

The AFAP-110 gene was first cloned from v-Src-transformed chicken embryo cells (5). AFAP-110 protein is a tyrosine phosphorylation substrate for activated Src (5) and a Src SH2 and SH3 domain binding partner (5, 7, 8, 14, 35). Two putative SH3 binding motifs have been identified in the AFAP-110 sequence, one of which has been confirmed to be essential for AFAP-110 binding to activated Src (7). AFAP-110 also contains four putative SH2 binding motifs, of which one at the NH2 terminus and another at the carboxy terminus have been confirmed to have Src binding properties (8). Thus AFAP-110 and Src may form a stable complex that is dependent on both SH2 and SH3 interactions. Immunofluorescence staining revealed that AFAP-110 is colocated with actin filaments, the cortical actin matrix, and the leading edge of the cell (5). Deletion of the α-helix in the carboxy terminus of AFAP-110 hinders its association with actin filament and the cell membrane (33). These structural features suggest that AFAP-110 is likely to mediate Src trans-
location to the cytoskeletal fraction and participate in Src activation. However, because AFAP-110 was originally cloned from the chicken, to study the interaction between AFAP-110 and c-Src in mammalian cells it is essential to obtain detailed information about its mammalian homologs.

Recently, we have cloned and identified the AFAP-110 gene from a human lung epithelial line (hAFAP) (9). Because most experimental studies of stretch-induced signal transduction have been conducted in primary cultured fetal rat lung cells, herein we cloned this gene from fetal rat lungs and named it rAFAP. Several novel features are noted from the rAFAP gene and its product. We demonstrate that mechanical stretch induces the binding of rAFAP to c-Src and increases its tyrosine phosphorylation within minutes. With detailed sequence information of rAFAP protein, a novel mechanoreception model is proposed.

MATERIALS AND METHODS

**Molecular cloning.** Total RNA (5 μg) isolated from whole fetal rat lungs was used for RT-PCR. The RT-PCR primers (P110F) 5’ GCC ATG GAA GAG TTA ATA GT 3’ and (GSP2R) 5’ CCT CTT TGG TCT GTA GGC GCT TG 3’ were designed from a highly conserved region within chicken AFAP-110 and human hAFAP sequences around the translation starting site. The RT-PCR was performed for 25 cycles at an annealing temperature of 43°C with a programmable thermal cycler, PCT-100 (MJ Research, Watertown, MA). The RT-PCR products were then ligated into pCR 2.1 vector (Invitrogen, San Diego, CA) and sequenced. The Marathon cDNA amplification kit (Clontech, Palo Alto, CA) was used to extend the sequence toward the 3’-end. Briefly, polyA+ RNA was isolated from fetal rat lung tissue with an Oligotex purification system (Qiagen, Hilden, Germany). After first and second strand synthesis from 1 μg of poly A+ RNA template, cDNA was ligated to Marathon cDNA adaptors. The 3’-rapid amplification of cDNA ends (3’-RACE) reactions were performed with 5’-gene-specific primer (rat GSP2) 5’ CCG GGT AGA AGC AGA TGC CAA GCG GC 3’, designed within the PCR product, and 3’-nested adaptor primer (from the kit; 30 cycles, annealing temperature 58°C). The RACE fragments were then cloned into pCR 2.1 vector (Invitrogen) and sequenced.

DNA and amino acid sequences were analyzed with the sequence analysis software (Hitachi, San Francisco, CA) and advanced basic local alignment search tool (BLAST) search. The final sequence data (shown in Fig. 1) has been deposited into the GenBank database and assigned accession number 432958.

**Northern analysis.** Total RNA (15 μg/lane) extracted from different rat tissues was size-fractionated with 1% (wt/vol) agarose gels containing 1% (wt/vol) formaldehyde in 1× MOPS buffer, transferred to Hybond N+ nylon membranes, and immobilized by ultraviolet cross-linking. The 750-bp PCR product of rAFAP was used as probe and labeled with deoxyxycytidine 5'-[α-32P]triphosphate with a random priming kit (Amersham, Arlington Heights, IL). Prehybridization and hybridization were performed in 0.1% (wt/vol) BSA, 30% (vol/vol) deionized formamide, 0.35 M sodium phosphate, and 6% (wt/vol) sodium dodecyl sulfate (SDS) at 42°C. After hybridization, blots were washed in 150 mM sodium phosphate, 0.1% SDS, and/or 30 mM sodium phosphate and 0.1% SDS and exposed to Kodak XAR-5 film using DuPont intensifying screens at −70°C.

**Glutathione S-transferase fusion protein pull-down assay.** Briefly, vectors pGEX-2T (Pharmacia) containing Src-SH2, Src-SH3, or Src-SH2/Sh3 cDNA were prepared as previously described (9). Bacterium-encoded glutathione S-transferase (GST) fusion proteins were induced with isopropylthiogalactopyranoside and isolated from the soluble fraction of sonicated bacteria. We carried out AFAP binding experiments by incubating cell lysates with GST alone, GST-SH2, GST-SH3, or GST-SH2/Sh3 immobilized on glutathione-Sepharose beads. The precipitates were washed, solubilized in sample buffer, and resolved by SDS-PAGE (8% gels). The resolved

**Immunoprecipitation and immunoblotting.** Detailed procedures have been described previously (21, 29). Briefly, cells or tissues were lysed with radioimmunoprecipitation assay buffer, and protein content was determined by a standard protein assay (Bio-Rad Laboratories). Direct Western blotting, equal amounts of total protein were boiled with SDS sample buffer and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to nitrocellulose membranes. Nonspecific binding was blocked by incubation of membranes with 5% (wt/vol) nonfat milk in PBS for 60 min. Blots were incubated with a designated antibody at 1:1,000 dilution overnight at 4°C (monoclonal anti-Src, clone GD11; monoclonal anti-phosphotyrosine, clone 4G10; Upstate Biotechnology, Lake Placid, NY). The blots were then washed with PBS and incubated for 60 min at room temperature with horseradish peroxidase-conjugated goat anti-rabbit (1:3,000 dilution) or anti-mouse (1:20,000 dilution) IgG (both from Amersham, Oakville, Ontario, Canada). After being washed, blots were developed with an enhanced chemiluminescence detection kit (Amersham, Oakville). Autoradiographs were quantified using a densitometer (GS-690; Bio-Rad Laboratories).

For immunoprecipitation, cell lysates were adjusted to an equal amount of protein (1,000 μg) and equal volume. Designated antibody was added, and samples were incubated at 4°C overnight. Immune complexes were recovered by the addition of 50 μl of protein A Sepharose beads (20% wt/vol) for polyclonal antibodies or 100 μl of protein G Sepharose beads (10% wt/vol) for monoclonal antibodies. Samples were incubated for 1 h with gentle agitation at 4°C. The immunoprecipitates were washed with lysis buffer. We eluted the proteins by boiling the precipitates in sample buffer followed by SDS-PAGE and Western blotting as described above.
Fig. 1. The nucleotide and predicted amino acid sequences of rat actin filament-associated protein (rAFAP). The putative ATG start codon is presented as bp1. *Terminiation codon (at bp 2,190). The 3′/H11032 untranslated region ends at a putative poly A/H11001 tail.

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proteins were transferred to nitrocellulose membrane and probed for the presence of rAFAP using anti-AFAP antibody F1.

Fetal lung cell culture and mechanical strain. Isolation and culture of fetal rat lung epithelial cells and fibroblasts have been described previously (25, 27, 45). Briefly, pregnant Wistar rats (200–250 g; Charles River, St. Constant, Quebec, Canada) were killed on day 19 of gestation (term = 22 days). Fetal lungs were pooled from at least two litters. Fetal rat lungs were dissected out, minced, and resuspended in Hanks' balanced salt solution. The minced lung tissue was trypsinized [0.125% (wt/vol) trypsin and 0.4 mg/ml DNase], filtered, and centrifuged. Fibroblasts were collected by sequential adhesion, and epithelial cells were further purified by low-speed centrifugation (25, 27, 45).

Organotypic cultures of fetal lung cells were established as previously described (22, 29). Fetal rat lung cells were inoculated on 2 × 2 × 0.25-cm Gelfoam sponges at a density of 3 × 10⁶ cells/sponge and incubated overnight in DMEM + 10% (vol/vol) fetal bovine serum. The sponges were washed with serum-free DMEM, and the medium was replaced by DMEM. Cells were cultured in serum-free DMEM for 2 days with medium changed daily. The mechanical strain device used in these studies has been described in detail (19). It is

Fig. 2. Comparison and alignment among human actin filament-associated protein (hAFAP), rAFAP, and chicken AFAP-110 peptide sequences. The similarity among peptide sequences of hAFAP, rAFAP, and chicken AFAP-110 is compared by DNASIS (Hitachi). Overlapping amino acids are printed in the shaded areas. Two putative Src SH3 binding motifs, 4 Src SH2 binding motifs, and 1 leucine zipper motif are underlined sequentially.
composed of a computerized Bio-Stretch controller with a set of solenoids. A culture dish with a Gelfoam sponge was placed in front of each solenoid. One end of each sponge was glued to the bottom of the dish, and the other end was attached to a movable metal bar, which was wrapped and sealed in sterile plastic tubing. A magnetic force, generated through the solenoids, acted on the metal bar to apply strain to the organotypic cultures. Sponges were subjected to a 5\% elongation from their original length at 60 cycles/min for 1–15 min. The recoil properties of the sponge allow it to return to its normal length after each episode of elongation.

Statistical analysis. The stretch experiments were carried out with materials collected from at least three separate cell cultures. The values of means ± SE from separate experiments were analyzed by one-way analysis of variance, followed by Student-Newman-Keuls test with significance defined as \( P < 0.05 \).

RESULTS

Cloning of a novel gene from rat fetal lungs. The sequence of rAFAP was obtained in a two-step process. First, we designed a pair of PCR primers from a highly conserved translational starting region in hAFAP and chicken AFAP-110. RT-PCR was performed with total RNA extracted from fetal rat lungs. The obtained RT-PCR product was ligated into pCR 2.1 vector and sequenced. Second, to extend the sequence toward the poly A\(^+\) tail, we performed a 3′-RACE reaction with a 5′-gene-specific primer designed from the RT-PCR sequence and a 3′-nested adaptor primer. The obtained RACE fragments were subcloned and sequenced. All the sequences were confirmed by double-stranded sequencing. Through the overlap of the two fragments a complete open reading frame was obtained. The 3′-untranslated region (UTR) ended at a stretch of 17 adenosines, as a putative poly A\(^+\) tail (Fig. 1). This novel rat gene was named rAFAP; it contains a 2.2-kb open reading frame encoding 731 amino acids.

The novel gene is a rat homolog of hAFAP and chicken AFAP-110 genes. We compared the predicted peptide sequence of rAFAP with the GenBank database. The results of the comparison revealed that rAFAP shares high similarity with hAFAP (9), chicken AFAP-110 (5), and AFAP-120 (4). AFAP-120 is a variant form of AFAP-110 detected in the chicken brain, and it contains an additional internal insert sequence adjacent to the carboxy terminus (4). The rAFAP gene does not contain this additional sequence. The homology of either nucleotide or peptide sequence among rAFAP, hAFAP, and chicken AFAP-110 is \( \geq 80\% \). rAFAP shares identical functional motif and domain organization as described for chicken AFAP-110 protein (33) (Fig. 2). However, there is little similarity in the 3′-UTRs among the three AFAP homologs.

Fig. 3. Northern blotting analysis of rAFAP tissue distribution. A: total RNA extracted from various adult rat tissues was analyzed by Northern blotting. rAFAP is differentially expressed in various rat tissues. B: the ethidium bromide staining of the RNA gel is presented to show the quality of RNA isolated and relatively equal loading of RNAs. Blots are examples from 1 of 3 animals.

Fig. 4. Western blotting analysis of rAFAP proteins in various rat tissues and cells. A: tissue lysates were prepared from various adult rat tissues and analyzed by Western blotting. rAFAP protein is differentially expressed in various rat tissues. The blot is an example from 1 of 3 animals. B: lysates from fetal rat lung epithelial cells and fibroblasts were subjected to Western blotting. The expression level of rAFAP protein in the fibroblasts is higher than that in the epithelial cells. Each lane represents a separate sample.
Differential expression of rAFAP among various rat tissues. To determine the expression of rAFAP mRNA among various rat tissues, we performed Northern blotting with total RNA isolated from adult rats. The molecular size of rAFAP mRNA is ~4.4 kb (Fig. 3A). The steady-state mRNA levels of rAFAP are relatively higher in the brain, intestine, kidney, and lung compared with that of the heart, liver, and muscles (Fig. 3A). Ethidium bromide staining of the original gel is presented to show the quality of the isolated RNA as well as relatively equal loading (Fig. 3B).

Protein content of rAFAP in rat tissues was analyzed by Western blotting. The expression of rAFAP in the brain is remarkably higher than that of other organs we examined.

Expression levels were also high in the lung, intestine, and heart (Fig. 4A). To take a further look at the protein expression levels of rAFAP in different cell types, we isolated fetal rat lung epithelial and fibroblast cells. The protein expression of rAFAP in fetal rat lung epithelial cells is notably lower than that of the fibroblasts (Fig. 4B). The tissue-dependent expression of rAFAP mRNA and protein in the rat indicates that the function and regulation of its expression is specific to various organs. Interestingly, although the mRNA level of rAFAP in the brain is similar to several other organs, the amount of rAFAP protein present in the brain is much higher. On the other hand, the steady-state mRNA level of rAFAP in the kidney is higher than in several other organs, but its protein content is relatively low. This discrepancy between the expression of mRNA and the protein levels suggests that the expression of rAFAP could be regulated at the posttranscriptional level.

Intracellular colocalization of rAFAP with actin filaments. AFAP-110 is associated with actin stress fibers in normal chicken embryo cells (5), as is hAFAP in human lung epithelial cells (9). To examine the relationship between rAFAP and actin filaments, we double-stained fetal rat lung epithelial cells and fibroblasts with F1 antibody for rAFAP followed by FITC-conjugated anti-rabbit IgG and rhodamine-conjugated phalloidin for actin stress fibers. Immunofluorescent-stained cells were examined by confocal microscopy.

![Colocalization between rAFAP protein and actin filaments](image)

*Fig. 5.* Colocalization between rAFAP protein and actin filaments. Fetal rat lung epithelial cells (*left*) and fibroblasts (*right*) were costained with antibody F1 for rAFAP protein and rhodamine phalloidin for actin filaments. Cells were examined by confocal microscopy with different channels. *Top:* actin filament staining; *middle:* rAFAP protein staining; and *bottom:* overlapping between actin filaments and rAFAP protein. Scale bars = 50 μm for epithelial cells and 10 μm for fibroblasts.
The distribution of rAFAP in both cell types is very well associated with actin filaments (Fig. 5).

Mechanical stretch induces rapid tyrosine phosphorylation of rAFAP and its association with c-Src. In our previous study, we noted that mechanical stretch of fetal rat lung cells induced association between rAFAP and c-Src and activation of c-Src within the first 15 min (21). In fact, other signaling events also occurred during this period (21, 24, 26). To determine whether stretch-induced rAFAP/c-Src association is an early signaling response, we subjected fetal rat lung cells in organotypic culture to stretch for 1, 5, and 15 min. The collected cell lysates were immunoprecipitated with antibodies against phosphorytosine or c-Src protein; precipitates were then resolved by SDS-PAGE followed by Western blotting with anti-AFAP antibody. Mechanical stretch induced a rapid coimmunoprecipitation of rAFAP protein with c-Src within the first few minutes (Fig. 6A), which is associated with a rapid tyrosine phosphorylation of rAFAP (Fig. 6B). The time courses of these two events are very similar (Fig. 6), suggesting that stretch-induced tyrosine phosphorylation of rAFAP and its association with c-Src may be mediated through an autoregulatory mechanism.

GST-fusion protein pull-down assays utilizing cell lysates from chicken embryo cells (5) demonstrate high binding efficiency of AFAP-110 to Src GST-SH3/SH2 domain. Furthermore, it has been shown that AFAP-110 from normal chicken embryo cells binds to Src GST-SH3 fusion protein with higher affinity than to the Src GST-SH2 domain, whereas in v-Src-transformed cell extracts the binding affinity of AFAP-110 to GST-SH2 fusion protein is higher than that of GST-SH3 protein (5). We examined whether mechanical stretch-induced c-Src activation can affect the binding affinity and preference between rAFAP and c-Src in fetal rat lung cells. Similar to normal chicken embryo cells, in fetal rat lung tissue, the binding affinity of rAFAP protein to Src GST SH2/SH3 fusion protein is much higher than that to GST fusion proteins containing Src SH3 or Src SH2 domain alone (Fig. 7A). This suggests that the binding of rAFAP to c-Src requires the structural integrity of both SH3 and SH2 domains of c-Src. In our stretch regimen we observed that the binding pattern of rAFAP to Src homology domains is highest when both SH2/SH3 domains are present. Less efficient binding is seen to Src SH3 alone; however, this binding pattern is still higher than that seen for the SH2 domain. (Fig. 7B). Therefore, although mechanical stretch can activate c-Src (21) and increase tyrosine phosphorylation of rAFAP, the binding preference of c-Src to rAFAP protein is similar to that in static cultured cells, where rAFAP preferentially binds to Src SH3 domain, and contradictory to that observed in v-Src transformed cells (5). Mechanical stretch slightly increased the binding of rAFAP protein to Src SH2/SH3 and Src SH2 fusion proteins but did not reach statistical significance.

DISCUSSION

The novel gene is a rat homolog of AFAP-110 gene. In the present study, a novel gene was cloned from fetal rat lung tissues. A search of the GenBank database with the BLAST program revealed that the coding region of this novel gene has little similarity with any known genes except for that of chicken AFAP-110, AFAP-120, and hAFAP. The identity of this gene as a rat homolog of chicken AFAP-110 is ensured by very high sequence similarity, biochemical features (such as

![Fig. 6. Mechanical stretch induces rapid tyrosine phosphorylation of rAFAP protein and its association with c-Src in fetal rat lung cells. Fetal rat lung cells were starved from fetal bovine serum overnight and then subjected to mechanical stretch. Cell lysates were immunoprecipitated with monoclonal anti-Src antibody, mAb GD11 (IP: α-Src, left), or monoclonal antiphosphotyrosine antibody, 4G10 (IP: α-pTyr, right). Immunoprecipitates were resolved by gel electrophoresis, and the blot was analyzed with polyclonal anti-AFAP antibody, F1. Blots from 3 separate experiments were quantified using a densitometer. The obtained optical density values were normalized with a nonstretched control (0 min). *P < 0.05 compared with 0 and 1 min of stretch.]

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tyrosine phosphorylation and binding with c-Src), and its intracellular colocalization with actin filaments.

The different 3'-UTRs of these genes are interesting. It is known that 3'-UTR is involved in the regulation of mRNA translation and stability (1, 17). Therefore, although the peptide sequence is conserved, the regulatory mechanisms of its translation and mRNA stability may be different. In the present study, differential expression of rAFAP was found at both the mRNA and protein levels, which suggests posttranscriptional regulations of the rAFAP gene. Knowing the genetic information of rAFAP gene in rats, we will be able to further study its expression, translation, and other features with animal models or primary cultured cells.

In both fetal rat lung epithelial cells and fibroblasts, the distribution of rAFAP is closely associated with actin filaments. This could be an important feature that allows for the transmission of a physical force from cytoskeleton to other signal transduction proteins, through AFAP as an adaptor. Extracellular matrix-integrin-cytoskeleton interactions are one of the most commonly studied pathways for mechanotransduction (13). The transmembrane integrins, cytoskeletal filaments, and nuclear scaffolds can be considered as a “hard-wired” network (12, 13). Mechanical loads are transmitted across the cell surface and into the cell by these physically interconnected structures. The carboxy terminus of AFAP-110 is both necessary and sufficient for actin filament association (34). In its amino terminus, AFAP-110 also contains several Src-binding motifs (5, 7, 8). These structural features are also found in rAFAP and hAFAP proteins (Fig. 2). Therefore, AFAP protein could function as an adaptor molecule to link c-Src to actin filaments.

Protein-protein interaction, a novel mechanism of mechanoreception. In addition to fetal rat lung cells, mechanical stretch also activated c-Src in human umbilical vein endothelial cells (HUVECs) (31) and in 3Y1 fibroblasts (37). Pressure overload induced c-Src activation in the rat heart (6). Shear stress stimulated
c-Src activation in HUVECs (32, 39, 47). Therefore, c-Src activation appears to be a common phenomenon in different cell types responding to various mechanical stimuli.

On the basis of crystallographic structure studies (38, 42, 46), it has been suggested that the kinase activity of c-Src is maintained at a low basal level by two major intramolecular interactions. One is the binding of its SH3 domain to the linker between the SH2 domain and the kinase domain, and the other is the binding of its SH2 domain to the phosphorylated tyrosine residue 527 in its carboxy-terminal tail (38, 42, 46). Interruption of these intramolecular interactions with high-affinity ligands for either the Src SH2 domain or the SH3 domain may activate the enzyme. We have recently shown that both hAFAP and AFAP-110 can activate c-Src when coexpressed with c-Src in Cos-7 cells and that purified recombinant AFAP-110 protein activated c-Src directly in vitro (9). We also have evidence that this function of AFAP protein is mediated through its binding to Src SH2 or SH3 domain (9). In the present study, we found that the initiation of mechanical stretch increased the association of rAFAP with c-Src within minutes and that the tyrosine phosphorylation of rAFAP also rapidly increased. The rapid binding between rAFAP and c-Src could be critical for c-Src activation by rAFAP. Using the GST fusion protein pull-down assay, we showed that although mechanical stretch activated c-Src, the binding of rAFAP to c-Src is still mainly through the Src SH3 domain. This may represent the transient activation of c-Src under physiological conditions. In contrast, transformation of cells with v-Src leads to persistent elevation of Src PTK activity, which significantly increases the tyrosine phosphorylation of AFAP-110 and many other Src substrates. Therefore, the binding of v-Src to AFAP-110 was mainly through Src SH2 domain (5).

The molecular structure of rAFAP protein and the fact that mechanical stretch rapidly increased rAFAP/c-Src binding imply that mechanical stretch-derived cytoskeletal deformation may increase the physical contact between rAFAP protein and c-Src. The high-affinity Src SH2/SH3 binding motifs of rAFAP may competitively bind to c-Src and activate it. Activated c-Src may increase the tyrosine phosphorylation of AFAP protein and further increase its ability to bind to c-Src. We suspect that physical force can be transmitted along the stress fibers to rAFAP; the latter then can competitively bind to and activate c-Src. Activated c-Src may send signal to other proteins by increasing their tyrosine phosphorylation (Fig. 8).

Although it has been shown that many intracellular signal transduction pathways can be activated by physical forces (2, 3, 13, 23, 40, 41), how cells sense these physical forces and convert them into biochemical reactions of signaling events is unknown. Herein, we propose that mechanical stretch-induced cytoskeleton deformation might activate signal transduction via protein-protein interactions. The interaction between rAFAP and c-Src could serve as an example; other signal transduction mechanisms could be initiated similarly. With the molecular cloning of AFAP genes from mammalian cells, we will be able to examine this hypothesis further.

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