Activation of pp60^{src} is critical for stretch-induced orienting response in fibroblasts

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SUMMARY

When subjected to uni-axial cyclic stretch (120% in length, 1 Hz), fibroblasts (3Y1) aligned perpendicular to the stretch axis in a couple of hours. Concomitantly with this orienting response, protein tyrosine phosphorylation of cellular proteins (molecular masses of approximately 70 kDa and 120-130 kDa) increased and peaked at 30 minutes. Immuno-precipitation experiments revealed that paxillin, pp125^{FAK}, and pp130^{CAS} were included in the 70 kDa, and 120-130 kDa bands, respectively. Treatment of the cells with herbimycin A, a tyrosine kinase inhibitor, suppressed the stretch induced tyrosine phosphorylation and the orienting response suggesting that certain tyrosine kinases are activated by stretch. We focused on pp60^{src}, the most abundant tyrosine kinase in fibroblasts. The kinase activity of pp60^{src} increased and peaked at 20 minutes after the onset of cyclic stretch. Treatment of the cells with an anti-sense S-oligodeoxynucleotide (S-ODN) against pp60^{src}, but not the sense S-ODN, inhibited the stretch induced tyrosine phosphorylation and the orienting response. To further confirm the involvement of pp60^{src}, we performed the same sets of experiments using c-src-transformed 3Y1 (c-src-3Y1) fibroblasts. Cyclic stretch induced a similar orienting response in c-src-3Y1 to that in wild-type 3Y1, but with a significantly faster rate. The time course of the stretch-induced tyrosine phosphorylation was also much faster in c-src-3Y1 than in 3Y1 fibroblasts. These results strongly suggest that cyclic stretch induces the activation of pp60^{src} and that pp60^{src} is indispensable for the tyrosine phosphorylation of pp130^{CAS}, pp125^{FAK} and paxillin followed by the orienting response in 3Y1 fibroblasts.

Key words: Uni-axial cyclic stretch, Protein tyrosine phosphorylation, c-src transformed 3Y1 fibroblast, Ca^{2+}, Gd^3

INTRODUCTION

All tissues in the body are subjected to physical forces originating either from themselves or the environment. In response to these forces, cells exhibit a variety of responses including increased gene expression, protein synthesis, and morphogenesis.

As for fibroblasts, it is known that their shape is dramatically altered in response to mechanical stimulation. Arterial fibroblasts subjected to uni-axial cyclic stretch aligned perpendicularly to the stretch axis (Kanda and Matsuda, 1993). In ligamentous fibroblasts subjected to repetitive stretch, they aligned perpendicularly to the deformation axis of the substrate (Desrosiers et al., 1995). However, signaling mechanisms as well as mechanotransduction processes are largely unknown in mechanically induced shape change in fibroblasts. Similar stretch-induced morphological changes were also observed in different cell types (Dartsch and Haemmerle, 1986; Terracio et al., 1988; Hamasaki et al., 1995) and data on mechanotransduction processes have been accumulated recently. We have been particularly interested in the morphological changes in response to uni-axial cyclic stretch in vascular endothelial cells. A previous study from our

laboratory in human vascular endothelial cells (HUVECs) indicated that tyrosine phosphorylation of focal adhesion proteins is critical for stretch induced morphological changes such as orientation and elongation perpendicular to the stretch axis (Naruse et al., 1998a). Treatment with tyrosine kinase inhibitors, such as genistein and herbimycin A, inhibited the stretch induced morphological changes. Moreover, treatment with an antisense S-oligodeoxynucleotide against pp125^{FAK}, which down-regulated the expression of the protein, also suppressed the morphological changes (Naruse et al., 1998b).

These results prompted us to further investigate whether a similar signaling mechanism in HUVECs is involved in the mechanically induced morphological changes in fibroblasts. Compared to HUVECs, fibroblasts have advantages for investigating the intracellular signaling cascade because they have a good proliferation rate, and are suitable for molecular biology because of the availability of transfectants.

The aim of this study was to explore the signaling events downstream of the initial mechanoreception on the plasma membrane of fibroblasts, with the main emphasis on the role of tyrosine phosphorylation. Furthermore, to clarify the role of the tyrosine kinase pp60^{src} in the stretch induced orienting response and protein tyrosine phosphorylation, the alteration

in morphology and protein tyrosine phosphorylation were analyzed in 3Y1 fibroblasts treated with an antisense or sense S-ODN against pp60^{src} expression.

MATERIALS AND METHODS

Cell culture

Rat 3Y1 fibroblasts (Kimura et al., 1975) and c-src-transformed 3Y1 (c-src-3Y1) fibroblasts were maintained in D-MEM (Dulbecco's minimum essential medium) supplemented with 10% fetal calf serum and 100 units/ml penicillin G sodium, 100 µg/ml streptomycin sulfate and 250 µg/mg amphotericin B. Cells were incubated in a tissue culture incubator at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Stretch apparatus

The cells were stretched as previously described (Naruse et al., 1998a,b; Kato et al., 1998; Sokabe et al., 1997; Suzuki et al., 1997). Briefly, cells were removed from the dish with 0.01% EDTA-0.02% trypsin and transferred onto a silicon chamber coated with 50 µg/ml fibronectin. One end of the chamber is firmly attached to a fixed frame, while the other end is held on a movable frame. The movable frame is connected to a motor driven shaft. The amplitude and the frequency of stretch were controlled by a programmable microcomputer. The silicon membrane was uniformly stretched over the whole membrane area and lateral thinning did not exceed 1% at 20% stretch.

Analysis of cell morphology

The morphology of cells on the silicon chamber was analyzed in photographs of phase contrast images obtained through a ×10 objective (Olympus, Tokyo Japan). The photographs were digitized by a film digitizer (Nikon Cool Scan, Tokyo Japan) and the captured images were analyzed using an image analysis program (Mocha Jandel Scientific) running on Windows.

Immuno-blotting

After the application of cyclic stretch, the cells were washed with ice-cold phosphate-buffered saline (PBS: 137 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 7.40) containing 0.5 mM Na_3VO_4 and were lysed with a sample buffer (62 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol, 0.003% Bromophenol Blue, 5% glycerol). Total cell lysate from
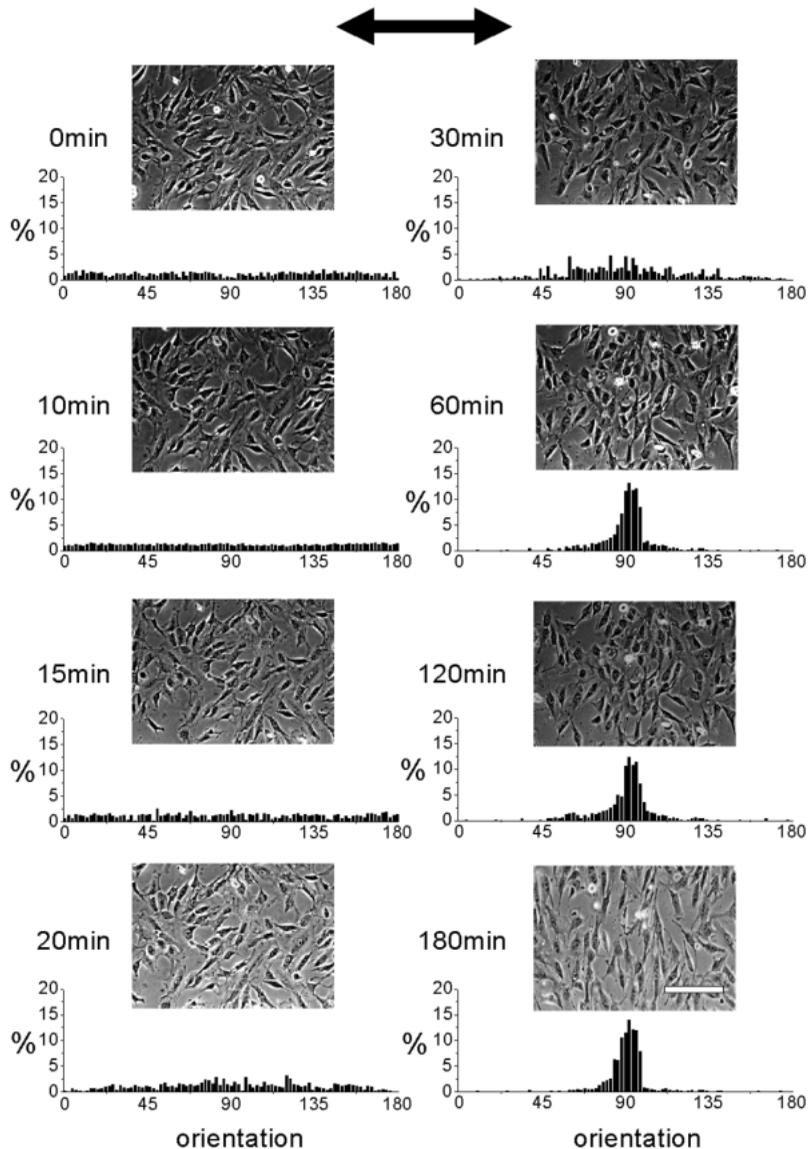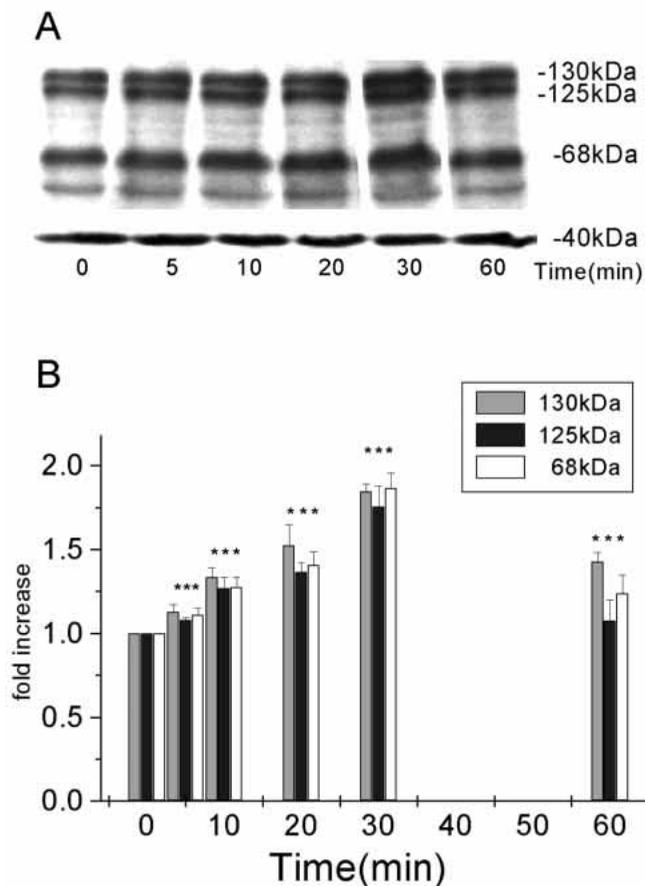

Fig. 1. Time course of the orienting response to uni-axial cyclic stretch. Sets of phase contrast images show the orienting response of 3Y1 fibroblasts to stretch for the times indicated. The double-headed arrow indicates the cyclic stretch axis. The cells began to orient perpendicularly to the stretch axis and the orientation of the vast majority of the cells is close to 90° with respect to the stretch axis. All the images were taken from the same place in the same silicon chamber. More than 600 cells from six independent experiments make up 100%. Bar, 50 µm.

Fig. 2. Time course of protein tyrosine phosphorylation of a cell lysate from 3Y1 fibroblasts during cyclic stretch. (A) Upper: immuno-blot probed with anti-phosphotyrosine monoclonal antibody. Cells were cyclically stretched for the times shown, extracted, and immuno-blotted as described in the text. The levels of tyrosine phosphorylation of broad bands of 130 kDa, 125 kDa, and 68 kDa were increased in response to cyclic stretch. Lower: immuno-blot probed with anti-actin monoclonal antibody shows that an equal amount of protein was loaded in each lane. The data are representative from five independent experiments. (B) Time course of protein tyrosine phosphorylation of broad bands of 120 kDa-140 kDa, and 70 kDa. Data are presented as fold increase of the static control. The level of the tyrosine phosphorylated proteins peaked at 30 minutes. Data are expressed as mean ± s.e. for six independent experiments. Asterisks denote those values that are significantly different (*$P<0.05$) from control (0 minutes).

Antisense S-ODN treatment

The cells were removed with 0.01% EDTA, 0.02% trypsin and were suspended at a density of 1×10^6 cells/ml in PBS. 100 ng of S-ODN was added to 400 μl of cell suspension and the suspension was transferred to an electroporation chamber, and was square electroporated at 75 V, 40 milliseconds, 1 pulse, using an Electro Square Porator-T820 (BTX Inc., San Diego, CA). Then the S-ODN treated cells were transferred into a 4 cm² silicon chamber, and were incubated for 24 hours. The anti-sense sequence against chicken pp60src (5′-GTC GGG GGC TGC TGT CTT-3′) and the sense sequence (3′-CAG CCC CCG ACG ACA GAA-5′) were designed using Genetyx (Softwear Development Company, Tokyo, Japan) and were synthesized by Rikaken Co., Ltd, Nagoya Japan.

Materials

Mouse anti-pp130[CAS] monoclonal antibody, mouse anti-pp125[FAK] monoclonal antibody, mouse anti-paxillin monoclonal antibody, and PY20 were purchased from Transduction Laboratories (Lexington, KY). Mouse anti-pp60src monoclonal antibody was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Rabbit anti-mouse IgG and goat anti-rabbit IgG were obtained from Sigma (St Louis, MO). Herbimycin A were purchased from Wako (Tokyo, Japan). Gadolinium (Gd^{3+}) chloride hexahydrate was purchased from Aldrich Chem. Co. Human plasma fibronectin was purified according to the method of Regnault et al. (1988). Other chemicals used were of special grade.

RESULTS

Cyclic stretch induced orienting response and protein tyrosine phosphorylation in 3Y1 fibroblasts

Application of sinusoidal cyclic stretch to the cells induced significant morphological changes particularly in their orientation. Without cyclic stretch, the cells showed no particular orientation (Fig. 1, 0 minutes). The cells began to orient perpendicularly to the stretch axis and the percentage of oriented cells increased with time. After 180 minutes, most of the cells aligned almost perfectly transversely across the stretch axis. A series of histograms in Fig. 1 show a time course of the distribution of cell orientation. Without stretch, cells showed no changes in their morphology at least for 3 hours. In contrast, the cells under cyclic stretch showed a clear indication of alignment, around 90° at 60 minutes after the onset of stretch.

To investigate the involvement of protein tyrosine phosphorylation in the stretch induced orienting response, tyrosine phosphorylation of cellular proteins was examined by

equivalent cell numbers was separated by 12.5% SDS-PAGE (Laemmli, 1970). The proteins were transferred electrophoretically onto polyvinylidene fluoride (PVDF) membranes, (Immobile P, Millipore Crop, Bedford, MA). The PVDF membranes were blocked with 3% ovalbumin in PBS, the membranes were subsequently probed with monoclonal antibodies in PBS containing 3% ovoalbumin for 1 hour. The antibody-antigen complexes were detected by using horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000 dilution). Immuno-reactivity was determined using the ECL chemi-luminescence reaction (Amersham Corp., Arlington Heights, IL), according to the instructions provided by the manufacturer and was measured using densitometry.

Immuno-precipitation and in vitro kinase assay

For immuno-precipitation, the cells were washed with ice-cold PBS and were lysed by A buffer (10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, pH 7.40). Cell lysates were centrifuged twice at 16,000 *g* for 20 minutes at 4°C and 50 μl of Protein A-Sepharose anti-mouse IgG were added to pre-clear the lysate. 1 μl of monoclonal anti-pp60src were added and the samples were incubated at 4°C, for 1 hour. Then 30 μl of Protein A-Sepharose anti-mouse IgG were added, and the samples were incubated at 4°C for 1 hour with gentle agitation. The resulting immuno-precipitates were washed five times with A buffer, and three times with kinase buffer (10 mM Tris-HCl, pH 7.40, 5 mM MnCl₂). The kinase reaction was started with the addition of 5 μCi of [γ-32P]ATP, 1 μM ATP and 2.5 μg of enolase. The reaction was terminated after 10 minutes with the addition of an equal volume of SDS-sample buffer and the samples were boiled for 5 minutes. The samples were separated on 12.5% SDS-PAGE and the autoradiogram was analyzed by BAS-1500 radioactivity imaging system (Fuji Photo Film, Tokyo, Japan).
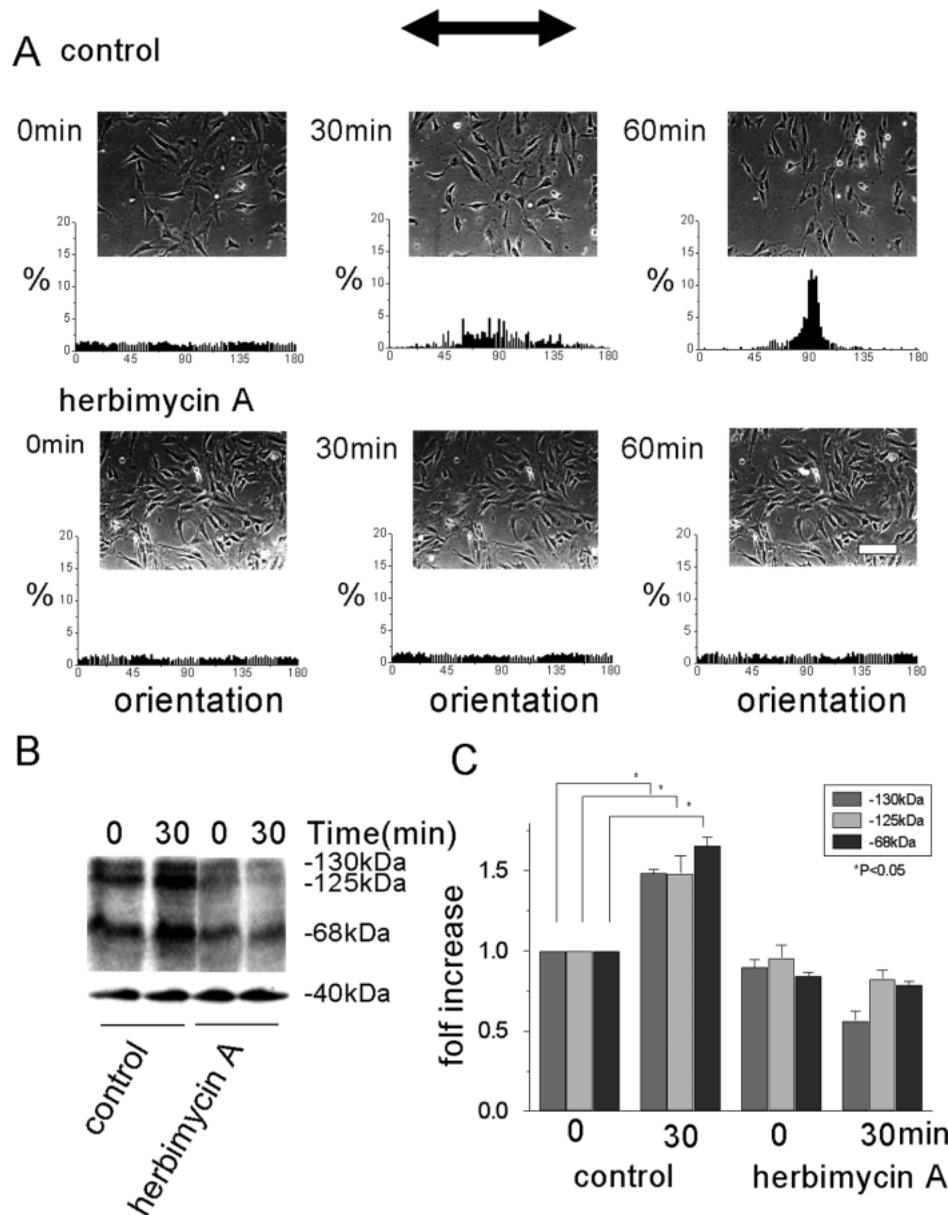
Fig. 3. Effects of herbimycin A on cyclic stretch induced protein tyrosine phosphorylation. (A) Phase contrast images of 3Y1 fibroblasts. The cells were cyclically stretched for the indicated times. The double-headed arrow indicates the cyclic stretch axis. Upper: control cells. Lower: preincubated with 3 μM herbimycin A for 10 hours. (B) Immuno-blot probed with anti-phosphotyrosine monoclonal antibody. The level of tyrosine phosphorylation increased in response to cyclic stretch for 30 minutes, however, when the cells were preincubated with 3 μM herbimycin A for 10 hours, cyclic stretch induced little change in the tyrosine phosphorylation. (C) The level of protein tyrosine phosphorylation was detected by using ECL, quantified by densitometry, and is presented as fold increase of the static control. Cyclic stretch induced 1.46-, and 1.72-fold increases in protein tyrosine phosphorylation for 130, 125 kDa, and for 68 kDa proteins, respectively. The increases were significantly inhibited by the preincubation with 3 μM herbimycin A.

immuno-blotting using anti-phosphotyrosine monoclonal antibody. In response to cyclic stretch, a time dependent tyrosine phosphorylation of multiple proteins was observed (Fig. 2A). Those proteins included broad bands of 130 kDa, 125 kDa, and 68 kDa. The elevation of the level of tyrosine phosphorylation was detected from 5 minutes after the onset of cyclic stretch, peaked at 30 minutes and declined gradually thereafter (Fig. 2B). Even without cyclic stretch, a certain level of protein tyrosine phosphorylation was observed, probably because integrin-mediated protein tyrosine phosphorylation occurred as the cells adhered to the extracellular matrix (Burridge et al., 1992).

To ensure if the anti-phosphotyrosine monoclonal antibody used here specifically recognized the tyrosine phosphorylated proteins, immuno-blotting was performed in the presence of an excess amount of phosphorylated tyrosine phosphorylated serine and phosphorylated threonine. Phosphorylated tyrosine inhibited the band, which was detected by anti-

phosphotyrosine monoclonal antibody to tyrosine phosphorylated proteins, while phosphoserine and phosphothreonine had no effect on immuno-detection patterns (data not shown). As herbimycin A has been demonstrated to be an effective inhibitor of tyrosine kinase (Satoh et al., 1992), we examined the effect of herbimycin A on the stretch induced tyrosine phosphorylation. When the cells were pre-incubated with 3 M herbimycin A for 10 hours to block tyrosine kinase, the cells did not show any orienting response even 60 minutes after the onset of cyclic stretch (Fig. 3A) and the increase in the amount of tyrosine phosphorylated proteins was not significant (Fig. 3B,C). Those results strongly suggest that protein tyrosine phosphorylation is necessary for the stretch induced orienting response in fibroblasts.

Paxillin, pp125[FAK], and pp130[CAS] were included in tyrosine phosphorylated proteins

Since major tyrosine phosphorylated proteins were detected at

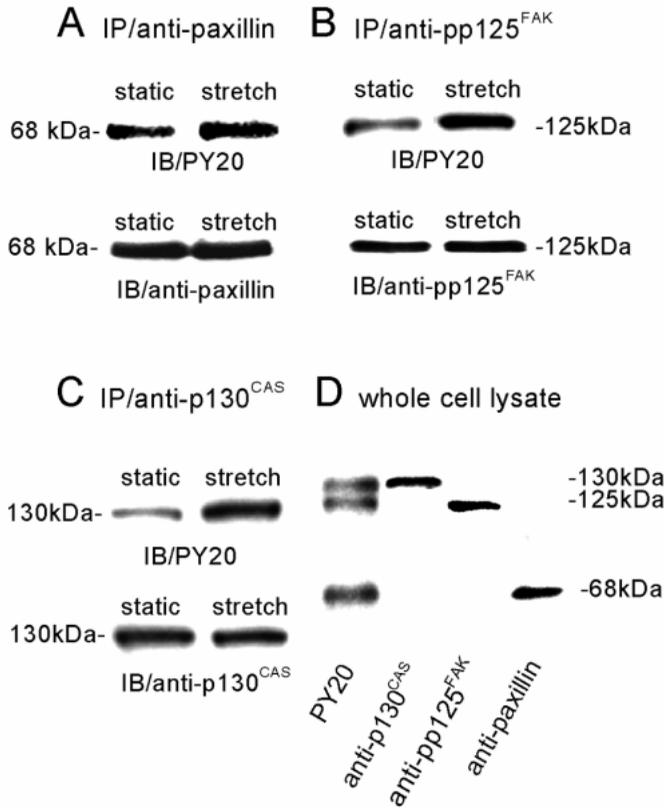**Fig. 4.** Involvement of paxillin, pp125FAK, and p130CAS in the tyrosine phosphorylated proteins. Equal protein amounts of cell lysate were immunoprecipitated with anti-paxillin, anti-pp125FAK, and anti-p130CAS, immuno-blotted on PVDF membrane, and the proteins probed with anti-phosphotyrosine monoclonal antibody. (A) A band at 68 kDa corresponding to paxillin increased the level of tyrosine phosphorylation by cyclic stretch (upper). Immuno-blotting with anti-paxillin shows equal amounts of protein were loaded in both lanes (lower). (B) A band at 125 kDa corresponding to pp125FAK increased the level of tyrosine phosphorylation by stretch (upper). Immuno-blotting with anti-pp125FAK shows equal amounts of protein were loaded in both lanes (lower). (C) A band at 130 kDa corresponding to pp130CAS increased the level of tyrosine phosphorylation by cyclic stretch (upper). Immuno-blotting with anti-p130CAS shows equal amounts of protein were loaded in both lanes (lower). (D) Whole cell lysate was immuno-blotted with anti-phosphotyrosine, anti-p130CAS, anti-pp125FAK, and anti-paxillin monoclonal antibody.

70 kDa and 120-140 kDa, we examined the involvement of tyrosine phosphorylation of pp130CAS (130 kDa; Sakai et al., 1994), pp125FAK (125 kDa; Schaller et al., 1992), and paxillin (68 kDa; Turner et al., 1990) in 3Y1 fibroblasts. Immunoprecipitation using the anti-paxillin monoclonal antibody revealed a single band of tyrosine phosphorylated protein of 68 kDa (Fig. 4A, upper). The level of tyrosine phosphorylation increased (1.7-fold) in response to cyclic stretch without any change in the total amount of paxillin present (Fig. 4A, lower). Pp125FAK was immuno-precipitated from lysates of static and stretched cells, and probed with anti-phosphotyrosine monoclonal antibody. The level of tyrosine phosphorylation of pp125FAK immunoprecipitated from stretched cells was much higher (1.8-fold) compared with that
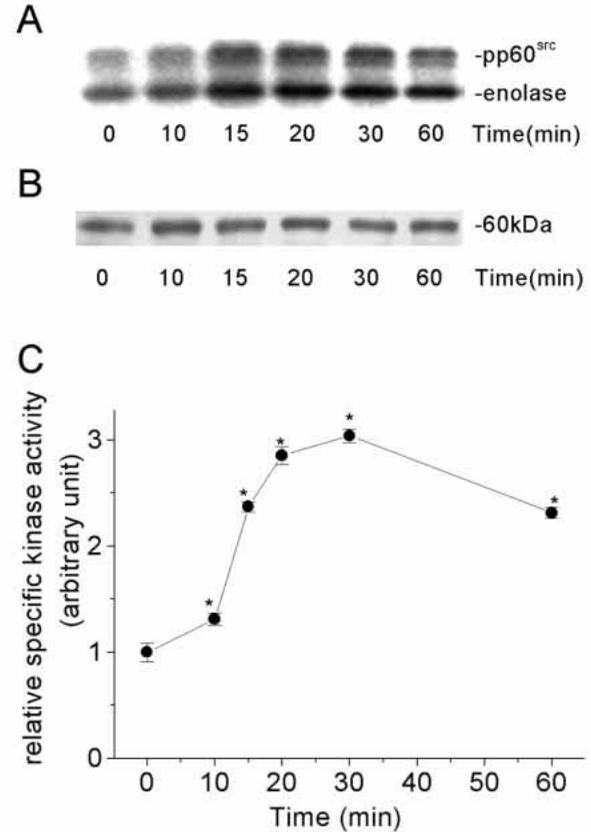
Fig. 5. Activation of pp60src in response to cyclic stretch. 3Y1 fibroblasts were stretched for the indicated times and were subjected to in vitro kinase assay. (A) The specific activity of pp60src was determined by measuring the incorporation of $^{32}PO_4$ into enolase relative to the protein level of pp60src. Upper bands correspond to phosphorylated pp60src and the lower bands correspond to phosphorylated enolase. (B) An immuno-blot probed with anti-pp60src monoclonal antibody shows that equal amounts of proteins were loaded in each lane. (C) The bands were quantified by densitometry and are presented as fold increase of the static control. The level of phosphorylated enolase peaked at 30 minutes. Data are expressed as mean $\pm$ s.e. from four independent experiments. Asterisks denote those values that are significantly different (*$P<0.05$) from control (0 minutes).

in static cells (Fig. 4B). Pp130CAS was examined in the same way and the level of tyrosine phosphorylation of pp130CAS immunoprecipitated from stretched cells was much higher (2.1-fold) compared with that in static cells (Fig. 4C). Fig. 4D summarizes the tyrosine-phosphorylated proteins in 3Y1 fibroblasts.

Pp60src is responsible for the stretch induced tyrosine phosphorylation

As cyclic stretch increased the level of protein tyrosine phosphorylation in 3Y1, we next investigated the involvement of pp60src in the cells, since pp60src is one of the major tyrosine kinases (Golden and Brugge, 1989), and pp125FAK is known as one of the substrates of pp60src. Pp60src was immunoprecipitated from lysates of 3Y1 fibroblasts subjected to uni-axial cyclic stretch. The specific kinase activity of pp60src was measured as the amount of phosphorylation of
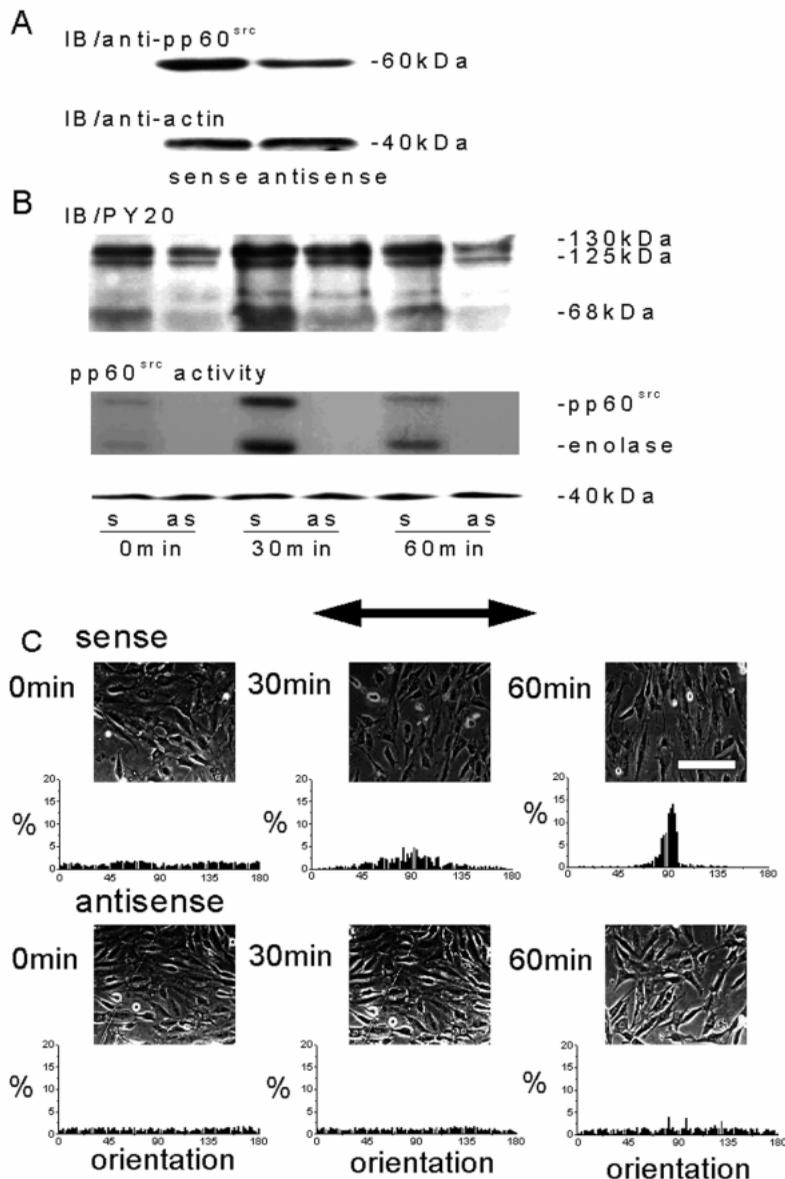
Fig. 6. Effects of the antisense S-ODN against pp60[src] on the cyclic stretch induced responses. (A) A band at 60 kDa corresponding to pp60[src] significantly decreased in the antisense-SODN treated cells (upper), however, the amounts of actin was not changed between the sense and antisense treated cells (lower). (B) Immuno-blot of the antisense and the sense S-ODN treated cells probed with anti-phosphotyrosine monoclonal antibody (upper). The level of tyrosine phosphorylation of the sense S-ODN treated cells increased with time, whereas that of the antisense S-ODN treated cells remained at a low level and did not increase significantly. Kinase activity of pp60[src] in response to cyclic stretch in the sense-SODN and the antisense-ODN treated cells (middle). The kinase activity increased with time in the sense S-ODN treated cells, whereas we could not detect any kinase activity in the antisense S-ODN treated cells. An immuno-blot of the same membrane probed with anti-actin monoclonal antibody shows an equal amount of protein was loaded in each lane (lower). (C) Sets of phase contrast images and histograms of the S-ODNS treated fibroblasts in response to cyclic stretch. The sense S-ODN treated cells began to orient perpendicularly to the stretch axis and the orientation of the vast majority of the cells is close to 90° with respect to the stretch axis (upper), whereas the anti-sense S-ODN treated cells showed almost no orienting in response to cyclic stretch (lower). More than 600 cells from five independent experiments make up 100%. Bar, 50 μm. The double-headed arrow indicates the cyclic stretch axis.

acid-treated enolase by immuno-complex followed by SDS-PAGE and autoradiography. The autoradiogram of phosphorylated enolase at 40 kDa revealed the increase in kinase activity of pp60[src] as early as 10 minutes after the stretch onset (Fig. 5A). The immuno-blotting pattern in Fig. 5B indicates that the same amounts of pp60[src] were loaded in each lane. The activity of pp60[src] peaked at 20 minutes (3.0±0.2-fold, mean ± s.e., n=5) and decreased gradually (Fig. 5C). The phosphorylation of pp60[src] was also observed and the time course of the phosphorylation was almost parallel with that of the tyrosine phosphorylation level of acid treated enolase. We also measured the kinase activity of src and the morphological changes in response to a static and constant deformation instead of the oscillatory deformation. However, we did not observe any changes in the kinase activity of pp60[src] nor in its morphology. To further investigate the role of pp60[src] in the stretch-dependent orienting response and protein tyrosine phosphorylation, we employed an antisense S-ODN against pp60[src] to down-regulate the expression of pp60[src]. The

expression level of pp60[src] protein in the antisense SODN treated cells decreased less than 24% as compared to that in the sense S-ODN treated cells, whereas the expression level of other proteins such as actin was not significantly changed (Fig. 6A). Without stretch stimulation, we could not observe any significant morphological changes in the S-ODN treated cells. The S-ODN treated cells were subjected to uni-axial cyclic stretch, and the levels of tyrosine phosphorylation and pp60[src] kinase activity were examined. In the sense S-ODN treated cells, the increase in tyrosine phosphorylation was observed in three major protein bands at 68, 125, and 130 kDa, as was observed in the untreated cells and an increase in the kinase activity was also observed (Fig. 6B), whereas in the antisense S-ODN treated cells, the basal tyrosine phosphorylation level was lower compared to the sense S-ODN treated cells, cyclic stretch did not increase the protein tyrosine phosphorylation level significantly, and kinase activity was not observed at all (Fig. 6B). Finally, uni-axial cyclic stretch induced the cell alignment perpendicular to the stretch axis in the sense S-ODN
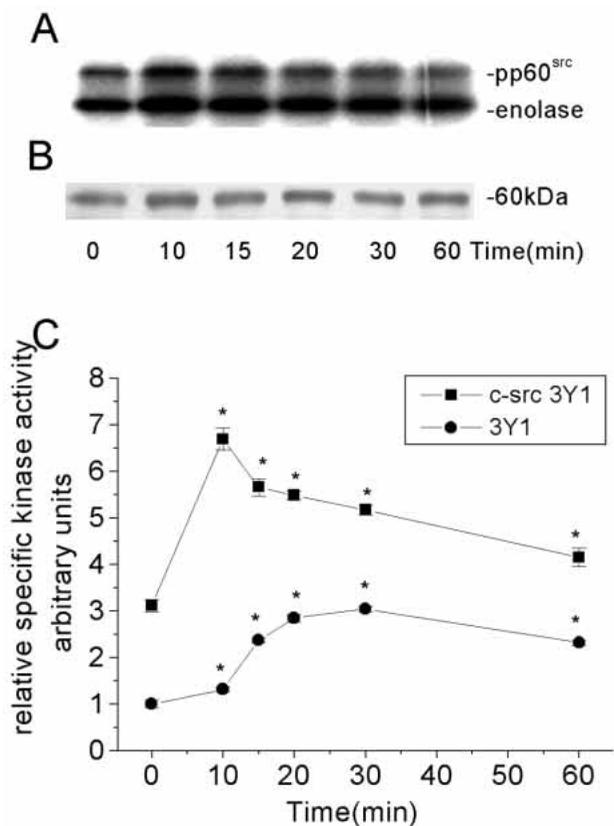
Fig. 7. Kinase activity of pp60^{src} in response to cyclic stretch in c-src transformed 3Y1(c-src-3Y1) fibroblasts. C-src-3Y1 was stretched for the indicated times and subjected to in vitro kinase assay. (A) The specific activity of pp60^{src} was determined by measuring the incorporation of ^{32}PO$_4$ into enolase relative to the protein level of pp60^{src}. Upper bands correspond to phosphorylated pp60^{src} and the lower bands correspond to phosphorylated enolase. (B) Immuno-blot probed with anti-pp60^{src} monoclonal antibody shows that equal amounts of proteins were loaded in each lane. (C) The incorporation of ^{32}PO$_4$ into enolase was quantified by densitometry and was presented as fold increase of the static control. The level of phosphorylated enolase peaked at 10 minutes and gradually decreased. Results are representative of four separate experiments. Data are expressed as mean ± s.e. Asterisks denote those values that are significantly different (*P<0.05) from control (0 minutes) values.

treated cells (Fig. 6C, upper), whereas in the antisense S-ODN treated cells, the orienting response was strongly inhibited (Fig. 6C, lower).

Pp60^{src} transformed 3Y1 fibroblasts showed an accelerated orienting response

We have shown that pp60^{src} was activated by cyclic stretch and that its depletion by antisense S-ODN suppressed the stretch induced orienting response. It is of interest to see whether pp60^{src} transformed cells show the acceleration in the morphological response to uni-axial cyclic stretch. First, we examined the kinase activity of pp60^{src} of c-src transformed 3Y1(c-src-3Y1) fibroblasts during uni-axial cyclic stretch. After the application of uni-axial cyclic stretch, pp60^{src} was immunoprecipitated and the specific kinase activity of pp60^{src} was measured as the phosphorylation of acid-treated enolase

by the immuno-complex. In c-src-3Y1 fibroblasts, the tyrosine kinase activity increased and peaked at 10 minutes in response to cyclic stretch (Fig. 7A). Immuno-blotting against pp60^{src} (Fig. 7B) revealed that the same amounts of protein were loaded in each lane, and that in comparison with Fig. 5B, c-src-3Y1 fibroblasts express a 3-fold higher level of pp60^{src} than do wild-type 3Y1 fibroblasts. Fig. 7C shows the comparison of the kinase activity of pp60^{src} in wild-type 3Y1 fibroblasts and c-src-3Y1 fibroblasts during cyclic stretch. We also examined the orienting response of c-src-3Y1 fibroblasts to cyclic stretch. In response to uni-axial cyclic stretch, c-src-3Y1 fibroblasts showed an alignment perpendicular to the stretch axis (Fig. 8). The striking difference between wild type and the transformed fibroblasts was that the orienting response of c-src-3Y1 fibroblasts was much faster than that of wild-type 3Y1 fibroblasts. In order to compare the difference in the time course more precisely, the standard deviations (s.d.) of the orientation distribution in the histograms were plotted against time, because the s.d. may be a good indication of the degree of orienting response. Fig. 9 shows the time course of the s.d. from orientation distributions at each time point for wild-type 3Y1 and c-src-3Y1 fibroblasts, showing that c-src-3Y1 fibroblasts aligned much faster than wild-type 3Y1 fibroblasts. Again, these data confirmed that pp60^{src} plays a crucial role in the stretch dependent orienting response in fibroblasts.

DISCUSSION

Stretch-induced activation of pp60^{src}

In the present study, we have demonstrated that uni-axial cyclic stretch induced the activation of pp60^{src} in fibroblasts and that pp60^{src} is critical for the stretch induced tyrosine phosphorylation of focal proteins and for the orienting response. Several observations are consistent with the notion that phosphotyrosine accumulation is involved in the process of the stretch-induced orienting response. First, the time course of the level of phosphorylation closely resembles that of the orienting response both in 3Y1 and c-src-3Y1. Uni-axial cyclic stretch increased pp60^{src} activity and the activity peaked at 20 minutes (Fig. 5) in 3Y1 fibroblasts. The time course of the tyrosine phosphorylation level of pp125^{FAK}, paxillin, and pp130^{CAS} coincided with that of pp60^{src} activation (Figs 2, 5). Second, treatment with herbimycin A inhibited not only the stretch-induced tyrosine phosphorylation but also the orienting response. Finally, depletion of pp60^{src} by the antisense-SODN suppressed the stretch-induced orienting response (Fig. 3). Moreover, overexpression of c-src in 3Y1 accelerated the stretch induced orienting response (Figs 8, 9). Taken together, it is highly likely that pp60^{src} is involved and is playing a crucial role in the stretch-induced orienting response in fibroblasts.

Involvement of SA channels in the activation of pp60^{src} tyrosine kinase

One probable mechanism for the stretch induced activation of pp60^{src} may be an activation of Ca^{2+}-permeable stretch-activated (SA) channels followed by intracellular Ca^{2+} concentration ([Ca^{2+}]$_i$) increase. Ca^{2+}-permeable SA channels have been reported in fibroblasts by patch clamp experiments (Stockbridge and French, 1988; Glogauer et al., 1995). The
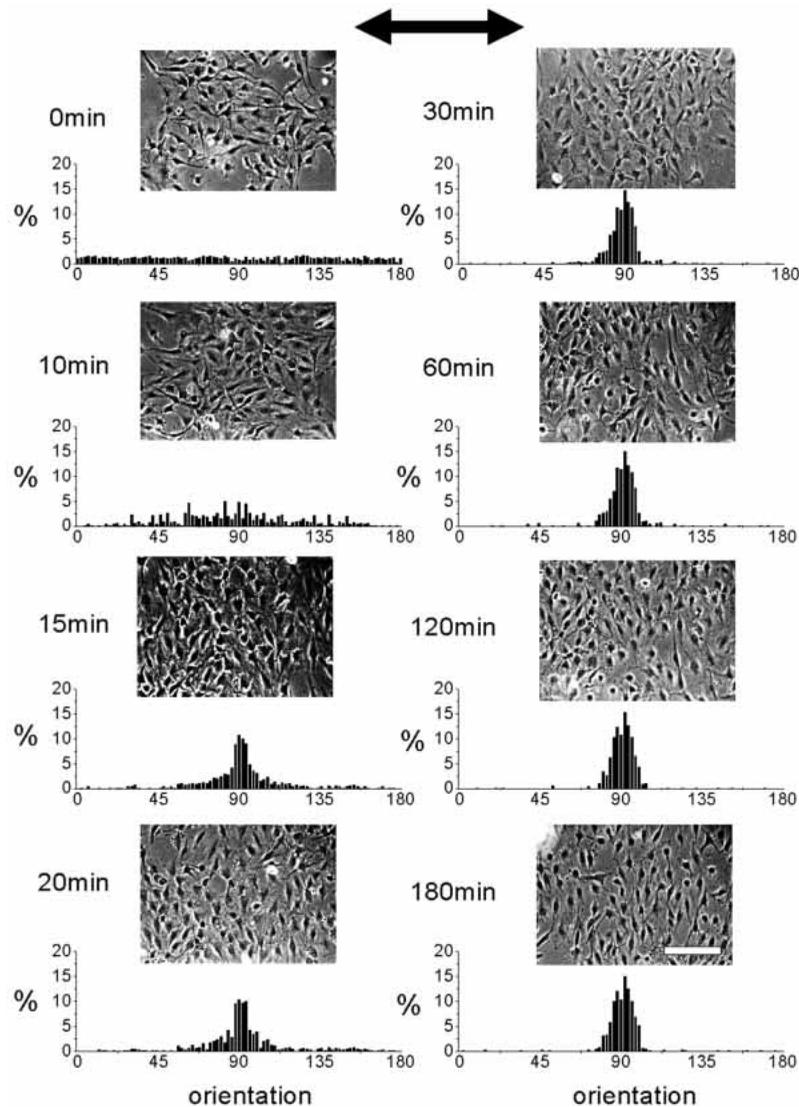
Fig. 8. Time course of the orienting response of 3Y1 fibroblasts in response to cyclic stretch. A series of phase contrast images show the time course of the orienting response of c-src-3Y1 during cyclic stretch. The double-headed arrow indicates the cyclic stretch axis. The cells began to orient perpendicularly to the stretch direction and the orientation of the vast majority of the cells is close to 90° with respect to the stretch axis at as early as 15 minutes after the stretch onset. A series of histograms show time dependent change in the percentage of the cell population plotted against the angle of individual long axes with respect to the stretch axis. More than 600 cells from six independent experiments make up 100%. Bar, 50 μm.
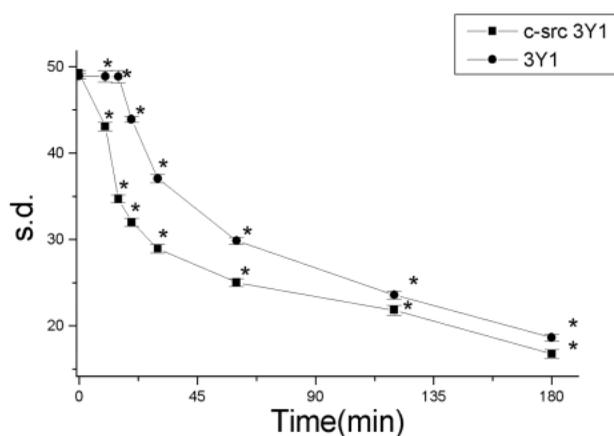

Fig. 9. Comparison of the time course of the orienting response between 3Y1 and c-src-3Y1 fibroblasts. S.d.s of the distribution of the histograms (Fig. 1 for 3Y1 and Fig. 8 for c-src-3Y1 fibroblasts) were plotted against time. The orienting response of c-src-3Y1 fibroblasts was significantly faster than that of 3Y1 fibroblasts. Data are expressed as mean ± s.e. Asterisks denote those values that are significantly different (*$P<0.05$) from control (3Y1) values.

involvement of SA channels in the stretch-induced morphological response was reported in HUVECs. In HUVECs, uni-axial cyclic stretch induced intracellular Ca^{2+} mobilization, which was blockable by Gd^{3+}, and tyrosine phosphorylation of focal proteins followed by morphological changes (Naruse et al., 1998b). The present study in fibroblasts implies the involvement of the same mechanism as in HUVECs, as a result of the following observations: (1) treatment of the cells with EGTA or BAPTA/AM inhibited the stretch-induced kinase activity of pp60^{src}, suggesting that Ca^{2+} influx followed by an increase in $[Ca^{2+}]_i$ contributes to the activation of pp60^{src}. (2) Application of Gd^{3+}, a blocker for the SA channel, inhibited the stretch-induced kinase activity of pp60^{src} as well as the stretch-induced tyrosine phosphorylation of focal proteins and the stretch-induced morphological changes.

At present, there is no evidence that pp60^{src} is activated directly by the increase in $[Ca^{2+}]_i$. However, a recent observation made by our laboratory suggested the involvement of calcineurin, Ca^{2+}/CaM dependent phosphatase in the stretch-induced pp60^{src} activation in HUVECs (Naruse et al., 1998c), since the stretch induced activation of pp60^{src} was inhibited by FK506, a specific inhibitor for calcineurin. It has

been proposed that pp60[src] is maintained in a low activity state by an interaction between phosphotyrosine 527 and the pp60[src] homology 2 (SH2) domain and that pp60[src] is activated upon tyrosine 527 dephosphorylation (Roussel et al., 1991). It is possible that calcineurin dephosphorylates the phosphotyrosine 527 of pp60[src]. Dephosphorylation of phosphorylated 527 would expose the SH2 domain freely, thus allowing it to bind to specific phosphotyrosine containing proteins (Fukui et al., 1991; Koch et al., 1991).

Role of tyrosine phosphorylation of focal proteins

In fibroblasts, tyrosine phosphorylation of paxillin and pp125[FAK] has been implicated during actin assembly (Burridge et al., 1992). In bovine endothelial cells, cyclic strain induces the tyrosine phosphorylation of pp125[FAK] and paxillin and these proteins are suggested to be involved in the morphological changes (Yano et al., 1996; Naruse et al., 1998a). Paxillin has been suggested to be a direct substrate of pp125[FAK] (Rankin and Rozengurt, 1994) and found to bind in vitro to the SH3 domain of pp60[src] (Weng et al., 1993). In 3Y1 fibroblasts, morphological alteration and transforming ability were regulated through increasing tyrosine phosphorylation of the p130[CAS] (Ogawa et al., 1994). It is likely that tyrosine phosphorylation of focal proteins may serve as a proximate signal for actin polymerization (Burridge et al., 1992). Treatment of the cells with herbimycin A suppressed the stretch-induced tyrosine phosphorylation of those proteins and inhibited the orienting response in 3Y1 fibroblasts, suggesting that tyrosine phosphorylation of focal proteins is indispensable for the stretch induced orienting response. How cells sense the direction of stretch remains unclear. One possibility is that cell orientation requires remodeling of focal adhesions; disassemby of existing complexes and formation of new ones to anchor reoriented stress fibers. Support for this idea is provided by the fact that when formation of new focal adhesions is inhibited cyclic stretching induces cell detachment as shown in Fig. 3A.

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