The Role of Actin-binding Protein 280 in Integrin-dependent Mechanoprotection*

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To survive in a mechanically active environment, cells must adapt to variations of applied membrane tension. A collagen-coated magnetic bead model was used to apply forces directly to the actin cytoskeleton through integrin receptors. We demonstrate here that by a calcium-dependent mechanism, human fibroblasts reinforce locally their connection with extracellular adhesion sites by inducing actin assembly and by recruiting actin-binding protein 280 (ABP-280) into cortical adhesion complexes. ABP-280 was phosphorylated on serine residues as a result of force application. This phosphorylation and the force-induced actin reorganization were largely abrogated by inhibitors of protein kinase C. In a human melanoma cell line that does not express ABP-280, actin accumulation could not be induced by force, whereas in stable transfectants expressing ABP-280, force-induced actin accumulation was similar to human fibroblasts. Cortical actin assembly played a role in regulating the activity of stretch-activated, calcium-permeable channels (SAC) since sustained force application desensitized SAC to subsequent force applications, and the decrease in stretch sensitivity was reversed after treatment with cytochalasin D. ABP-280-deficient cells showed a >90% increase in cell death compared with ABP-280+ve cells after force application. We conclude that ABP-280 plays an important role in mechanoprotection by reinforcing the membrane cortex and desensitizing SACs.

A large body of recent work has focused on how cells convert applied mechanical tension into cytoplasmic signals that regulate cell metabolism and transcription (mechanotransduction) (1–3). Important elements of mechanotransduction include cellular adaptation and survival in the face of increased environmental force (4). Indeed, cells undergo dramatic internal structural changes in response to increased environmental forces (5). These mechanoprotective, structural adaptations enable cells to maintain membrane integrity, cell shape, and adhesion to extracellular matrix molecules (4, 6). For example, periodontal ligament fibroblasts function in a much more mechanically stressed environment than gingival fibroblasts and have a nearly 2-fold higher proportion of actin in filamentous form (7). This example indicates that cells are able to sense and adapt to environmental tension in part through cytoskeletal adaptations. NIH/3T3 fibroblasts up-regulate their attachment strength to extracellular matrix ligands if increased tension is applied through integrins (8), indicating that cells not only sense changes in applied extracellular loads but can rapidly reinforce cytoskeletal linkages locally at force application sites. Consistent with these data we have demonstrated that fibroblasts undergo localized actin assembly during isolated force application through focal adhesion complexes (6).

Localized cortical actin mechanoprotective responses presumably involve actin-binding proteins. The cross-linking and bundling activities of actin-binding proteins can increase the structural strength and integrity of the cortical actin network (9). Among the most efficient actin cross-linking proteins is actin-binding protein 280 (ABP-280),1 a 540-kDa dimeric protein first identified in macrophages (10) and present in other tissues including most non-muscle cells (11). A homologous protein, filamin, first purified from skeletal muscle (12, 13) shares extensive sequence homology with ABP-280 but is encoded by a separate gene and displays different abilities to cross-link or bundle F-actin. ABP-280, filamin, dystrophin, spectrin, α-actinin, ABP-120, and fimbrin are part of an actin cross-linking superfamily (14) that share a common actin-binding site (11). Some members of this superfamily (spectrin and dystrophin) may act to mechanically reinforce the cell membrane (15) and thereby enhance membrane stability during increased membrane tension. Notably, ABP-280 cross-links actin and links actin to integral membrane proteins (16, 17), thereby providing increased cellular cortical rigidity (18). Thus ABP-280 and other actin cross-linking and bundling proteins may be key structural elements that stabilize the cell membrane by facilitating interactions between the cortical actin cytoskeleton and the plasma membrane.

The importance of structural interactions between the cortical actin cytoskeleton and the plasma membrane in ion channel regulation has been recognized previously (3, 19, 20). Specifically, ABP-280 has been implicated in regulating the conductance of ion channels activated by cell swelling (21). Since chronically high Ca\(^{2+}\) entry is known to be toxic to cells (22), it is likely that cells in mechanically active environments must have evolved adaptive mechanisms to regulate the sensitivity

1 The abbreviations used are: ABP, actin-binding protein; SAC, stretch-activated, calcium-permeable channels; TRITC, tetramethylrhodamine isothiocyanate; Pipes, 1,4-piperazinediethanesulfonic acid; BSA, bovine serum albumin; PBS, phosphate-buffered saline; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; BIM, bisindolylmaleimide; MARCKS, myristoylated alanine-rich C kinase substrate; PKC, protein kinase C.
of SACs to chronic or prolonged high membrane tension. We have suggested previously that the open probability of SACs is dependent on the rigidity of the membrane cortex which is determined by specific cytoskeletal proteins and their organization (6). In this study we have characterized a localized, force-induced actin recruitment to focal adhesions and have examined the dependence of this recruitment on ABP-280. In this article, we test the hypothesis that ABP-280 mediates a mechanoprotective mechanism that desensitizes SACs and is important for cell survival in the face of applied physical stress.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Anti-filamin mouse monoclonal antibody (clone FIL2), cross-reactive with ABP-280, anti-filamin rabbit polyclonal antibody, anti-vinculin mouse monoclonal antibody (clone VIN-1, a-actinin mouse monoclonal antibody (clone BM 75.2), β-actin monoclonal anti-body (clone AC-15), fluorescein isothiocyanate-conjugated goat anti-mouse antibodies, and TRITC-phallloidin were purchased from Sigma. Mouse monoclonal antibody to α5-integrin was purchased from Calbiochem (clone P1E6). Anti-phosphoserine antibody was purchased from Zymed (San Francisco, CA). Anti-villus mouse monoclonal antibody (clone ID2C3) was purchased from Biodesign International (Kennebunk, ME). Anti-MARCKS mouse monoclonal antibody was obtained from Upstate Biotechnology (Lake Placid, NY). Fura-2/AM was purchased from Molecular Probes (Eugene, OR). All antibodies were pre-determined by specific cytoskeletal proteins and their organization (6). In this study we have characterized a localized, force-induced actin recruitment to focal adhesions and have examined the dependence of this recruitment on ABP-280.

**Cell Culture**—Human gingival fibroblasts were derived from primary explant cultures as described (7). Cells from passages 6–19 were grown as monolayers in T-75 flasks (3). Twenty-four hours before each experiment cells were harvested with 0.01% trypsin, and 2 × 10⁶ cells were plated into 60-mm diameter culture dishes (Falcon, Becton Dickinson, Mississauga, ON). The cells were sub-confluent prior to all experiments.

Melanoma cell lines were grown as described previously (3) in α-minimal essential medium (Life Technologies, Inc.) supplemented with 5% newborn calf serum, 2% fetal calf serum, and 0.5 mg/ml G418 (Life Technologies, Inc.) to maintain selection. The ABP-280- melanoma cells (A7) were originally derived from the transfection of a parent ABP-280(−) cell line, M2T, with a mammalian expression vector (LK444) that either did (A7) or did not contain the cDNA for full-length ABP-280. Cells grown on coverslips were fixed with 3.7% formaldehyde in PBS, washed three times, and resuspended in calcium-free buffer. Beads were added to attached cells in PBS for 10 min, and the cells were washed three times to remove unbound beads. Cells were exposed to force in phosphate-buffered saline (PBS), washed three times, and incubated in calcium-free buffer. Beads were added to attached cells in PBS for 10 min, and the cells were washed three times to remove unbound beads. Cells were exposed to force in phosphate-buffered saline (pH 7.4).

**Electron Microscopy**—Fibroblasts were permeabilized with 10% PHEM (0.6 mM Pipes, 0.25 mM Heps, 0.1 mM EGTA, 20 mM MgCl2), 0.75% Triton X-100, and 1% glutaraldehyde. After 30 min, samples were embedded in Lowicryl-K4M, and thin sections were placed on nickel grids. Gold-conjugated (15 nm diameter) goat anti-mouse IgG was obtained from Zymed (San Francisco, CA). Sections were blocked with a 0.2% gelatin, 0.1% BSA in TBS solution for 1 h. The grids were washed in PBS, 0.1% BSA buffer and placed on a 25-μl drop of anti-filamin/ABP-280 monoclonal IgG antibody (10 mg/ml, diluted in PBS, 0.1% BSA buffer) and incubated for 1 h at room temperature. Samples were washed as described above. The grids were placed on a 25-μl drop of the secondary gold-conjugated goat anti-mouse IgG (1:20, diluted in PBS, 0.1% BSA). The grids were stained with uranyl acetate and lead citrate and observed under an electron microscope (Hitachi-60).

**Fluorescence Quantification of Actin**—Images of TRITC-phallolidin-stained fibroblasts acquired in Winview were assessed for F-actin accumulation/enrichment at bead binding sites using the pixel fluorescence function in the Winview 1.61 software. Paraformaldehyde-fixed cells from no force and force-treated samples were stained with rhodamine-phallolidin; the cells were imaged, and the rhodamine fluorescence (F-actin) level (average pixel intensity) at bead sites on a given cell were divided by the average pixel intensity for the entire cell. This provided a measure of the percent F-actin enrichment at bead binding sites.

**Intracellular [Ca2+]i**—Measurement of intracellular calcium ion concentration ([Ca2+]i) was conducted as described previously (3). Briefly, cells on coverslips were incubated at 37°C with 3 mM fura-2/AM (Molecular Probes, Eugene, OR) for 20 min and then at room temperature for 10 min. Whole cell [Ca2+]i measurements were obtained with a dual excitation, microscope-based spectrofluorimeter (Photon Technology Int., London, ON). A variable aperture, intra-beam mask was used to restrict measurements to single cells. Estimates of the precise intracellular concentration of fura-2 were calculated from dual excitation emitted fluorescence as described (24). As demonstrated previously by image analysis (3) and by microscopic evaluation between exposures, repeated applications of force did not remove attached beads. Force application during calcium measurements was applied as described previously (3). Briefly, an electromagnet with a pole extension was used to focus and direct the magnetic field to the cell of interest.

**Isolation of Bead Complexes**—Proteins enriched in bead-associated focal adhesion complexes were assessed as described previously (6, 25). Briefly, cells and attached beads were collected by scraping cells into ice-cold cytoskeleton extraction buffer (CSKB, Triton X-100, 50 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 20 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 10 mM Pipes (pH 6.8)). The isolation procedure was carried out at 4°C using a side-pull magnetic isolation apparatus (Dynal, Lake Placid, New York). The cell-bead suspension was sonicated for 10 s (output setting, 3, power 15%; Sonifier 185, Branson) and homogenized in a 2-ml Dounce homogenizer (20 strokes). The magnetic beads were pelleted and washed 3 times with CSKB prior to protein analysis. Equal numbers of beads from both “force” and “no force” samples were suspended in equal volumes of sample buffer and boiled for 5 min to remove protein from the beads. Protein levels were estimated using densitometry of Western blots. Standard curves were performed for each protein from standards obtained from beads, and a large concentration range (1–20 μg) of protein was loaded on each lane. The standard curve demonstrated a linear relationship between protein loading levels, and densitometry measurements for all the proteins that were examined between 0 and 20 μg (Pearson correlation R² values were between 0.89 and 0.97 for all proteins tested). The optimal protein concentration loaded per gel was 10 μg maximum, which was well within the linear range as determined from each of the standard curves.

**Inhibitors**—We assessed the dependence of actin rearrangement on calcium ions by incubating cells with BAPTA/AM at 3 μM for 45 min at 37°C prior to force application. Previous pilot experiments have shown that this reduces [Ca2+]i to <50 nM and blocks ligand-induced calcium fluxes. We assessed the dependence of actin rearrangement on actin assembly by incubating cells with cytochalasin D at 1 μM for 30 min at 37°C prior to force application.

**Bisindolylmaleimide (BIM) (26) and calphostin C (27) were used to...
horseradish peroxidase (Amersham Corp.) for 1 h, washed 5 times. Protein was separated from beads by heating at 65 °C in 2·Laemmli buffer.

Blots were washed three times with 0.5% Tween/PBS for 10 min, PBS and incubated in the indicated antibody for 1 h at room temperature. Blots were blocked for 1 h with 5% skim milk in PBS/Tween, and developed by chemiluminescence (Amersham Corp.).

FIG. 1. A. Western blots of proteins isolated from collagen-coated magnetic beads before and after application of force to gingival fibroblasts. Blots demonstrate that beads bind through α2-integrins and associate with focal adhesion proteins (vinculin). Force induces increased actin to associate with integrin bound beads without increasing levels of the adhesion complex proteins. Protein from equal numbers of beads was loaded in each lane. B, schematic diagram demonstrating the approximate dorsal-apical level of micrographs. 1, electron micrographs of the most dorsal surface of gingival fibroblasts bound with magnetic beads. Micrographs of force samples clearly demonstrate increased filament assembly associating with the bead complex compared with no force samples. Note that beads were removed during sectioning (clear zone). A bead fragment is still present in the right corner of the force micrograph (bar = 0.5 μm). 2, lower magnification micrographs demonstrate increased fiber bundles in force-exposed cells (bar = 40 μm).

Western blots demonstrating that the force-induced increase of actin at bead binding sites is dependent on calcium ions and actin assembly. Proteins from gingival fibroblasts were obtained by the bead isolation technique (see “Experimental Procedures”). Elimination of intracellular and extracellular calcium ions (3 μM BAPTA/AM and calcium-free buffer) prevented force-induced actin accumulation. Prevention of actin assembly with cytochalasin D (1 μM) also prevented the force-induced actin accumulation.

FIG. 2. Western blots demonstrating that the force-induced increase of actin at bead binding sites is dependent on calcium ions and actin assembly. Proteins from gingival fibroblasts were obtained by the bead isolation technique (see “Experimental Procedures”). Elimination of intracellular and extracellular calcium ions (3 μM BAPTA/AM and calcium-free buffer) prevented force-induced actin accumulation. Prevention of actin assembly with cytochalasin D (1 μM) also prevented the force-induced actin accumulation.

Eugene, OR) were coated with collagen as described above. Cells were incubated with beads at a ratio of 4 beads per cell for 3 h. Cell were trypsinized to create a cell suspension and remove externally attached beads. Ten minutes prior to analysis propidium iodide (50 mg/ml) was added to cell suspensions to estimate the proportion of non-viable cells. Internalization of beads and cell viability was assessed by dual color flow cytometry as described previously (30). Briefly, cell samples were analyzed with a FACStar Plus flow cytometer (Becton Dickinson FAC Systems, Mountain View, CA) at a sheath pressure of 11 p.s.i. and with excitation from an Innova 70 argon laser (Coherent Laser, Palo Alto, CA) at light regulation mode setting of 250 milliwatts and a wavelength of 488 nm. Emitted fluorescence was split through two detectors by a short pass 560 nm beam splitter (all filters and beam splitters from Omega Optical Inc., Brattleboro, VT) and a 530DF30 filter for green fluorescence (phagocytosed beads) and a 625DF38 filter for red fluorescence. Photomultiplier tube voltage settings were determined for each experiment on the basis of thresholds established from appropriate negative and positive control samples.

Motility Assay—Confluent monolayer cultures of gingival fibroblasts on 60-mm dishes were “wounded” by creating a uniform cell-free wound using a scalpel (31). The wound length was approximately 1 mm in length. The maximum migration distance for untreated control cells was 500 μm over the assay period. Hepes-buffered α minimum essential medium containing 15% fetal bovine serum was added to the culture, and migration of the cells into the wound was visualized by time lapse cinemicrography (Panasonic AG6050 recorder; Nikon microscope) of the wound area for 15 h with a 20 × objective (Nikon). Tracings of the wound area were made before and after force application, and the cell-free area was calculated using NIH IMAGE (version 1.6) on a Macintosh computer. The migratory index was expressed as the percent area of the original wound that was repopulated with cells.

Statistical Analysis—For all assays, three or more separate experiments were performed; means ± S.E. were calculated for continuous variables, and comparisons were made by unpaired t tests or analysis of variance as indicated.

RESULTS

F-actin Accumulation—We studied force-induced actin assembly locally at the force transfer sites by isolating the proteins associated with collagen-coated magnetic beads. Identification by Western blot of cell membrane proteins bound to the magnetic beads showed that the bead attachment sites were enriched with vinculin, actin, and α2-integrin but that the levels of vinculin and α2-integrin did not change over time of force exposure (20 min; Fig. 1A). In contrast, there was a marked increase (1.5–2-fold) in levels of actin associating with the beads (Fig. 1A). The actin accumulation following force application was confirmed by electron micrographs of bead-containing sections from fibroblasts exposed to force. Prominent filament bundles were observed in close proximity to collagen beads from force-treated samples but were absent in samples incubated only with the collagen beads (Fig. 1B).
Lower magnification micrographs demonstrated increased density of actin fiber bundles in cells exposed to force with some of the bundles oriented toward the substrate surface (i.e., parallel to the applied force). To verify the Western blot data, a fluorescence-based image analysis technique was developed to visualize and quantify the enrichment of F-actin at bead attachment sites. The average pixel intensity around beads of rhodamine-phalloidin-stained samples was enriched 1.5-fold in force samples compared with no force samples (No force: 1.15 ± 0.074; Force: 1.79 ± 0.089; p < 0.001; mean ± S.E.).

We determined if the force-induced actin accumulation was dependent on calcium ions and on actin assembly. Cells were loaded with 3 μM BAPTA/AM and exposed to force in a calcium-free buffer. These samples demonstrated no increase in actin at the bead adhesion sites. Similarly, cells incubated with 1 μM cytochalasin D showed no force-induced actin accumulation (Fig. 2).

Actin-binding Proteins—Western blots of the proteins isolated from the beads were screened for a number of actin-binding proteins that have been implicated in F-actin cross-linking and F-actin membrane association. Proteins that were probed included ABP-280 (18), α-actinin (32), villin (33), α2-integrin, and MARCKS (34). Densitometry of Western blots demonstrated that only ABP-280 increases at the bead-membrane complex during constant force exposure, whereas the levels of the other actin-binding proteins and the α2-integrin of the collagen receptor remain constant (results are representative of at least three separate experiments; error bars are S.E.; asterisks indicate p < 0.01 comparing Force to No Force accumulation).
FIG. 4. A, gingival fibroblasts were exposed to the indicated treat-
ment combinations of force (20 min) and the PKC inhibitor bisindolyl-
maleimide (BIM; 5 μM), and ABP-280 was immunoprecipitated from
fresh cell lysates. The blot was probed with an anti-phosphoserine
antibody. Note the marked increase in ABP-280 serine phosphoryla-
tion following force application which is inhibited by treatment with the
PKC inhibitor. Equal ABP-280 was loaded in each lane as determined
by parallel Western blot. B, proteins isolated from the beads were
probed with the indicated antibody. Note the increase of serine-phos-
phorylated ABP-280, actin, and a serine-phosphorylated 70-kDa pro-
tein following force application. Inhibition of ABP-280 serine phos-
phorylation by BIM decreased the association of ABP-280 and actin
association with the beads but not the 70-kDa protein which may be
phosphorylated by a different force-activated kinase.

We determined if the force-induced ABP-280 accumulation was dependent on calcium ions and on actin assembly. Cells loaded with 3 μM BAPTA/AM and exposed to force in calcium-
free buffer demonstrated no increase in ABP-280 at the bead adhesion sites. Similarly, cells incubated with 1 μM cytochalas-
in demonstrated no force-induced ABP-280 accumulation (Fig.
3C).

Regulation of ABP-280—ABP-280 has been described as a phosphoprotein (35), and the regulatory mechanism for the localization and actin association of ABP-280 involves serine phosphorylation (36, 37). To determine if ABP-280 was serine-phosphorylated in gingival fibroblasts, we immunoprecipitated ABP-280 and Western blotted for serine-phosphorylated pro-
tins in whole cell lysates. ABP-280 was serine-phosphorylated
during force application (Fig. 4A). Control experiments showed
that force did not increase total cell ABP-280 levels over the
experimental time frame (data not shown). We also examined
ABP-280 levels and the serine phosphorylation of ABP-280 in
proteins prepared from beads (Fig. 4B). Force increased ABP-
280 levels 4-fold in bead preparations. Serine-phosphorylated
ABP-280 was increased 8-fold by force indicating that the in-
creased phosphorylated ABP-280 seen at bead sites was not
simply due to the presence of more ABP-280 but also because of
an increased number of phosphorylated serine residues. As we
hypothesized that serine phosphorylation of ABP-280 may be a
critical regulatory step in force-induced actin cross-linking, the
protein kinase C (PKC) inhibitor bisindolylmaleimide (BIM; 5
μM) (26) was used to determine if force-induced serine phos-
phorylation was mediated through PKC (28). BIM decreased
the force-induced serine phosphorylation of ABP-280 (Fig. 4A)
and reduced the level of force-induced ABP-280 accumulation
at bead sites (Fig. 4B). BIM also reduced the amount of actin
accumulating at the force transfer sites. Notably, the only other
serine-phosphorylated protein present in the bead isolates was
approximately 70 kDa, but the level of serine phosphorylation
and accumulation of this 70-kDa protein at bead binding sites
appeared to be independent of PKC since BIM did not affect its
accumulation or phosphorylation state. To verify the role of
PKC in the force-induced actin reorganization, a second specific
PKC inhibitor calphostin C was used (27). Calphostin inhibited
the force-induced serine phosphorylation, ABP-280, and actin
accumulation to the same extent as the BIM (data not shown).

ABP-280 Knockouts—To verify that the actin accumulation
was dependent on ABP-280, a melanoma cell line that does not
express ABP-280 (M2) was used to examine the force-induced
actin redistribution. By using the single cell fluorescence
method described above, we found a >95% increase in force-
induced actin accumulation at bead sites from ABP-280+ cells
(97; p < 0.01) compared with only a 12% increase in the
ABP-280-deficient cells (p > 0.1; Fig. 5).

SACs—As suggested previously (3, 6), the cortical actin and
the force-induced actin accumulation in particular may play a
role in regulating stretch-activated ion channel (SAC) activity.
To assess this potential downstream mechanoprotective effect,
we measured calcium ion influxes through SACs following a
brief (1-s) force pulse. Net calcium influx levels in fibroblasts
following the actin/ABP-280 reorganization demonstrated a
68% decrease in the stretch-induced calcium ion influx (Fig. 6A;
No preforce versus Preforce: p < 0.01). The force-induced cyto-
skeletal dependent decrease was abolished when the cells
were treated with cytochalasin D which as we have demon-
strated previously prevents force-induced actin accumulation
(7) (Fig. 6A). We determined if there was a force time/dose-de-
pendent reduction in the decrease of the SAC response to force.
Increasing the length of the force exposure induced a time-de-
pendent decrease in SAC responses (Fig. 6B).
The regulation of SAC activity by ABP-280 was determined using the ABP-280-deficient cell line (M2) and the ABP-280+ control (A7). Preliminary experiments demonstrated that both cell lines exhibited nearly identical increases of [Ca\(^{2+}\)], when treated with 1 \(\mu\)M thapsigargin (to deplete internal calcium stores) or with 3 \(\mu\)M ionomycin, confirming that the absence of ABP-280 did not alter thapsigargin-sensitive internal stores or membrane pumps. The M2 line demonstrated a 70% greater calcium influx in response to a single brief (1-s) force application compared with the A7 cell line (Fig. 6C). Following a 20-min force treatment the M2 cell line exhibited a 50% reduction in the calcium ion influx, and the A7 line exhibited a 87% reduction in the calcium ion influx (Fig. 6C).

Since ABP-280 appeared to play an important role in force-induced cytoskeletal reorganization, we asked if ABP-280 may play a role in protecting cells from tension-induced damage. Cellular viability after 1 h of force exposure was determined for the M2 (ABP-280−) and the A7 (ABP-280+) cell lines using flow cytometry and propidium iodide exclusion. Exclusion of propidium iodide denotes cell viability due to an intact plasma membrane. We found that the cell line lacking ABP-280 had a greatly increased susceptibility to force-induced membrane leakage compared with the wild-type (M2 versus A7: \(p < 0.01\); Fig. 6C). Since the elastic modulus of the ABP-280− M2 cells is lower than that of A7 cells (18), application of equal stresses may be important for signal transduction and cellular mechanoprotective processes. We have used a magnetic bead model that allowed the application of defined, localized forces directly through integrin receptors to the actin cytoskeletal complex. This method also permits isolation of proteins that localize to these force transfer sites and thereby facilitates analyses of local changes during increased membrane and cytoskeletal tension. By using atomic force microscopy, we have previously demonstrated that this model induces localized changes in actin assembly that result in increased local rigidity. The localized changes in actin assembly occurred without causing detectable changes in global actin architecture or the global cellular balance of actin monomer/filament (6).

**DISCUSSION**

The important question of how cells protect themselves and adapt to increased environmental tension remains largely unanswered. By using a novel force application model (6), we studied actin recruitment to focal-like contacts through which the tension was applied. Our principal finding is that force induces a local actin accumulation at force transfer sites that is dependent on the co-localization and modification of ABP-280. Previous studies have focused on whole cell cytoskeletal changes during force application (5). These types of studies do not provide information on local changes in the cell cortex that may be important for signal transduction and cellular mechanoprotective processes. We have used a magnetic bead model that allowed the application of defined, localized forces directly through integrin receptors to the actin cytoskeletal complex. This method also permits isolation of proteins that localize to these force transfer sites and thereby facilitates analyses of local changes during increased membrane and cytoskeletal tension. By using atomic force microscopy, we have previously demonstrated that this model induces localized changes in actin assembly that result in increased local rigidity. The localized changes in actin assembly occurred without causing detectable changes in global actin architecture or the global cellular balance of actin monomer/filament (6).
these same cells, the localized mechanoprotective actin response was restored. The ABP-280 accumulation explains in part the localized increase in membrane rigidity following force application. ABP-280 has been shown to increase the rigidity of actin solutions in vitro (39). Furthermore, cells expressing ABP-280 have more than a 2-fold greater elastic modulus (cor-
tical rigidity) compared with corresponding ABP-280-deficient cells (18).

The requirement of ABP-280 for the force-induced actin accumulation implicates ABP-280 as an important mechanoprotective protein. The mechanism by which ABP-280 could locally increase actin accumulation involves at least three possible

**Fig. 7.** A, viability of melanoma cells as assessed by flow cytometry and propidium iodide (PI) staining. Viable cells excluded propidium iodide and were therefore not detected in the red channel. Application of force induced a 93% increase of propidium iodide-stained cells in ABP-280 negative (M2) cell line compared with a 46% increase in ABP-280 expressing (A7) cells ($p < 0.01$). B, actin-dependent processes. Phagocytosis is reduced during force application in gingival fibroblasts. Phagocytosis was measured using a collagen fluorescent bead assay and flow cytometry to assess the proportion of collagen phagocytosing cells. The reduction of phagocytosis was dependent on the magnitude of the force. C, force-dependent reduction of motility in gingival fibroblasts. The effect was dependent on the magnitude of the applied force. Motility was assessed using an in vitro wound system in which the ability of cells to migrate into a cell-free zone was measured over 15 h with or without force. The migratory index is expressed as the percent area of the cell-denuded wound which is repopulated during the experiment.
routes. First, as ABP-280 can increase actin polymerization/gelation rates in vitro (40), it is conceivable that localized increases of ABP-280 mediate an accumulation of F-actin. Second, ABP-280 is associated with F-actin binding to integral membrane proteins (41). Filamin has been localized to the membrane-associated ends of stress fibers in chicken fibroblasts (42) and ABP-280 may interact directly with integrins (17). Thus ABP-280 may increase the number of F-actin connections with the plasma membrane and thereby increase the number of membrane-bound polymerization sites. Third, since ABP-280 is a potent actin filament cross-linking protein (11), ABP-280 may simply increase the local F-actin content by cross-linking or attaching smaller filaments into an enlarged, local cortical complex.

**ABP-280 Regulation**—We demonstrated that the force-induced cytoskeletal response is dependent on calcium ions and actin polymerization since both chelation of free cytoplasmic calcium ions and cytochalasin D treatment inhibited the accumulations of both ABP-280 and actin. However, since ABP-280 was required for actin accumulation, we sought to determine what specific regulatory pathway was involved in the force response. As ABP-280 is a phosphoprotein with more than 380 serine/threonine residues (11), we hypothesized that phosphorylation may be an important regulatory process for localized accumulation in the bead complex (37). The data showed that ABP-280 was phosphorylated on serine residues following force application. From the amino acid sequence of ABP-280, 33 potential PKC sites have been deduced (11), and this suggested that ABP-280 may be phosphorylated by PKC. Indeed 10 of the 33 PKC phosphorylation sites are clustered near the N terminus which contains the actin-binding domain (36). This observation supports the idea that PKC may be involved in regulating the ability of ABP-280 to bind actin. We found that BIM and calphostin C, potent inhibitors of PKC, reduced the serine phosphorylation induced by force and also reduced the amount of ABP-280 localizing to the bead/force application site. This finding suggests that serine phosphorylation plays an important role in regulating ABP-280 force-induced actin binding and that PKC is one of the kinases involved in this event. Support for this regulatory mechanism comes from Wu and co-workers (36) who demonstrated the existence of four phosphorylated forms of ABP-280 in platelets and showed that the more phosphorylated form is able to cross-link twice as much actin as the lesser phosphorylated forms.

**SAC**—Mechanoprotective responses likely involve regulation of stretch-activated ion channels (SAC) since chronic force application without SAC desensitization could lead to pathologically high calcium levels (22). As we have previously demonstrated (3, 6), the actin cytoskeleton does play a regulatory role in SAC activation. In the present report, cells with localized cytoskeletal accumulation exhibited decreased SAC activity, an effect that was reversed by cytochalasin D. To determine if ABP-280 plays a role in regulating SAC sensitivity, we studied the stretch-induced calcium influx in ABP-280-deficient cells. There was a markedly increased calcium influx in the ABP-280-deficient cells compared with the ABP-280+ cells suggesting that ABP-280 reduces the open probability of SACs possibly through a tension absorption mechanism. Previous work with the same ABP-280-deficient cell line demonstrated increased basal permeability to K+ ions and the lack of a regulatory volume decrease in response to osmotically induced stretch. These alterations were thought to be caused by deficient linkages between the actin cortex and the membrane (43). Consistent with this hypothesis we suggest that ABP-280 and actin interactions are part of a sensing mechanism required to regulate ion transport at the plasma membrane.

**Membrane Stabilization**—Apparatus force applied appears to shift the actin monomer/filament equilibrium in cortical regions toward gelation which in turn promotes the formation of a protective shell. The protective nature of this response is suggested by the observation that compared with ABP-280+ cells, ABP-280-deficient cells demonstrated significant elevations of propidium iodide staining after increasing membrane tension indicative of plasma membrane disruption. The increased gelation in the cortical region also affects downstream actin-dependent events such as motility and phagocytosis. In an *in vitro* motility model, increased gelation due to higher levels of ABP-280 is associated with inhibition of filament velocity and reductions in the number of moving filaments (44). ABP-280-induced gelation of the actin cytoskeleton also dramatically inhibits the rate of gel contraction (45). Based on this previously mentioned work and our data, we suggest that the force-induced cross-linking of cortical actin filaments decreases actin filament turnover which is required for rapid ruffling and pseudopod extension (46).

The main finding in this report is that ABP-280 is recruited into cortical areas under increased tension, and in bead-associated sites ABP-280 promotes actin gelation and membrane stabilization. ABP-280-dependent actin accumulations may influence membrane deformability by the applied force, thereby damping deformation-based signaling and SAC activity. We conclude that ABP-280 plays an important role in cellular adaptation during increased environmental tension by structurally protecting the cell and by helping to modulate and regulate mechanotransduction signals.

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