Fascin is a 55–58-kDa actin-bundling protein, the actin binding of which is regulated by phosphorylation (Yamakita, Y., Ono, S., Matsumura, F., and Yamashiro, S. (1996) J. Biol. Chem. 271, 12632–12638). To understand the mechanism of fascin-actin interactions, we dissected the actin binding region and its regulatory site by phosphorylation of human fascin. First, we found that the C-terminal half constitutes an actin binding domain. Partial digestion of human recombinant fascin with trypsin yielded the C-terminal fragment with molecular mass of 32, 30, and 27 kDa. The 32- and 27-kDa fragments purified as a mixture formed a dimer and bound to F-actin at a saturation ratio of 1 dimer:11 actin molecules with an affinity of 1.4 × 10⁶ M⁻¹. Second, we identified the phosphorylation site of fascin as Ser-39 by sequencing a tryptic phosphopeptide purified by chelating column chromatography followed by C-18 reverse phase high performance liquid chromatography. Peptide map analyses revealed that the purified peptide represented the major phosphorylation site of in vivo phosphorylated fascin. The mutation replacing Ser-39 with Ala eliminated the phosphorylation-dependent regulation of actin binding of fascin, indicating that phosphorylation at this site regulates the actin binding ability of fascin.

Bundles of actin filaments, found in many cellular structures, including stress fibers, contractile rings, microvilli, and microspikes at the leading edges of motile cells, are formed mainly due to the functions of actin-bundling or cross-linking proteins. To date, a number of actin-bundling or cross-linking proteins have been characterized and demonstrated to play different roles in each of the cellular structures (for reviews, see Refs. 1 and 2).

The 55-kDa actin-bundling protein from HeLa cells has been purified as a major component of actin gels, which are formed by adding exogenous actin into cytoplasmic extracts (3). It binds to actin in a stoichiometry of 1:4 and cross-links F-actin into bundles in vitro. The protein is a globular and monomeric protein (3). Immunocytological study has revealed that the 55-kDa protein is localized both in stress fibers and microspikes in cultured cells (4), suggesting that this protein is involved in the formation of actin bundles in vivo.

The in vitro characteristics of the HeLa 55-kDa protein is very similar to that of sea urchin fascin. Sea urchin fascin, isolated from cytoplasmic extracts of sea urchin eggs (5, 6), is the first actin-bundling protein to be characterized in vitro (7). Fascin is recruited to the cortical cytoskeleton to form microvilli at fertilization of eggs (8) and filopodia of coelomocytes, phagocytic cells from the coelomic cavity (9, 10). Those structures have actin bundle cores with an 11-nm transverse cross-banding pattern (11, 12), which is also observed in actin bundles formed with fascin in vitro (7). Thus, those studies have demonstrated that echinoid fascin plays crucial roles in forming actin bundles.

Molecular cloning of sea urchin fascin by Bryan et al. (13) has revealed that fascin has sequence similarity to the Drosophila singed gene (sn) product (14), whereas fascin shows no apparent homology with the other known actin-binding proteins. Subsequently, the HeLa 55-kDa actin-bundling protein has been demonstrated to be a human homologue of the sn product and sea urchin fascin (15, 16). Therefore, we will henceforth designate the HeLa 55-kDa actin-bundling protein as human fascin. Recently, fascin cDNAs have been cloned from Xenopus (17) and mouse (18), indicating that fascin constitutes a widely distributed gene family.

Drosophila singed mutants exhibit gnarled bristle development and female sterility (19). In normal Drosophila bristles, 7–11 bundles of microfilaments are attached to the plasma membrane. However, in developing bristles of sn mutants, the number of actin bundles is greatly reduced and disorganized (20, 21). Cant et al. (22) have shown that the recombinant sn gene product (Drosophila fascin) can produce actin bundles with 12-nm transverse cross-banding, which is also observed in the microfilament bundles in bristles. In addition, Drosophila fascin is present in normal bristles, but the absence of Drosophila fascin in sn mutants correlates with the development of gnarled bristles. Female sterility of sn mutants is caused by the defects in the transfer of cytoplasm from nurse cells to the oocytes. The nurse cells of sn mutants lack cytoplasmic actin bundles, so that the nurse cell nuclei are allowed to plug up the ring canals to block cytoplasmic transfer (22).

There must be mechanisms that control fascin-actin interactions in a variety of biological activities. For example, actin bundles containing fascin are formed in response to cell adhesion to thrombospondin 1 (24), fertilization of sea urchin eggs...
(25), and treatment of starfish oocytes with the maturation-inducing hormone 1-methyladenine (25). Filopodia of fibroblasts that contain fascin-actin bundles are known to be highly motile structures, constantly extending and retracting during cell locomotion. We have found that fascin is phosphorylated in vitro on 12-O-tetradecanoylphorbol-13-acetate treatment and also in vitro by protein kinase C (PKC) \(^1\) at the same sites (26). Phosphorylation of fascin results in the reduction of its actin binding ability, suggesting that phosphorylation may be one of the mechanisms that regulate fascin-actin interactions (26).

To further understand the fascin-actin interaction and its regulation, we dissected the actin binding domain and PKC phosphorylation site of fascin. We found that at least one of the actin-binding sites was located in the C terminus, and that the major PKC phosphorylation site was in the N terminus (Ser-39). We generated a mutant fascin replacing the phosphorylation site with Ala. Actin binding of the mutant was no longer regulated by phosphorylation, indicating that phosphorylation at the N terminus regulates the fascin-actin interaction.

**EXPERIMENTAL PROCEDURES**

**Materials**—The HeLa 55-kDa protein (human fascin) was prepared from HeLa cells as described (3). Actin was prepared from rabbit skeletal muscle as described (27). PKC was purchased from Biomol (Plymouth Meeting, PA). \(^{32}P\)ATP was from Amersham Corp. Trypsin (treated with 1-tosylamide-2-phenylhydrazine) was from Worthington. Human thrombin was from Boehringer Mannheim.

**Preparation of Recombinant Human Fascin**—The bacterial expression vector for human fascin was constructed by cloning the Smal fragment of human fascin cDNA (1.7 kilobases) into pGEX-2T to make a fusion protein with glutathione S-transferase (GST) (15). E. coli strain JRB600 carrying the plasmid was grown in Luria Bertani medium containing 50 \(\mu\)g/ml ampicillin at 37°C until the \(A_{660}\) reached 0.6. Protein expression was induced by adding 0.1 mM isopropyl \(\beta\)-D-thiogalactopyranoside at 25°C for 3 h. Cells were harvested by centrifugation and disrupted by sonication in phosphate-buffered saline plus 1 mM dithiothreitol (DTT). The lysate was centrifuged at 10,000 rpm for 20 min (Beckman Instruments JA-20 rotor), and the supernatant was applied to a glutathione-Sepharose 4B column (Pharmacia Biotech Inc.), which had been equilibrated with phosphate-buffered saline plus 1 mM dithiothreitol (DTT). The column was washed with 0.1 M Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0. The eluate was dialyzed at pH 6.1, which contained most of the radioactivity (Fig. 5), was lyophilized, dissolved in 0.06% trifluoroacetic acid, and applied to a C-18 reverse phase high performance liquid chromatography column (Delta-Pak C18, 300 Å, 2 × 150 mm, Millipore Waters), which had been equilibrated with eluent A (0.06% trifluoroacetic acid). The column was developed with a linear gradient of 0–100% eluent B (80% acetonitrile, 0.052% trifluoroacetic acid) over 105 min at 0.2 ml/min. Absorbance data were processed and analyzed with Millennium 2010 (Millipore Waters). The radioactivity was counted in the Gerenkov manner.

**Site-directed Mutagenesis of Human Fascin**—The Smal fragment of human fascin cDNA (1.7 kilobases) was cloned into pTZ18U and then used for oligonucleotide-directed *in vitro* mutagenesis using a Mutagen phagemid in *E. coli* (Bio-Rad). An oligonucleotide, 5′-GAACGCGTCCGCCAGCGCCCTGAAGAAGAAGCAG-3′, was used as a primer to mutate Ser-39 to Ala. The mutation was confirmed by DNA sequencing. The mutated insert was subcloned into a pGEX-2T vector and used for protein expression. The mutant fascin was expressed and purified as described above for wild-type r-fascin.

**Other Procedures**—Two-dimensional tryptic phosphopeptide mapping was performed as described (34). Protein concentrations were determined by BCA protein assay reagent (Pierce) using bovine serum albumin as a standard. SDS-PAGE was performed by the method of Blatter et al. (29) with 12.5% polyacrylamide slab gels, except that the buffer system of Laemmli (30) was used. Microsequencing of fascin C-terminal fragments and the purified phosphopeptide was as described (16). For electron microscopy, one drop of sample solution was put onto a carbon-coated Formvar grid and negatively stained with 1% uranyl acetate. Samples were observed by JEOI 100CX II at an accelerating voltage of 80 kV.

**RESULTS**

**Production and Characterization of Recombinant Fascin**—We have modified a method using bacterial expression of r-fascin to obtain active protein. In a previous attempt to produce r-fascin as a fusion protein with GST, no soluble protein was recovered (15). However, we found that the GST-fascin fusion protein was expressed as a soluble protein in the same system when the expression was induced at a low temperature (25°C). Then, r-fascin was successfully purified by glutathione affinity column chromatography, followed by cleavage of the GST portion with thrombin digestion. Although r-fascin is an extra-peptide (GSPGPRSGLSSTAT) at the N terminus, which was derived from the 5′-untranslated region, r-fascin showed an actin bundling activity similar to that of authentic fascin purified from HeLa cells. As Fig. 2 shows, when purified r-fascin was incubated with F-actin, F-actin became pelletable in low speed centrifugation (Fig. 24), and bundles of F-actin were

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1 The abbreviations used are: PKC, protein kinase C; GST, glutathione S-transferase; DTT, dithiothreitol; r-fascin, recombinant fascin; PAGE, polyacrylamide gel electrophoresis; MES, 2-(N-morpholino)ethanesulfonic acid; MARCKS, myristoylated alanine-rich C-kinase substrate.
observed by electron microscopy (Fig. 2B). It should be noted that the GST-fascin fusion protein also showed similar actin-bundling activity, indicating that the addition of extra sequences to the N terminus does not seem to affect the actin-bundling activity of fascin.

The C-terminal Tryptic Fragments of r-Fascin Bind to F-actin—Previous studies (3) have shown that fascin is a globular and monomeric actin-bundling protein, suggesting that it has at least two actin binding sites in a single molecule. To define the actin binding sites of fascin, we first constructed several N- or C-terminal deleted forms of fascin in a bacterial expression system to test whether those truncated forms bind to actin. However, all efforts to produce truncated proteins with binding activity have failed, despite our success in producing fully active, full-length fascin in bacteria. Even a small truncation (less than 20 amino acids) at either the N or C terminus resulted in the complete loss of actin binding activity. Because such negative results are fruitless, we applied a classic proteolysis method for the dissection of the fascin domain structure.

Partial digestion of r-fascin with trypsin yielded three fragments with Mr values of 27,000, 30,000, and 32,000, (Fig. 3A). The same fragments were observed by digesting authentic HeLa fascin with trypsin (data not shown), suggesting that r-fascin has the same conformation as authentic HeLa fascin. The N terminus sequencing of these fragments showed that all three fragments were derived from the C terminus of fascin; both 32- and 30-kDa fragments started from residue 248, and the 27-kDa fragment started from residue 277 (Fig. 3B). These results indicate that they constitute approximately the C-terminal half of the molecule, and it is noted that the 30-kDa fragment appears to lack a small portion of the C terminus. The N-terminal fragments appear to be digested quickly, because no N-terminal fragments were obtained by trypsin digestion.

We found that all three tryptic C-terminal fragments coprecipitated with F-actin together with undigested r-fascin when the trypsin digests were incubated with F-actin. This observation suggests that the C terminus had at least one actin binding site. The actin binding of 30-kDa fragments suggests that the very end of the C terminus may not be required for actin binding. To exclude the possibility that the coprecipitation of the C-terminal fragments with F-actin is due to complex formation between the fragments and undigested fascin, we separated the C-terminal fragments from undigested fascin and examined their actin binding abilities. Both 32- and 27-kDa fragments were purified as a 1:1 (molar ratio) mixture, whereas the 30-kDa fragment was lost apparently due to digestion during purification, as no band of 30 kDa was detected by SDS-PAGE in any fractions.

An actin binding assay revealed that both 32- and 27-kDa fragments bound to F-actin in the absence of undigested fascin (Fig. 4A). The mixture of the 32- and 27-kDa C-terminal fragments also showed actin bundling activity. The fragments appeared, however, to exist as a dimer, as judged from gel filtration chromatography, and therefore, we could not conclude whether the fragments had one or two actin binding sites,

![Fig. 1. Purification of recombinant human fascin. Lysates of bacteria expressing GST-fascin (lane a) were applied to a glutathione-Sepharose 4B column. GST-fascin eluted from the glutathione-Sepharose 4B column (lane b) was digested with thrombin (lane c). r-Fascin (arrowhead F) was purified by the adsorption of cleaved GST to the same glutathione-Sepharose 4B column (lane d). Molecular mass markers in kDa are indicated on the left.](image1)

![Fig. 2. F-actin bundling activity of r-fascin. A, F-Actin (0.5 mg/ml) was incubated without (−) or with (+) r-fascin (0.1 mg/ml) for 1 h at room temperature. The mixtures were centrifuged for 10 min at 12,000 × g (Eppendorf Microfuge). Supernatants (s) and pellets (p) were analyzed by SDS-PAGE. In the presence of r-fascin, F-actin formed bundles and became pelletable. Molecular mass markers in kDa are indicated on the right. B, Electron micrograph of actin bundles formed with r-fascin (0.2 mg/ml) and F-actin (0.5 mg/ml). Bar, 0.2 μm.](image2)
although further digestion yields no smaller fragments that retained actin binding ability. These results indicate that the C-terminal half of fascin has at least one actin binding domain.

The stoichiometry of actin binding was determined by varying concentrations of purified fragments. Because both 32- and 27-kDa fragments bound to F-actin indistinguishably, these two fragments were treated as the same molecule and as a dimer with a $M_r$ of 59,000. As Fig. 4 shows, actin binding of the C-terminal fragments was saturated at a molar ratio of 1 dimer:11 actin molecules, and the apparent binding constant was estimated to be $1.4 \times 10^6 \text{ M}^{-1}$, which is 2.5 times lower than that of intact r-fascin (see Fig. 9).

We also used other proteolytic enzymes, including Lys-C, V8 protease, and chymotrypsin, to yield fragments containing different domains with partial actin binding ability. Although such digestion yielded different proteolytic patterns, the proteolytic fragments did not show significant actin binding ability.

Identification of the PKC Phosphorylation Site of Fascin—We have previously shown that (1) fascin is phosphorylated in vitro on 12-O-tetradecanoylphorbol-13-acetate treatment; (2) the same sites are phosphorylated in vitro by PKC; and (3) PKC phosphorylation inhibits actin binding of human fascin (26). The sites of PKC phosphorylation appeared to be within the N terminus of fascin, because the C-terminal fragments did not contain $^{32}$P radioactivity when phosphorylated fascin was digested by trypsin as described above.

To determine the phosphorylation sites, we purified phosphopeptides following complete digestion of PKC-phosphorylated fascin and found, by microsequencing of purified phos-
phopeptides, that Ser-39 is the site of phosphorylation by PKC. The chelating column chromatography (Fe^{3+}-charged iminodiacetic acid-Sepharose 6B) was used to adsorb phosphopeptides selectively (31). The bound peptides were eluted by a stepwise pH gradient. In the case of PKC-phosphorylated fascin, most of the phosphopeptides were eluted at pH 6.1 (Fig. 5). This does not seem to be a typical elution pattern for phosphopeptides, since previous reports have shown that phosphopeptides are eluted at more basic pH, around pH 9.0 (31, 32). The eluate at pH 6.1 was separated by C-18 reverse phase high performance liquid chromatography (Fig. 6). The peak (Fig. 6, *arrowhead*) matched with the peak of radioactivity and was pure enough to be sequenced, as judged from the chromatogram and spectrum. As a result of microsequencing of peak a, the peptide was found to correspond to residue 33–42 of the fascin sequence (Fig. 6b). The recovery of serine residue 39 was poor, which is characteristic of phosphoserine after Edman degradation.

Phosphopeptide map analyses were performed to determine whether the purified phosphopeptide represents the major phosphorylation site of fascin. A peptide map of the purified peptide (Fig. 7A) gave two spots, a and b, probably because one was generated during peptide mapping by digestion of the other at one of lysine residue of its C terminus. The pattern of the purified peptide was similar to that of authentic fascin phosphorylated in vitro by PKC (Fig. 7C). This is confirmed because the two spots, a and b, exactly comigrated with the two major spots of fascin phosphorylated in vitro by PKC (Fig. 7B). We have previously shown that both in vivo and in vitro phosphorylation of fascin occur at the same site (26). These results thus indicate that Ser-39 is the major site of in vitro as well as in vivo phosphorylation.

**Characterization of a Fascin Mutant Lacking the PKC Phosphorylation Site**—To verify the importance of the phosphorylation site in regulating the actin binding ability of fascin, we produced and characterized a fascin mutant lacking the PKC phosphorylation site of Ser-39. Using site-directed mutagenesis, Ser-39 was replaced with Ala. The resultant mutant fascin (called A-fascin) was bacterially expressed and purified (see "Experimental Procedures"). We found that PKC phosphorylated A-fascin to an extent 10 times lower (0.03 mol P/mol of protein) than wild-type r-fascin (0.3 mol P/mol of protein). A phosphopeptide map of PKC-phosphorylated A-fascin exhibited one minor spot, which was specific to the r-fascin (Fig. 8B, *arrowhead*; this spot is likely to be produced by the phosphorylation of the extra peptide at the N terminus of r-fascin derived from the 5′-untranslated region). On the other hand,
the wild-type r-fascin showed the same pattern as that of authentic fascin except for the presence of the additional spot specific to the recombinant fascin (Fig. 8A, arrowhead). These results confirm that Ser-39 was the major phosphorylation site of fascin.

We analyzed the effect of PKC phosphorylation on actin binding activities of A-fascin and wild-type r-fascin. As Fig. 9 shows, actin binding of r-fascin was regulated by phosphorylation with PKC in a way similar to the way authentic fascin is regulated (26). The apparent association constant was estimated to be 3.5 × 10^6 M^-1 for r-fascin, which is approximately two times lower than that (6.7 × 10^6 M^-1) of authentic fascin. The reason for this decrease is currently unknown. It may be due to the presence of the extra N-terminal sequence in r-fascin. Phosphorylation of r-fascin by PKC (phosphate incorporation, 0.3 mol P/mol of protein) reduced its apparent actin binding constant to less than 0.5 × 10^5 M^-1. In contrast, the actin binding of A-fascin was not regulated by PKC phosphorylation. The actin binding of A-fascin was the same as that of unphosphorylated r-fascin regardless of the treatment of A-fascin with PKC, indicating that Ser-39 is responsible for the phosphorylation-dependent regulation of fascin-actin binding.

**DISCUSSION**

Our results indicate that actin binding of human fascin is regulated by phosphorylation at the N terminus. The major phosphorylation site, Ser-39, has a PKC consensus motif, (S/T)(X(K/R))(35), which is present in all fascins, including mouse, *Drosophila*, *Xenopus*, and sea urchin fascins (23). It should also be noted that the site is located in one of the most conserved regions among all fascins, and in particular, the sequences surrounding this site are virtually identical among human, mouse, and *Xenopus* fascins, suggesting that phosphorylation may universally control actin binding of all vertebrate fascins.

The sequence surrounding the phosphorylation site may constitute an actin binding site in the N terminus. Ser-39 is located in a 15-amino acid sequence of fascin (residue 29–42, FGFKVNASASSLKKK) which, as pointed out by Mosialos et al. (16), shows similarity to the actin binding site of myristoylated alanine-rich C-kinase substrate (MARCKS) (the residue 151–166, FSPKKSFKLSGFSPKK) (36, 40). The actin binding site of MARCKS contains three PKC phosphorylation sites (37, 38), and importantly, phosphorylation by PKC at these sites inhibits the binding of MARCKS to actin in a way similar to the phosphorylation-dependent regulation of fascin-actin interactions. Furthermore, the cluster of basic amino acids have been identified to be responsible for actin binding of several actin-binding proteins (for review, see Ref. 39). In the case of fascin, the phosphorylation site is followed by three lysine residues. If this stretch of basic amino acids constitutes the actin binding site, phosphorylation will give negative charges, and the inhibition of actin binding through phosphorylation is neatly explained.

Fascin is a globular protein, and correct folding is important for its actin binding. It is thus quite possible that phosphorylation at the N terminus not only directly inhibits binding of the putative N-terminal actin binding site but also affects the actin binding of the C terminus. This notion is supported by the result that the actin binding constant of phosphorylated r-fascin was considerably lower than that of the purified C ter-
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Actin should have two actin binding domains in a single molecule (3). The purified C-terminal fragments were able to bind to actin and to make the F-actin aggregate into bundles. This observation, however, does not lead to the conclusion that the C terminus has two actin binding domains, because the C-terminal fragments formed dimers. Rather, we think it likely that the N and C termini each have one actin binding site from the following reasons. First, as described above, the N terminus has a sequence that shows similarity to the actin binding site of MARCKS. Second, the actin binding affinity of the C terminus was two to three times lower than that of intact r-fascin, suggesting that the N terminus may be involved in the binding of intact fascin to actin. Last, Edwards et al. (33) have recently made a similar approach using proteolysis of recombinant murine fascin, in which the 30-kDa C-terminal fragment binds to but does not bundle F-actin.

Proper folding is essential for fascin to show its actin binding ability. Full-length fascin with actin binding ability can be produced in bacteria at a low culture temperature of 25°C but not at 37°C. At 37°C virtually all fascins became insoluble, indicating that expressed fascin had adopted an incorrect conformation. Furthermore, bacterially expressed fascin truncates, which lack less than 20 amino acids of its N or C terminus, did not bind to actin, even though they were expressed as soluble proteins at a lower culture temperature. In addition, although the C terminus produced by proteolysis of intact r-fascin binds to actin, the same C-terminal fragment, when bacterially expressed, shows no actin binding ability, confirming that bacterially produced fascin truncates do not fold correctly.

A possible next step for the structural analyses is the identification of residues critical for actin binding through the expression of mutants with charged residues replaced with polar residues. Such studies must be carefully interpreted, however, because we currently have no basis to judge whether such mutants are conformationally altered. We are collaborating with Dr. S. Almo (Albert Einstein University) to obtain three-dimensional structural information through crystallography. We have successfully prepared excellent crystals of bacterially expressed, shows no actin binding ability, confirming that bacterially produced fascin truncates do not fold correctly.

We speculate that one of the actin binding domains is located in the C terminus, and the other is possibly in the N terminus. Phosphorylation at the N terminus by PKC is likely to inhibit both actin binding sites. We have generated an A-fascin mutant, the actin binding of which is not regulated by phosphorylation. To verify the physiological significance of phosphorylation at Ser-39, we plan to examine, through transfection, what effects this unphosphorylatable mutant will have on the organization of the microfilament cytoskeleton, in particular, motility of fascin-containing filopodia.

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