Phosphorylation of Human Fascin Inhibits Its Actin Binding and Bundling Activities*

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HeLa 55-kDa actin-binding protein and sea urchin fascin are similar in that they both cause aggregation of F-actin into bundles and that their molecular masses are similar. Sea urchin fascin is a 58-kDa protein, first isolated from sea urchin eggs (1). Fascin is thought to act in the formation of microvillar cores at fertilization (2) and is involved in the formation of filopodia by coelomocytes, phagocytic defense cells of echinoderms (2). We have purified a 55-kDa actin-bundling protein from HeLa cells (3). Immunofluorescent studies have revealed that this human protein is localized in filopodia and membrane ruffles as well as in stress fibers (4), suggesting the involvement of this protein in the organization of these structures.

The molecular cloning of sea urchin fascin by Bryan et al., (5) showed that fascin has 35% identity at the amino acid level to the product of the Drosophila singed gene, a 55-kDa protein of heretofore unknown function. The derived amino acid sequence of sea urchin fascin shows homology to three peptide sequences of HeLa 55-kDa protein, which suggested that HeLa 55-kDa protein is a human homologue of sea urchin fascin. Two groups have independently cloned a human cDNA homologous to Drosophila singed. They have demonstrated, in collaboration with us, that HeLa 55-kDa protein is a homologue of Drosophila singed protein and sea urchin fascin (6, 7). Subsequently, mouse and Xenopus fascins have also been cloned (8). The amino acid sequences of these fascins share no apparent homology with other actin-binding proteins (including villin and fimbrin), indicating that the fascins represent a new family of actin-binding proteins.

It appears that each of these three proteins functions as an actin-bundling protein in microspikes and stress fibers of fibroblasts (4, 9), in filopodia of coelomocytes (2), and in bristles and nurse cells of Drosophila (10). Many Drosophila singed mutants exhibit two phenotypes: gnarled bristle development and female sterility (11). A common feature shared between the two phenotypes involves the association of the singed protein with actin fibers (10). In bristles, actin-containing filament bundles that are oriented along the length of the shaft surround microtubules in the center of the bristle. In singed mutants, a decreased number of microfilament bundles are observed, resulting in a short, curved bristle shaft (10, 12). In Drosophila oogenesis, each developing oocyte is surrounded by and connected by intercellular bridges to 15 nurse cells (13). Nurse cell cytoplasmic contents flow into the oocyte along actin filaments traversing these cytoplasmic bridges. A singed allele associated with female sterility has been reported to affect the microfilament structure required for this nurse cell cytoplasmic flow (10).

It is not clear what regulates the actin binding and bundling activities of human fascin. This report shows that human fascin is phosphorylated in vivo up to 0.30 mol/mol of protein by TPA treatment. Human fascin can also be phosphorylated in vitro by PKC when the pH is lowered to 6.0. In vitro phosphorylation occurs at the same sites as observed in vivo as judged by phosphopeptide mapping. Phosphorylation greatly reduces the actin binding activity of human fascin, although lowering the pH to 6.0 alone does not affect fascin-actin binding. With the incorporation of 0.25 mol of phosphate/mol of protein, the actin binding affinity is reduced from 6.7 to 1.5 × 10^6 M^-1. The actin bundling activity is also decreased. These results suggest that phosphorylation of fascin plays a role in actin reorganization after treatment with 12-O-tetradecanoylphorbol-13-acetate.

MATERIALS AND METHODS

Cell Culture—Human neuroblastoma cells (ATCC HTB11 SK-N-SH) were used in this study. Cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum in an atmosphere of 5% CO2 and 95% air at 37°C. Cells were used in this study. Cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum in an atmosphere of 5% CO2 and 95% air at 37°C.

32P or 35S Labeling and Immunoprecipitation—For 32P labeling, cells (one 100-mm dish, 70–80% confluency) were incubated for 2.5 h in phosphate-free Dulbecco’s modified Eagle’s medium containing 0.18 mCi/ml [32P]orthophosphoric acid and 10% dialyzed newborn calf serum, and then TPA (800 μM dissolved in dimethyl sulfoxide) was added to a final concentration of 240 nM. Cells were continuously labeled until the indicated times. After washing three times with phosphate-free Dulbecco’s modified Eagle’s medium, cells were lysed in 50 μl of hot 2

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The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; MES, 4-morpholineethanesulfonic acid.
In vivo phosphorylation of human fascin upon treatment with TPA. Human neuroblastoma cells were labeled with $^{32}$P for 2.5 h and then treated with TPA for 2 h. TPA was not added to control cells. Human fascin (indicated by 55k) was immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis. Lane1, fascin from control cells; lane2, fascin from TPA-treated cells. Molecular mass markers are indicated to the left (from top to bottom: 200, 116, 96, 68, 42, 30, and 21 kDa).

× SDS sample buffer (2% SDS, 15% glycerol, 100 mM dithiothreitol, 80 mM Tris-HCl, 0.003% bromphenol blue (pH 6.8) and stored at –70°C. Control cells were labeled under the same conditions except for the addition of TPA and were processed in the same way.

For $^{35}$S labeling, cells (one 100-mm dish, 70–80% confluency) were incubated in methionine-free Dulbecco's modified Eagle's medium containing 55 µCi/ml Tran$^{35}$S-label (ICN) and 10% dialyzed newborn calf serum. Cells were treated with TPA in the same way as described above.

Immunoprecipitation was performed with two monoclonal antibodies (clone 55k-2 and 55k-14), which have been characterized previously (4). After being thawed quickly, the samples lysed in the SDS sample buffer were first diluted 10 times with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 8 mM Na$_2$HPO$_4$ (pH 7.3) containing 50 mM NaF, homogenized by several passages through a 25-gauge needle, and then centrifuged at 100,000 × g for 30 min. To the supernatants containing the same total radioactivities was added 0.025 volume of asites fluids of the mononclonal antibodies. After incubation at 4°C for 1.5 h, rabbit antibodies against mouse IgG that had been conjugated to protein A-Sepharose were added and further incubated for 4°C. After washing, one-half of the immune complexes were loaded on one- or two-dimensional gels and analyzed by autoradiography. Human fascin is focused as multiple spots on two-dimensional gels (3).

To unambiguously identify fascin spots on two-dimensional gels, the other half of the immunoprecipitate samples were mixed with purified, nonradioactive HeLa fascin and separated on two-dimensional gels, and fascin spots were identified by comparison of the patterns detected by Coomassie Blue staining and by autoradiography. The amounts of radioactivity in $^{35}$S- or $^{32}$P-labeled protein spots separated on one- or two-dimensional gels were quantitated with a PhosphorImager. In some experiments, phosphorylated fascin was dephosphorylated by treatment with calcineurin under the following conditions: 50 mM imidazole HCl (pH 7.0), 0.1 mM MgCl$_2$, 0.1 mM CaCl$_2$, 1 mM MnCl$_2$, 0.5 mM dithiothreitol, 0.4 mM 4-(2-aminoethyl)benzenesulfon fluoride hydrochloride, and 10 µg/ml calcineurin.

Actin Binding and Bundling Assays—Human fascin purified from HeLa cells was phosphorylated at pH 6.0 as described above, and as a control, fascin was incubated in the pH 6.0 MES buffer in the same way except for the addition of PKC. Both samples were examined for their actin binding and bundling activities as described (3). Briefly, 12 µg F-actin was mixed with varying concentrations of phosphorylated or unphosphorylated human fascin in 20 mM imidazole buffer containing 100 mM KCl, and the pH values of the mixtures were adjusted to pH 7.0. After incubation at room temperature for 1 h, the mixtures were centrifuged in a Beckman Airfuge at 25,000 g for 30 min for the actin binding assay. For the actin bundling assay, the mixtures were centrifuged in an Eppendorf centrifuge for 15 min. Both supernatants and pellets were analyzed by SDS-polyacrylamide gel electrophoresis. The intensities of the 55-kDa bands on stained SDS gels and autoradiographs were analyzed with a densitometer (Chromoscan 3, Joyce-Loebl and Co.) as described (34). Actin bundling activities were confirmed by electron microscopy.

Other Procedures—Two-dimensional phosphopeptide mapping and phosphoamino acid analyses were performed as described previously (21). Two-dimensional gel electrophoresis was performed as described (3). Immunofluorescence was performed as described (4). Protein concentrations were determined using the Bradford assay (15).

RESULTS

In Vivo Phosphorylation of Human Fascin—We investigated, using human neuroblastoma cells, whether human fascin is phosphorylated under a variety of conditions including treatment with TPA (PKC activator), forskolin (protein kinase A activator), or platelet-derived growth factor. We have found that human fascin is phosphorylated to a considerable extent by treatment with TPA, but not with the other agents. As Fig. 1 shows, fascin in the immunoprecipitate is found to be phosphorylated by treatment with calcineurin under the following conditions: 50 mM imidazole HCl (pH 7.0), 0.1 mM MgCl$_2$, 0.1 mM CaCl$_2$, 1 mM MnCl$_2$, 0.5 mM dithiothreitol, 0.4 mM 4-(2-aminoethyl)benzenesulfon fluoride hydrochloride, and 10 µg/ml calcineurin.

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We examined the time course of fascin phosphorylation after TPA treatment. As Fig. 2 shows, fascin phosphorylation is not a rapid reaction, gradually increasing over 2 h after TPA treatment. To correlate fascin phosphorylation with changes in the localization of fascin, we have immunolocalized fascin during...
the time course of fascin phosphorylation (Fig. 3). Human neuroblastoma cells are heterogeneous, consisting of two kinds of cells: spindle-shaped cells with long neurite-like extensions (A) and well spread cells with stress fibers and short microspikes (B). Both structures are stained with the fascin monoclonal antibody. C and D, 10 min after TPA treatment. Long neurite-like extensions rapidly disappear; instead, numerous membrane ruffles (indicated by arrowheads) stained with the fascin antibody are induced (C). Stress fibers, which are stained with the fascin antibody, are more resistant to TPA (D). E and F, 1 h after TPA treatment. Fascin staining of membrane ruffles disappears, and cells become more rounded (E). Stress fibers also disappear (F).

Wenextdeterminedthetoichimetryofin vivo phosphorylation in the following way. Because human fascin is known to be focused as multiple spots on two-dimensional gels (3), we first identified, by in vivo $^{32}$P labeling, which spots represent phosphorylated isoforms of human fascin. Then, by labeling in vivo with $[^{35}S]$methionine followed by two-dimensional gel separation, the level of both phosphorylated and unphosphorylated isoforms of fascin can be determined by PhosphorImager analysis. Fig. 4A shows a Coomassie Blue-stained two-dimensional gel of purified, nonradioactive HeLa fascin for reference, which exhibits two major basic and two minor acidic spots (designated as spots a–d from basic to acidic). Fascin was immunoprecipitated from $^{32}$P-labeled cells after 2 h of TPA treatment. Fig. 4B shows an autoradiograph of a two-dimensional gel of the immunoprecipitates where five spots are observed (spots c–g). Co-electrophoresis of the $^{32}$P-labeled immunoprecipitates with unlabeled human fascin (data not shown) revealed that two (Fig. 4B, spots c and d) of the five phosho-
In vitro phosphorylation of human fascin by PKC. Human fascin (indicated by 55K) purified from HeLa cells was incubated with PKC under the conditions described under "Materials and Methods." A, one-dimensional gel analyses. Lanes 1 and 2, Coomassie Blue staining; lanes 3 and 4, corresponding autoradiography. Lanes 1 and 3, with PKC; lanes 2 and 4, without PKC. Molecular mass markers are indicated by the arrowheads to the left and are the same as described for Fig. 1. B, two-dimensional gel analyses of in vitro phosphorylated human fascin. Panel 1, Coomassie Blue staining; panel 2, corresponding autoradiography.

Fig. 5. In vitro phosphorylation of human fascin by PKC. Human fascin (indicated by 55K) purified from HeLa cells was incubated with PKC under the conditions described under "Materials and Methods." A, one-dimensional gel analyses. Lanes 1 and 2, Coomassie Blue staining; lanes 3 and 4, corresponding autoradiography. Lanes 1 and 3, with PKC; lanes 2 and 4, without PKC. Molecular mass markers are indicated by the arrowheads to the left and are the same as described for Fig. 1. B, two-dimensional gel analyses of in vitro phosphorylated human fascin. Panel 1, Coomassie Blue staining; panel 2, corresponding autoradiography.

In vitro phosphorylation of human fascin by PKC—TPA treatment is known to activate PKC in vivo (17). We thus investigated whether PKC phosphorylates human fascin in vitro. As Fig. 5A (lanes 1 and 3) shows, PKC does phosphorylate human fascin in vitro. HeLa fascin preparation alone has no kinase activity because human fascin is not phosphorylated without the addition of PKC (lanes 2 and 4). Two-dimensional gel analyses revealed that in vitro phosphorylated fascin yields the same phosphoprotein pattern as observed in vivo phosphorylated fascin. In Fig. 5B, panels 1 and 2 show the Coomassie Blue-stained gel and its corresponding autoradiograph, respectively. Again, five phosphoprotein spots (spots c–g) are identified on the two-dimensional gel (panel 2), of which two (spots c and d) comigrate with acidic minor forms of purified human fascin (panel 1), and two major basic spots (spots a and b) are not phosphorylated. The more acidic spots (spots e–g) are either barely detected or not detected on the Coomassie Blue-stained gel (panel 1). It should be noted here that phosphorylation was performed at pH 7.0, under which condition human fascin is poorly phosphorylated as described below, and thus, an increase in the level of phosphorylated spots (spots c–e) is not evident.

In vitro phosphorylation of fascin by PKC depends greatly on pH (Fig. 6). At pH 7.0 and above, only 5% of fascin is phosphorylated, which initially made us think that fascin is a poor substrate of PKC. However, the stoichiometry of fascin phosphorylation is greatly improved as the pH is lowered, while the activity of PKC itself is decreased. At pH 6.0, ~38% of fascin is phosphorylated within 20 min, and 60% of fascin is phosphorylated at pH 5.0. In contrast, phosphorylation of myosin light

rylated spots comigrate with the two minor acidic forms (Fig. 4A, spots c and d), but not with the two major basic forms (spots a and b), of purified HeLa fascin. The three most acidic spots (Fig. 4B, spots e–g) are too minor to be detected on the Coomassie Blue-stained two-dimensional gel pattern of the purified protein (Fig. 4A). These are, however, isoforms of human fascin because Western blotting using enhanced chemiluminescence (Amersham Corp.) detected these minor spots on two-dimensional gels (data not shown).

With the identification of phosphorylated isoforms of human fascin on two-dimensional gels, we determined the stoichiometry of in vivo phosphorylation of human fascin after a 2-h treatment of human neuroblastoma cells with TPA. Cells were labeled with [35S]methionine in the presence or absence of TPA, and fascin was immunoprecipitated and separated on two-dimensional gels. Fig. 4 (C and D) shows the two-dimensional gel patterns of human fascin in the absence and presence of TPA, respectively. Again, the identification of these spots as fascin was confirmed by co-electrophoresis of radioactive spots of TPA, respectively. Again, the identification of these spots as human fascin because Western blotting using enhanced chemiluminescence (Amersham Corp.) detected these minor spots on two-dimensional gels (data not shown).

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chain, a good substrate of PKC, is decreased by 30% when the pH is decreased from 7.0 to 5.0. The increase in phosphorylation of fascin at lower pH values is probably not due to the activation of contaminant kinases because a PKC peptide inhibitor (Sigma) inhibits phosphorylation reactions of both fascin and myosin light chain to the same extent at pH 6.0. Inhibition of Actin Binding by Phosphorylation—We examined the effects of phosphorylation on fascin-actin binding and found that phosphorylation by PKC inhibits actin binding of fascin. Fascin was first phosphorylated at pH 6.0 with PKC to a molar ratio of 0.25 mol of phosphate/mol of protein, and then actin binding was examined at pH 7.0. As a control, fascin was treated in the same way except that PKC was not added to the phosphorylation reaction mixtures.

Fig. 8 shows actin binding of unphosphorylated (lanes 1–4) and phosphorylated (lanes 5–8) fascin at two different fascin concentrations (1 μM total fascin for lanes 1, 2, 5, and 6; 1.5 μM total fascin for lanes 3, 4, 7, and 8). Coomassie Blue staining (lanes 1–8) revealed that the levels of free fascin in the supernatants with phosphorylated fascin (lanes 5 and 7) are higher than those with control unphosphorylated fascin (lanes 1 and 3) (compare lane 5 with lane 1 and lane 7 with lane 3), indicating that phosphorylated fascin shows a lower actin affinity. Furthermore, the autoradiographs (lanes 9–12) corresponding to the Coomassie Blue-stained gels (lanes 5–8) show that most of the phosphorylated fascin remains in the supernatants (lanes 9 and 11).

We determined the level of radioactivity of fascin in the pellets and supernatants after actin binding. The phosphate incorporation of the original phosphorylated sample is 0.25 mol/mol of protein. After actin binding, the phosphate incorporation of fascin in the supernatant (Fig. 8, lanes 5 and 9) is increased to 0.71 mol of phosphate/mol of protein, while that in the pellet (lanes 6 and 10) is decreased to 0.07 mol/mol of protein. These results indicate that phosphorylated fascin has a greatly reduced actin binding activity.
We used 25% phosphorylated fascin to estimate its actin binding constant. Fig. 9 shows a comparison of actin binding between unphosphorylated and phosphorylated fascin. Phosphorylated fascin shows reduced actin binding. The reduction in actin binding is not due to denaturation caused by pH 6.0 as judged from the following results. First, the actin binding affinity of fascin is not affected by the pH range from 7.5 to 6.0. Second, dephosphorylation of phosphorylated fascin by treatment with calcineurin (protein phosphatase 2B) restores actin binding. Although fascin is not completely dephosphorylated by this treatment, phosphate incorporation is decreased from 0.25 to 0.1 mol of phosphate/mol of protein. As Fig. 9 shows (open square marked with an arrowhead), fascin treated with phosphatase shows a higher affinity than does untreated phosphorylated fascin, indicating that phosphorylation indeed regulates actin binding of fascin.

Fig. 9. Effects of phosphorylation by PKC on actin binding of human fascin. Fascin was phosphorylated at pH 6.0 to a ratio of 0.25 mol/mol of protein, and then actin binding of phosphorylated fascin (●) was assayed at pH 7.0 as described under "Materials and Methods." As a control, fascin was incubated in the same way except for the addition of PKC and assayed for actin binding (○). To examine the effect of dephosphorylation, 1 μM phosphorylated fascin was dephosphorylated by treatment with calcineurin and examined for actin binding (indicated by the open square marked with an arrowhead). Note that dephosphorylation restores actin binding of fascin.

Fig. 10. Scatchard plotsof actin binding of phosphorylated (●) and control (○) fascin. Concave-down shapes for the curves indicate that actin binding of both phosphorylated and unphosphorylated fascin is cooperative.

Finally, we examined the effects on actin bundling activities. As Fig. 11 shows, the actin bundling activity of phosphorylated fascin is considerably lower than that of the control. The concentrations required for the half-maximal bundling activity are 1.1 μM for unphosphorylated fascin and 1.6 μM for phosphorylated fascin (0.25 mol of phosphate/mol of protein).

DISCUSSION

We have shown in this paper that 1) fascin is phosphorylated in vivo up to 30% after TPA treatment; 2) PKC is able to phosphorylate fascin in vitro at the same sites as observed in vivo; and 3) in vitro phosphorylation greatly reduces fascin-actin binding. TPA treatment is known to induce movement of membrane ruffles and to cause disassembly of stress fibers in many cells (16). Human neuroblastoma cells also show similar but complicated effects upon TPA treatment (Fig. 3). In a short time (10 min) after TPA treatment, when the phosphorylation level is still low, long neurite-like extensions are rapidly retracted; instead, numerous membrane ruffles are induced, which can be strongly stained with fascin antibody. Stress fibers are disassembled gradually over 2 h of incubation, which apparently causes rounding of most cells. Concomitantly, fascin staining of stress fibers as well as membrane ruffles disappears, resulting in more diffuse staining in the cytoplasm. The maximum level of phosphorylation roughly corresponds to these alterations of fascin distribution. These observations suggest that fascin phosphorylation may play a role in the dissociation of fascin from stress fibers and membrane ruffles, but does not appear to be involved in the initial rapid retraction of neurite-like extension upon TPA treatment.

In vivo phosphorylation of fascin is substoichiometric (~30%). However, the actin binding experiments with partially...
phosphorylated fascin (Fig. 9) have shown that the actin binding affinity is reduced to one-fourth of its normal level, suggesting that phosphorylation at this level can have a considerable effect on fascin-actin interactions. Furthermore, there have been some reports that substoichiometric phosphorylation produces biological effects. For example, phosphorylation of only 5 or 20% (depending on the source of smooth muscle) of myosin light chain is sufficient for tension development in smooth muscle (18, 19). We have also reported (22) that 15–30% of myosin light chain is phosphorylated at Ser-1/Ser-2 during mitosis, which is suggested to inhibit myosin from premature activation before cytokinesis.

It is interesting to note that phosphorylation of human fascin with PKC depends greatly on pH. The optimal pH of PKC is known to be pH 7.5, and indeed, myosin light chain is better phosphorylated by PKC at pH 7.0 or above. Thus, the pH dependence of fascin phosphorylation should not be attributed to the pH-dependent activity of PKC itself. Rather, it is likely that fascin phosphorylation sites become more accessible to PKC at a lower pH. Lowering the pH to 6.0 alone does not affect the actin binding ability, suggesting that fascin is not denatured at this pH. The observation that actin binding ability is recovered by dephosphorylation with calcineurin (protein phosphatase 2B) treatment confirms that phosphorylation actually regulates actin binding of fascin.

At present, we cannot conclude that PKC is responsible for phosphorylation of fascin in vivo. It is possible that local changes in pH may occur where both fascin and PKC are present. Indeed, some isoforms of PKC are known to be localized in microfilament structures (20). However, it is not known whether pH drops upon TPA treatment. Furthermore, the time course of PKC activation upon TPA treatment does not appear to coincide with the time course of fascin phosphorylation. Further studies are required to identify whether PKC or another kinase (or kinases) is involved in fascin phosphorylation in vivo.

Phosphorylation appears to have a drastic effect on actin binding of fascin. The actin binding assay (Fig. 11) shows that the actin binding constant was decreased 4-fold with the incorporation of 0.25 mol of phosphate/mol of human fascin. However, this actin binding constant was obtained with a mixture of 75% unphosphorylated and 25% phosphorylated human fascin. Densitometry of the Coomassie Blue-stained gel shown in lanes 5 and 6 of Fig. 8 revealed that ~72% of fascin binds to actin. On the other hand, densitometry of the autoradiograph of the same lanes showed that only 20% of radioactive fascin binds to actin. Thus, if we were able to use 100% phosphorylated fascin, we would find a much reduced actin binding constant, perhaps >10 times lower than that of unphosphorylated fascin.

We have recently identified one of the sites of human fascin phosphorylation as Ser-39.2 This site is well conserved among many fascins, including human, mouse, Xenopus, and Drosophila (sea urchin fascin has a Thr residue instead). Furthermore, the sequence around this site is one of the most conserved domains of these fascins, suggesting its importance in the functions of fascin. We are in the process of generating fascin mutants with the Ser residue replaced by Ala or Asp. It will be very interesting to see whether the expression of such mutants changes the organization of filopodia, membrane ruffles, and stress fibers. Such studies will facilitate the understanding of the physiological significance of phosphorylation of fascin in microfilament organization and cell motility.

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