Replacement of Threonine 558, a Critical Site of Phosphorylation of Moesin in Vivo, with Aspartate Activates F-actin Binding of Moesin

REGULATION BY CONFORMATIONAL CHANGE*

(Received for publication, January 20, 1999, and in revised form, February 5, 1999)

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Point and deletion mutants of moesin were examined for F-actin binding by blot overlay and co-sedimentation, and for intra- and intermolecular interactions with N- and C-terminal domains with yeast two-hybrid and in vitro binding assays. Wild-type moesin molecules interact poorly with F-actin and each other, and bind neither C- nor N-terminal fragments. Interaction with F-actin is strongly enhanced by replacement of Thr558 with aspartate (T558D), by deletion of 11 N-terminal residues (DelN11), by deletion of the entire N-terminal membrane-binding domain of both wild type and T558D mutant molecules, and by exposure to phosphatidylinositol 4,5-diphosphate. Activation of F-actin binding is accompanied by changes in inter- and intramolecular domain interactions. The T558D mutation renders moesin capable of binding wild type but not mutated (T558D) C-terminal or wild type N-terminal fragments. The interaction between the latter two is prevented. DelN11 truncation enables binding of wild type N and C domain fragments. These changes suggest that the T558D mutation, mimicking phosphorylation of Thr558, promotes F-actin binding by disruption of interdomain interactions between N and C domains and exposure of the high affinity F-actin binding site in the C-terminal domain. Oscillation between activated and resting state could thus provide the structural basis for transient interactions between moesin and the actin cytoskeleton in protruding and retracting microextensions.

Moesin is one of several closely related and widely expressed proteins (1–3). They include ezrin and radixin and share ~75% overall sequence identity and regions, denoted A–H, of even higher structural conservation (4). A common secondary structure is predicted to consist of a 320-residue globular N-terminal domain, a central ~200-residue predominantly α-helical region, and a ~50-residue highly charged C-terminal domain. Based on a large body of evidence, these proteins bind to components of the cell membrane with the N-terminal domain (5–11). Candidate membrane-binding targets include CD44; intercellular adhesion molecules 1, 2, and 3 (12–14); phosphatidylinositol 4,5-disphosphate (5–11). Candidate membrane-binding targets include CD44; intercellular adhesion molecules 1, 2, and 3 (12–14); phosphatidylinositol 4,5-disphosphate (15); cyclic AMP-dependent protein kinase (16); and intermediary adapters of the NHERF family of proteins (17, 18). The C-terminal domain contains a major high affinity actin binding site, presumed to be located in region H, a sequence that is identical in ezrin and radixin (19–23). This sequence differs in the tumor suppressor merlin, and while F-actin binds to the moesin sequence, it does not bind to merlin under the same conditions (23).

Moesin and related proteins are required for the formation of membrane microextensions (5, 7, 8, 24) and for the GTPase-mediated formation of stress fibers, focal adhesion complexes, and microextensions (25–27). Interactions of proteins in the cell cortex underlie a variety of cellular responses, such as changes in cell shape, adhesion, movement, and signal transduction. The function of moesin in these dynamic processes at the membrane/cytoskeletal interface very likely depends on regulation of its binding activities (28, 29).

Several proposals have been made, but it has not been firmly established as yet how the linkage function of moesin operates and how it is regulated in cells. In vitro experiments have demonstrated homotypic and heterotypic associations between ezrin and moesin and small amounts of dimeric molecules have been isolated from cell lysates by immunoprecipitation (30, 31). This, combined with the fact that isolated N and C domains of these proteins interact, has led to the suggestion that oligomers are functionally important and that they are formed by disruption of an intramolecular interaction between N-terminal and C-terminal domains (30, 32, 33). Consistent with this idea is that the binding sites mediating this head-to-tail association are masked in the full-length protein, when tested for interaction of full-length molecules with either C or N domains (30).

A second proposal has been that phosphorylation activates binding functions of moesin. In human platelets, for instance, thrombin activation leads to a rapid, but transient, increase in the phosphorylation of a single threonine, Thr558, in the C-terminal domain and near the presumptive location of the F-actin binding site (20, 28). This residue is conserved in moesin, ezrin, and radixin, and it is modified also in RAW macrophages (34), Swiss3T3 cells (29),1 NIH3T3 cells,2 and RB2H3 mast cells.2 Phosphorylation at this site is regulated by the activity of Rho and Rho-associated kinase phosphorylates two residues in the C-terminal region, one of which is Thr558 (29). Thr558 can also be phosphorylated by θ-phosphokinase C (35). Phosphorylation of this conserved amino acid by Rho-associated kinase prevents interaction of the phosphorylated C domain with the N-terminal domain (29) but does not appear to influence binding of the isolated C domain to F-actin. Other than Thr558, the role of phosphorylation of specific tyrosine,

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* This work was supported in part by California Tobacco-related Disease Research Program Grant 4RT-0316. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by a President’s Undergraduate Scholarship Award from Stanford University.

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1 F. Nakamura and H. Furthmayr, unpublished observations.
2 L. Huang and H. Furthmayr, unpublished observations.
3 E. Ichimaru and H. Furthmayr, unpublished observations.
serine, or other threonine residues in response to stimulation by growth factors, histamine, or lysophosphatidic acid has not been established (36–40).

Phosphorylation of Thr558 of moesin is of significance in human platelets, because it is modulated by physiological activity, it can be manipulated by protein kinase and phosphatase inhibitors, and it is accompanied by changes in moesin distribution and platelet morphology (28). We believe that phosphorylation of Thr558 can be mimicked by replacement of threonine with aspartate, and the present study was undertaken to test the hypothesis that this modification activates the F-actin binding function of moesin. We show that Thr558 substitution greatly enhances F-actin binding activity of moesin by comparing the actin filament binding properties of wild type moesin and to generate pKG-MSNT558A (GST-MSNT558A).

Experimental Procedures

GST-Moesin Mutants—Mutations of moesin Thr558 within the sequence KYKT1 L were introduced into moesin cDNA by PCR using oligonucleotides as primers that specify both the desired mutation and carry a convenient restriction site to facilitate subcloning and isolation of mutant clones. The following oligonucleotides were synthesized: A, GEX5-A (5'-CGCGGACCAATACATTGCGG3'-); B, MSN T558D BglII- (5'-CGCGGATCCTCGGAGAAGATCCCGG3'-); C, MSN T558D BglII- (5'-GCCGCCAGACACACACGACATTTGATCGCC3'-); D, GST-MSN in vitro 1-310 HA-moesin N12–310, respectively. The longer Ndel– HindIII fragment, obtained from pKG-MSNT558D, was digested with HindIII and partial digest HI and excised from pACT2-MSN by partial digest and cloning into pCR32 to generate pCR32-MSN. To substitute Thr558 with Asp (D), two PCR reactions were performed using pGhuMo (pGEX-KG-human moesin, or GST-MSN (20)) plasmid DNA as template with oligonucleotides A and B as primers in reaction 1, and C and D in reaction 2. The product of reaction 1 was digested with BglII yielding a 1.1-kb fragment, and the product of reaction 2 gave a 0.5-kb fragment by digestion with BglII and AarII. The two fragments were then subcloned by three-way ligation into pGhuMo that had been predigested with BglII and AarII to delete the C-terminal portion of moesin. This resulted in pKG-MSN11 and pGEX-MSN11 and pACT2-MSN11 were digested with SmaI and EcoRV to delete GFP and recircularization.

Activation of Moesin

pKG-MSNT558D was digested with BglII to delete encoding residues 198–558 and recircularized to generate pKG-MSN11 (L198–T558) (GST-MSN11). pKG-MSNc (GST-moesin C-terminal region residues 404–577) was constructed by inserting the EcoRI–XhoI fragment from MSNCr3 (see below) into pGEX-KG.

pKG-EZR1 (GST-EZR1–310/P74, NBS) and pKG-EZRN (GST-EZR1–310/P74, NBS) were constructed by replacing the moesin Ndel–NcoI fragment in pKG-MSN with the human ezrin Ndel–NcoI or Ndel–SalI (from pSA1-MSNc3) fragments, respectively. pGEX-EZRN (GST-EZRN20–586) was made by inserting the ezrin C-terminal BamHI–BamHI fragment into pGEX-3X vector. PGEK-RDX (GST-RDX1–583) was as described by Pestonjamasp et al. (20). PGEK-RDXN (GST-RDX1–449) was generated by deleting the pig radixin C-terminal HindIII–HindIII fragment from pKG-RDX and recircularizing the plasmid. PGEK-RDX (GST-RDX373–585) was constructed by subcloning the C-terminal XhoI–XhoI fragment from pKG-RDX into pGEX-KG.

PGEY-NF2 (GST-merlin) and pGEX-NFc (GST-merlin C-terminal region, residues 254–595) were as described by Huang et al. (23). To confirm the correct sequence, all PCR-derived mutants were sequenced using the ABI PRISM DYE Terminator cycle sequencing system (Perkin-Elmer).

Untagged Moesin Mutants in pEt Vectors—Wild type and mutated moesin cDNAs were subcloned into derivatives of pEt vectors (Stratagene) carrying a T7 promoter for expression of untagged moesin proteins in Escherichia coli and in vitro (41). The Ndel–HindIII fragment from pGhuMo, pKG-MSNT558D, and pKG-MSN15K was cloned into pETnde to generate MSNc-pEt, MSN558D-pEt, and MSN15K-pEt, respectively. The longer Ndel–HindIII fragment, obtained from pKG-MSNc-pEt, was digested with HindIII and partial digest HI and cloned into pETnde to generate MSN558A/pEt. The shorter Ndel–HindIII fragment was cloned into pETnde to make MSNC19. The Ndel–NcoI fragment from UHI was cloned into pETneo to generate MSN11/pET.

Untagged Moesin Mutants in pCR3 Vector—The EcoRI–XhoI fragment encoding full-length moesin from pGhuMo was subcloned into pCR3 (Invitrogen) to generate MSNc-pCR3. The C-terminal PstI–XhoI fragment from pGhuMo was subcloned into pCR3 to make MSNCpCR3 encoding residues 421–577 with codon 421 as the initiation methionine. Likewise, the PstI–XhoI fragment from pKG-MSN558D was subcloned into pCR3 to generate MSN558D/pCR3. MSNCpCR3 encoding moesin’s N-terminal region residues 1–310 was digested by digesting MSNC-pCR3 with SmaI and EcoRV to delete GFP and recircularization.

G4AD-Moesin Hybrid Plasmids—The Ndel–NcoI fragment from pCR3 was inserted into pSA1 and pACT2 to create pSA1-MSN11 (G4BD-HA-moesin) and pACT2-MSN11 (G4AD-HA-moesin) (23). pACT2-MSNc19 (G4AD-HA-moesin C-(1–558)/19 residues truncated) were constructed by subcloning the C-terminal XhoI–XhoI fragment from pGhuMo and pKG-MSNT558D, respectively. To substitute Thr558 with Ala, two PCR reactions were performed using primer sets (see below) into pGEX-3X vector.

pACT2-MSNc (G4AD-HA-moesin) and pACT2-MSN558D (G4AD-HA-moesin T558D mutant) were constructed by subcloning into pACT2 the EcoRI–XhoI fragment from pGhuMo and pKG-MSNT558D, respectively. pACT2-MSNc (G4AD-HA-moesin) and pACT2-MSN558D (G4AD-HA-moesin T558D mutant) were constructed by subcloning into pACT2 the EcoRI–XhoI fragment from pGhuMo and pKG-MSNT558D, respectively. To delete KYKT1 L, two PCR reactions were performed with oligonucleotides A and H as primers in reaction 1 and I and D in reaction 2 using pGhuMo as template. The first reaction yielded a 1.1-kb BglII–PstI fragment, and the second yielded a 0.5-kb PstI–AarII fragment. The two fragments were subcloned by three-way ligation into BglII/AarII–PstI-digested pGhuMo to delete the C-terminal portion of moesin and to generate PKG-MSNT558A (GST-MSNT558A).

To delete KYKT1 L, two PCR reactions were performed with oligonucleotides A and H as primers in reaction 1 and I and D in reaction 2 using pGhuMo as template. The first reaction yielded a 1.1-kb BglII–PstI fragment, and the second yielded a 0.5-kb PstI–AarII fragment. The two fragments were subcloned by three-way ligation into BglII/AarII–PstI-digested pGhuMo to result in PKG-MSNc (GST-MSNK5). PKG-MSNT558A was digested with HindIII to delete the C-terminal 19 codons and recircularized to generate PKG-MSNc19 (GST-MSNK5). 4

4 The abbreviations used are: PCR, polymerase chain reaction; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; PI, phosphatidylinositol; PIP2, phosphatidylinositol 4,5-diphosphate; kb, kilobase pair(s).
ing the EcoRI-SalI fragment from pA51-MSN into pACT2 cut with EcoRI and XhoI. pA51-MSN (G4BD-HA-moesin N-terminal region 1-310) was constructed by subcloning the Smal-SmalI fragment from pACT2-MSN into pA51. It was not suitable for the study because of high background signal in yeast two-hybrid assays.

### RESULTS

#### F-Actin Binding of Moesin Mutants: Enhancement by T558D Mutation

As a first step, a series of mutations of Thr558 and flanking sequences were introduced into moesin by polymerase chain reaction, and the mutants were subcloned to yield GST fusion proteins. Mutant and wild-type GST-moesin were then expressed in *E. coli*, and effects of the mutation on F-actin binding were examined by F-actin blot overlay with 32P- (27) or 125I-labeled F-actin (20).

As shown in Fig. 1, control wild type (WT) and T558A mutant moesin fused to GST bind the F-actin probe relatively poorly. A significantly higher amount (5–7-fold increase) is observed for full-length moesin, when Thr558 is replaced with aspartate, a negatively charged residue that is widely used to mimic phosphorylation. Deletion of KYKTL, the pentapeptide that includes Thr558 (AKYKTL558L), reduces F-actin binding as compared with WT moesin, while truncation of the C-terminal 19 residues (ΔC19) downstream of Thr558 or of 361 residues (ΔL198-T558) upstream of Thr558 eliminates binding completely.

The weak signal obtained for WT moesin could have been due to the fusion of GST to the C terminus, and since GST can have potentially undesirable effects on structure and on the biological activity of moesin (48), we formally addressed this issue with untagged moesin. WT and mutant proteins were expressed in *E. coli* using pET vectors and examined for F-actin binding by blot overlay assay. Similar to data with GST fusions, untagged WT moesin binds F-actin poorly, the T558A mutation binds slightly better, and binding of the T558D mutant is greatly enhanced (Fig. 1). Deletion of the five residues KYKTL (AKYKTL) largely diminishes, and deletion of the C-terminal 19 residues in ΔC19 totally abolishes, F-actin binding, although four times the amount of protein was analyzed. This result clearly shows that the strong enhancement of F-actin binding is caused by the replacement of Thr558 with Asp and is similar in its effect to phosphorylation of this threonine residue in platelet moesin (28, 63). Furthermore, the addition of GST to moesin in the fusion protein does not affect the actin binding property of moesin. However, this result does not permit conclusive interpretations regarding mechanism. The mutation could have increased the affinity for F-actin of the previously determined binding site in the C-terminal domain (20) simply by the addition of a negative charge, it could have caused exposure of a previously masked binding site, or it could have even created binding sites de novo.

To distinguish between these possibilities, we have tested an additional mutant. Earlier observations suggested that in contrast to WT full-length moesin, the ΔN11 mutant, made by truncation of 11 N-terminal residues, binds N- and C-terminal fragments (see below). As shown in Fig. 1B (lane 6), this mutant binds approximately 9–4-fold higher amounts of F-actin in comparison with WT. This mutation is located opposite to where the high affinity F-actin binding site is presumed to reside on the molecule. Thus, it is highly unlikely that, at least for this mutant, the enhancement of F-actin binding is due to an increase in the affinity of the C-terminal binding site for F-actin. Additional binding site(s) for actin have been detected in the N-terminal domain of ezrin with a solid phase binding assay (48). In contrast to these data, we have not detected these sites by blot overlay assaying GST fusions or untagged N- and
C-terminal domains of moesin, ezrin, radixin, and merlin. As shown in Fig. 2 and as expected from previous results (20, 23), the C-terminal domains of moesin, ezrin, and radixin, but not merlin, bind F-actin, while none of the N-terminal domain fusions exhibits binding.

Co-sedimentation of F-actin and Moesin Mutants: Enhancement by T558D and DN11 Mutation and by PIP2—The effects of mutations on F-actin binding were examined independently with a co-sedimentation assay (43). To perform this experiment, WT moesin and two of the critical mutants were subcloned into a T7-driven expression vector and translated in vitro in the presence of [35S]methionine. Radiolabeled proteins were then incubated with actin filaments and after centrifugation analyzed by SDS-PAGE and autoradiography. As shown in Fig. 3a, only a very small amount (<2%) of WT moesin co-sediments with actin. In contrast, considerably higher amounts of moesin are recovered in the pellet with actin, when Thr558 is mutated to aspartate, and somewhat lesser amounts of the N-terminal deletion mutant (DN11).

PIP2, but not phosphatidyl 4-phosphate, binds to the N-terminal domain of ezrin (15). It has been proposed to form a complex with and to stabilize the interaction of moesin, ezrin, and radixin with the cytoplasmic segment of CD44 in physiological salt concentrations (49). To examine whether PIP2 affects F-actin binding, radiolabeled WT moesin was generated by in vitro translation and incubated with actin filaments in the presence or absence of PI or PIP2. As shown in Fig. 3b, enhanced binding to actin filaments is seen when PIP2, but not phosphatidyl 4-phosphate, is present. The amount of sedimented WT moesin is similar to that of the mutants shown in Fig. 3a.

Wild Type and T558D Mutant C-terminal Domains of Moesin Have Comparable F-actin Binding Activity—To determine if the T558D mutation affects the interaction with actin filaments directly, we tested mutant and wild type C domains, prepared by in vitro translation, in the co-sedimentation assay. As shown in Fig. 4, equal fractions of wild type and mutant C domains either untagged or fused to GFP, co-sediment with F-actin. Thus, the high affinity binding site associated with the C-terminal domain appears to be identical in WT and mutant moesin.

![Fig. 1. T558D mutation and N-terminal deletion of 11 amino acid residues result in enhancement of F-actin binding of moesin on blot overlay.](image)

a, Top left, Coomassie Blue-stained SDS gel of lysates from bacteria expressing GST fusion proteins of moesin (WT, T558A, T558D, ΔKYKTL (deletion of Lys555–Leu559), ΔC19 (deletion of C-terminal 19 residues), and ΔL198-T558). Bottom, 32P-labeled F-actin blot overlay of lysates shown in the top gel. Note the difference in signal intensity of T558D mutant moesin in comparison with WT, T558A, and ΔKYKTL. No binding is seen to C-terminal truncated forms of moesin (lanes 5 and 6). b, Top, Coomassie Blue-stained SDS gel of lysates of bacteria expressing untagged WT and mutant forms of moesin (lanes 1–5, same as in a; lane 6, DN11 (deletion of 11 N-terminal residues). Bottom, 32P-labeled F-actin blot overlay of lysates shown in the top gel. Numbers on the left denote molecular masses of standards. The difference in signal intensity between WT, T558A, ΔKYKTL, and T558D is similar to that in a. No binding is seen to ΔC19. ΔN11 binds as strongly as T558D mutant moesin.

![Fig. 2. The C domains of moesin, ezrin, and radixin bind 35S-labeled F-actin equally well on blot overlay.](image)

Top left, Coomassie Blue-stained SDS gel of lysates from bacteria expressing GST fusions of the C-terminal domain of merlin (NF2c), merlin (NF2), ezrin (EZR), radixin (RDX), and moesin (MSN). Top right, Coomassie Blue-stained SDS gel of lysates from bacteria expressing GST fusions of N- and C-terminal domains of ezrin, radixin, and moesin. Bottom, autoradiograms of 35S-labeled F-actin blot overlays of the gels shown on top. Relatively weak binding of the F-actin probe is seen with full-length protein, with radixin showing a stronger signal in comparison with ezrin and moesin (lanes 3–5), but none of the N domains binds the probe (lanes 6, 8, and 10). In contrast, the C domain of all of three proteins interacts strongly.
molecules. Once uncovered by removal of the N-terminal domain, T558D has no additional effect. Fig. 4 shows that the untagged form of C-moesin yields a double band during translation. This additional band of lower molecular mass can be detected also when this domain is expressed in fibroblasts, E. coli or yeast. The reason for this and the nature of the second product has not been investigated further.

**T558D Mutation Prevents Interaction between WT N- and Mutated C-terminal Domains**—The similar F-actin binding activity of WT and mutant C-terminal domains suggested that the binding site is present but not exposed in WT moesin and that the activity is uncovered by the T558D mutation. As originally shown by Gary and Bretscher (30), the two isolated domains of ezrin made by recombinant techniques bind to each other, but react poorly with full-length ezrin. This was consistent with a model in which the two domains interact within the structure of the full-length protein. To further investigate how the T558D mutation affects the interdomain and intramolecular interactions, we took advantage of this information and tested for binding using \(^{35}\)S-labeled N and C domains as probes. GST-N-moesin was immobilized on Sepharose beads and incubated with isotope-labeled WT N-terminal, WT C-terminal, and T558D mutant C-terminal domains generated by \emph{in vitro} translation. After incubation and extensive washing, SDS was added, and the solubilized proteins were analyzed by SDS-PAGE and autoradiography. Fig. 5 shows that, as expected, only WT C-moesin and not N-moesin bind to N-moesin. In contrast, T558D-C-moesin could not be recovered in the bound fraction, suggesting that the mutation disrupts the N-terminal binding activity of the C domain. Similar results were obtained with \emph{in vitro} translated GST-moesin domain fusions (not shown).

 extending these studies to full-length moesin, we asked the question whether immobilized GST-fusions of C and N domains bind \emph{in vitro} translated and radiolabeled WT and T558D mutant moesin. We observed only weak binding of both domains to full-length WT moesin (Fig. 6, lanes 3 and 9) but markedly enhanced binding of the C domain to T558D mutant moesin molecules (Fig. 6, lane 6). A similar enhancement was also seen in the binding of AN11-moesin to GST-N domain. These results suggest that WT moesin is in a “closed” configuration, in which the intramolecular interaction sites are blocked. The T558D mutation shifted molecules to an “open” form, which can bind an extramolecular C, but not N domain, presumably because the mutation disrupts the interdomain interaction present in WT moesin.

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**Fig. 3. Co-sedimentation of F-actin and \emph{in vitro} translated T558D and AN11 moesin mutants and of wild type moesin in the presence of PIP2.** a, \emph{in vitro} translated WT, T558D, or AN11 moesin was incubated with actin filaments and centrifuged, and supernatants (S) and pellets (P) were analyzed by autoradiography of the SDS gel. A larger fraction of T558D moesin co-sediments in comparison with WT and AN11. b, \emph{in vitro} translated \(^{35}\)S-labeled WT moesin was preincubated with PIP2 or PI and F-actin. After centrifugation, supernatants and pellets were analyzed by SDS gel electrophoresis and autoradiography. PIP2 but not PI enhances binding of WT moesin to F-actin.

**Fig. 4. T558D mutation abolishes the interaction between N- and C-terminal domains of moesin.** GST-N-moesin was immobilized on a glutathione matrix and \emph{in vitro} translated, and labeled N-moesin, C-moesin, or T558D-C-moesin was added. After washing the beads, the bound material was solubilized with SDS sample buffer and analyzed by gel electrophoresis and autoradiography. Lanes 1, 3, and 5 (lanes) show the translation products prior to incubation. The arrowheads indicate molecular masses of N-moesin (left) and C- or C-T558D-moesin (right). Lanes 2, 4, and 6 (+ lanes) show the bound material. N-moesin does not interact with itself (lane 2) but binds C-moesin (lane 4) unless Thr\(^{558}\) is mutated to Asp (lane 6).

DelN11 moesin is able to bind both N and C fragments (not shown here, but see below), suggesting that the intramolecular N-C interaction is sufficiently weakened or disrupted to favor binding of extramolecular fragments.

It is of additional interest that PIP2 enhances binding of the N but not C domain to WT moesin and inhibits the interaction between Thr\(^{558}\) moesin and GST-C-moesin (Fig. 6, lane 4). This is consistent with PIP2 blocking the site for C domain binding in the N-terminal domain.

To assess whether these interactions occur under perhaps more relevant \emph{in vivo} conditions as well, we employed the yeast two-hybrid assay and consistent data could be obtained regardless of whether fragments were expressed as GAL4BD or GAL4AD fusions (Table I). For instance, WT moesin shows no interactions with WT C-moesin, delN11-N-moesin, WT N-moesin, or delN11 moesin (Table I, line C, 1, 5, 6, 7, and 8). The reverse is true as well (Table I, column 7, A, B, C, and D). The control experiment shows strong interactions between WT N- and C domains in both directions (Table I, compare B5,
B6, and 1A), while T558D abolishes this interaction (Table I, 2A).

In full-length moesin, the T558D mutation enhances the interaction with WT C-moesin (Table I, E1 and B9), while both N and C domains interact with delN11-moesin molecules. Thus, both mutations affect binding between fragments and full-length molecules, albeit by different mechanisms.

**DISCUSSION**

The present study documents that T558D substitution, which is expected to mimic phosphorylation of Thr<sup>558</sup>, activates F-actin binding of moesin by exposure of the high affinity binding site in the C-terminal region. Activation is accomplished by a structural change that affects the arrangement of the N- and C-terminal domains within the molecule (Fig. 7). This conformational mechanism could be physiologically relevant in intact cells, since intra- and extracellular signals determine the state of phosphorylation of moesin (28, 29). Phosphokinases and phosphatases thus appear to regulate one important function of this protein.

Extending earlier observations on ezrin and moesin (19,20), we confirm with *in vitro* translated C-terminal fragments that a high affinity binding site for F-actin is localized in the C-terminal domain and that the T558D mutation does not significantly alter its binding activity (Fig. 4). Two F-actin binding tests indicate that full-length moesin molecules either made in bacteria or in a cell-free system interact with actin filaments, but do so only weakly. Substituting threonine with the negatively charged amino acid residue aspartate, but not alanine markedly enhances binding. The weak binding of the full-length protein is evidently due to the inaccessibility of the binding site because truncation of the entire N domain or of only 11 N-terminal amino acid residues leads to strong F-actin binding (Figs. 1 and 2). This result agrees with previous data demonstrating that radixin C domains made in insect cells prior to and after phosphorylation with Rho-associated kinase *in vitro* exhibit similar F-actin binding activity (29). Taken together, the current results provide compelling evidence in support of our conclusion that the negative charge introduced at the Thr<sup>558</sup> position controls F-actin binding not by changing affinity but rather by exposing the C-terminal binding site.

**T558D Mutation Exposes F-actin Binding Site by Disrupting N and C Domain Interaction**—Our data demonstrate a striking link between the unmasking of F-actin binding activity and of interdomain binding sites. Gary and Bretscher (30) have shown that isolated N and C domain fragments of ezrin bind each other and that sites for interdomain binding are not accessible in the intact molecule when probing bacterially made ezrin and ezrin isolated from tissues with N and C domain fragments or with specific peptide antibodies. Partial unfolding was required to recognize the binding sites for domain fragments. Likewise, we observe strong binding between N and C domains of moesin and only weak or absent interaction between these fragments and full-length moesin. The C domain fragment carrying the T558D mutation, on the other hand, does not bind the N domain fragment any longer (Figs. 5 and 6), while the full-length T558D mutant protein binds an additional C domain fragment. Both observations are in agreement with the proposal that N- and C-terminal domains interact within the native protein structure. The mutation disrupts the intramolecular domain interaction, and a conformational shift results that presumably is large enough to allow binding of the extramolecular C-terminal fragment (Fig. 7).

It is of interest in this context that full-length moesin is a poor substrate for Rho-associated kinase *in vitro*, while this enzyme phosphorylates two threonine residues, Thr<sup>558</sup> and Thr<sup>567</sup>, when the C domain fragment of moesin is used for the reaction (29). In its modified form, the C domain fragment is no longer capable of binding to the N domain. It is clear from our data that the modification of only one site, Thr<sup>558</sup>, is sufficient to inhibit binding.

We have identified two additional mechanisms that apparently accomplish activation as well: deletion of 11 amino acid residues at the N-terminal end and binding of phosphatidylinositol 4,5-diphosphate (PI(4,5)P<sub>2</sub>) (Fig. 7). PI<sub>(4,5)P<sub>2</sub></sub> binds to a GST fusion protein of ezrin consisting of the N-terminal 309 amino acid residues but not to the C-terminal fragment (positions 310–568) (19). If this is true for moesin as well, PI(4,5)P<sub>2</sub> may activate F-actin binding of moesin by interfering with interdomain binding as seen by the inhibition of the interaction between the C domain fragment with T558D moesin in its presence (Fig. 6). It is less clear at the moment, how N-terminal truncation activates F-actin binding. Very recent structural data derived for a co-crystal of N and C domains of moesin show that two of the three subregions of the N domain contact the C domain. This suggests that interdomain interaction in the molecule is complex and may depend on the structural integrity of regions of the protein that are separate from identified binding sites (49).

The binding sites for F-actin and the N domain appear to be...
Filter lift β-galactosidase assays were performed on yeast Y190 cells co-expressing pairs of GAL4-moesin fusions as described under "Experimental Procedures" to assess interdomain and intermolecular interactions of moesin. β-Galactosidase activity was scored visually, with ++++ denoting dark blue and — denoting white colonies. The hybrid constructs used were, pAS1-MSNΔ (A), pAS1-MSN (B), pAS1-MSN (C), pAS1-MSNΔN11 (D), pAS1-MSNT558D (E), pGBT9 (F), pACT2-MSN (1), pACT2-MSNΔC19 (2), pACT2-MSNΔK5 (3), pACT2-MSNΔC19 (4), pACT2-MSNΔ (5), pACT2-MSN (6), pACT2-MSN (7), pACT2-MSNΔN11 (8), pACT2-MSNT558D (9), and pACT2 (10).

TABLE I

Interactions of full-length and domains of moesin in yeast two-hybrid system: T558D and ΔN11 mutations disrupt intramolecular head (N) to tail (C) association

| GAL4BD fusion | GAL4AD fusion |
|---------------|---------------|
| 1            | WT            | C-moesin 404–577 |
| 2            | T558D         | C-moesin 404–577 |
| 3            | ΔKYKTL        | C-moesin 404–577 |
| 4            | ΔC19          | C-moesin 404–558 |
| 5            | ΔN11          | N-moesin 12–577 |
| 6            | ΔN11          | N-moesin 1–577 |
| 7            | WT            | moesin 1–577 |
| 8            | WT            | moesin 1–577 |
| 9            | T558D         | moesin 1–577 |
| 10           | None          | None |

A. ΔN11 N-moesin 12–310 +++++ — — — — — — —
B. WT C-moesin 404–577 — — — — — — — —
C. WT moesin 1–577 — — — — — — — —
D. ΔN11 moesin 12–577 + + + ++ + ++ + + + + + + + + + +
E. T558D moesin 1–577 + + + — — — — — — — — — — — — — —
F. None — — — — — — — — — — — — — — — —

* Five residues deleted.

FIG. 7. Model illustrating conformational change induced by mutations and PIP2. On the left, the N domain (amino acid residues 1–310) interacts with a segment of the C domain (residues 404–577). On the right, mutations (indicated by an asterisk) and PIP2 (open rectangle) disrupt this interaction to expose a high affinity binding site for F-actin and the sites of contact of the two domains (solid bars). The site on the N domain becomes accessible for an additional C domain, but the C domain site is blocked for N domain binding by T558D. The interaction of F-actin with the C domain site is not affected by this mutation.

rather close to each other, both located near the C terminus. We have shown that the ΔC19 mutation abolishes both activities, ΔKYKTLR largely diminishes the former and eliminates the latter, and T558D retains the former and abolishes the latter. This suggests that the respective binding sites do not completely overlap, but we do not know whether there is competition for binding.

Model for Activation of Moesin in Vivo—Binding between the C domain of moesin and actin filaments can be observed in cells most clearly by co-distribution with stress fibers (5, 6). This co-distribution is not seen with full-length moesin, suggesting that activation of the actin-binding site may not occur in the cytoplasm. On the contrary, recent evidence with GFP fusions of moesin in live NIH3T3 cells have shown that the protein is distributed along the plasma membrane, where it may be bound to a variety of receptor proteins (5). It is presumably at these sites and in the vicinity of the plasma membrane that activation of moesin occurs. In platelets, protein phosphokinase and phosphatase inhibitors can rapidly change the phosphorylation state of moesin, suggesting its dependence on a balance of their activities during cell stimulation (28). Similar findings have been made for other cells. Thus, lysophosphatidic acid stimulates a rapid but transient increase in the phosphorylation of moesin, ezrin, and radixin in Swiss 3T3 cells, and it has been suggested that Rho kinase is responsible for this increase (29). However, a change with similar kinetics can presumably occur by inhibition of a phosphatase. The myosin-binding subunit of myosin phosphatase binds moesin, and its activity is inhibited when phosphorylated by Rho kinase (50). Phosphokinase C-θ also phosphorylates moesin at the Thr558 site, but this kinase is expressed primarily in blood cells (35).

We demonstrate here that F-actin and interdomain binding characteristics of moesin change in the presence of PIP2. This is of interest, since it was previously shown that PIP2 stabilizes the interaction of moesin with the membrane protein CD44 (51). PIP2, however, has other effects as well. It induces actin polymerization in permeabilized platelets (55), presumably by uncapping barbed ends (56). In platelets, turnover of PIP2 is increased upon stimulation, while overall levels of PIP2 decrease (52). This raises the possibility that local changes in PIP2 concentration in the vicinity of the membrane influence moesin function. The role of PIP2 could thus be to sensitize moesin to become a target for phosphokinases. Moesin is necessary for and localized in protruding filopodia, and this is where most likely activation and linkage to actin filaments occurs (8, 50, 53, 54). Phosphorylated moesin is detectable in protrusions (34, 50). Moesin phosphorylation transiently increases in response to stimuli (28, 29), and the increase in number of phosphorylated molecules presumably reflects the average change and is the result of a simultaneous action of kinase and phosphatase on different molecules. According to this model, changes in the phosphorylation state may also reflect whether moesin is bound to the actin cytoskeleton.

Moesin, ezrin, and radixin appear to be necessary for functions in the cortex of cells. Perturbation of microvillus formation and adhesion was observed in moesin/ezrin/radixin antisense oligonucleotide-treated fibroblasts (53), radixin/moesin-suppressed neurons exhibit abnormal growth cones and short neurites and fail to develop an axon (54), and micro-CALI ablation of ezrin causes collapse of ruffles (57). Recent studies also suggest that expression of the N domain of ezrin in epithelial cells causes loss of microvilli, a change in localization of...
endogenous ezrin, and perturbation of human growth factor-induced differentiation (58). Expression of the N domains of moesin, ezrin, or radixin in NIH3T3 cells causes subtle changes in behavior that are characterized by more numerous and longer filopodia, which are poorly attached and are retracted abnormally, but even more prominent is a defect in retraction of lamellipodial membrane (5). This suggests that it is not merely the existence of these proteins at these sites that is critical, but the fact that they have to be present in a functionally active form. As the cells respond to stimuli by protruding and retracting different areas of the surface and as new actin filaments are formed and others disassemble, some moesin molecules are activated and are enabled to interact with filaments, while others return to an inactive state but remain bound to the membrane. Parts of this hypothesis have been tested recently by expression of constitutively active and inactive moesin molecules (50).

The concept proposed here of moesin changing its overall configuration is not completely novel. Such a mechanism has been proposed for other cytoskeletal proteins, such as vinculin (59), myosin II (60), and Src kinase (61), which phosphorylates tyrosine residues of proteins at cell-substratum contacts. It still remains to be seen whether the folded, jackknife configuration is not completely novel. Such a mechanism has been tested recently by expression of constitutively active and inactive moesin molecules (50).

Acknowledgments—We thank U. Francke from the Howard Hughes Medical Institute at Stanford University for valuable assistance with sequencing; A. Spudich from the Department of Biochemistry at Stanford Medical Institute at Stanford University for valuable assistance with subcloning and yeast two-hybrid sequencing; A. Spudich from the Department of Biochemistry at Stanford Medical Institute at Stanford University for valuable assistance with subcloning and yeast two-hybrid sequencing; A. Spudich from the Department of Biochemistry at Stanford Medical Institute at Stanford University for valuable assistance with subcloning and yeast two-hybrid sequencing; A. Spudich from the Department of Biochemistry at Stanford Medical Institute at Stanford University for valuable assistance with subcloning and yeast two-hybrid sequencing; A. Spudich from the Department of Biochemistry at Stanford Medical Institute at Stanford University for valuable assistance with subcloning and yeast two-hybrid sequencing.