BIOCHEMISTRY
including biophysical chemistry & molecular biology

RAPID REPORT

pubs.acs.org/biochemistry

Evidence for Multisite Ligand Binding and Stretching of Filamin by Integrin and Migfilin

Sujay Subbayya Ithychanda and Jun Qin*

Department of Molecular Cardiology, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, Ohio 44195, United States

Supporting Information

ABSTRACT: Filamin, a large cytoskeletal adaptor, connects plasma membrane to cytoskeleton by binding to transmembrane receptor integrin and actin. Seven of 24 filamin immunoglobulin repeats have conserved integrin binding sites, of which repeats 19 and 21 were shown to be autoinhibited by their adjacent repeats 18 and 20, respectively. Here we show using nuclear magnetic resonance spectroscopy that the autoinhibition can be relieved by integrin or integrin regulator migfilin. We further demonstrate that repeats 19 and 21 can simultaneously engage ligands. The data suggest that filamin is mechanically stretched by integrin or migfilin via a multisite binding mechanism for regulating cytoskeleton and integrin-mediated cell adhesion.

Many transmembrane receptors are tethered to the actin cytoskeleton, and this interaction determines the residence time of the membrane protein on the plasma membrane of animal cells. The tethering causes a variety of phenomena like receptor endocytosis, ligand affinity, receptor signaling, and cytoskeletal remodeling. Integrins make up a class of conserved heterodimeric transmembrane glycoproteins in multicellular animals that attach cells to the extracellular matrix and to other cells.[1] Upon activation,[2] the extracellular sides of integrins bind to collagen, laminin, fibronectin, fibrinogen, and other matrix proteins, whereas their generally short cytoplasmic tails (CTs) can link to the actin cytoskeleton by binding a variety of cytoplasmic proteins, thereby modulating cell adhesion and signaling outcomes.[3,4] One of the major integrin CT binding proteins is filamin, a large actin binding cytoskeletal protein. The filamin−integrin interaction was found to compete with the integrin activator talin,[5−7] which has led to a proposal that filamin is a negative regulator of integrin activation.[8,9] Interestingly, a LIM domain containing protein migfilin was found to bind to filamin[10] in the same manner as integrin,[8,11] thereby competing with filamin for binding to integrin CTs and facilitating integrin activation and cell matrix adhesion.[8]

The filamin monomer consists of an N-terminal actin binding domain followed by 24 immunoglobulin (Ig) repeat domains of ∼95 amino acids. Filamin dimerizes through the 24th Ig repeat.[12] Seven of these 24 repeats share a common interface where the ligands dock.[13] This interface consists of β-strands C and D of the Ig structure.[5,8,11,14] Filamin ligands themselves extend one of the β-sheets of the Ig fold. The C-terminus of filamin has four alternate repeats (17, 19, 21, and 23) whose conserved binding sites for integrin, migfilin, and GP1bα have been confirmed.[13] Interestingly, recent structural studies have shown that even-numbered repeats 18 and 20 mask the binding pocket of repeats 19 and 21, respectively, resulting in "autoinhibited" filamin.[15−17] This implies that filamin can function in a "closed" mode and an "active/stretched" mode during the regulation of integrin−actin dynamics. In the autoinhibited form, filamin repeats are globular and not in a "string of beads" alignment. It was thought that the mechanical tug of the actin cytoskeleton stretches filamin and exposes the masked CD groove.[17] A splice variant of filamin, filamin-$A_{var\text{-}1}$, that lacks amino acids 2127−2167 is a naturally occurring autoinhibition deficient filamin.[18,19] This mutant lacks 14 amino acids of repeat 19 and 27 amino acids of repeat 20. For Ig repeats 17 and 23, no autoinhibition phenomenon is reported. The segments of repeats 18 and 20 that cause autoinhibition weakly resemble integrin and migfilin (refs 15 and 16 and Figure S1 of the Supporting Information). Despite being autoinhibited, Ig repeat 21 is frequently identified in biological screens as the principal docking site for filamin ligands. We previously showed that Ig repeat 21 binds the ligand tightest and postulated that the presence of seven binding sites in a single filamin molecule could lead to membrane receptor clustering mediated by filamin. A key issue is how the autoinhibition of filamin is relieved. Moreover, can multiple Ig repeats truly engage with ligands simultaneously to mediate integrin clustering?[13] Using *Escherichia coli*-expressed and purified multirepeat filamin constructs, we show here that autoinhibition can be potently relieved by integrin or migfilin, suggesting that filamin is in a stretched mode when bound to integrin or migfilin. Further, we demonstrate that filamin can indeed simultaneously engage more than one integrin or migfilin, thus providing the first experimental evidence of multisite ligand binding by filamin.

To address our questions, we used the construct encoding Ig repeats 19−21 of human filamin A. The domain boundaries were amino acids 2045−2140 for Ig19, 2141−2235 for Ig20, and 2236−2329 for Ig21.[12] There are three reasons to use this construct. (i) It has a well-characterized autoinhibitory unit (i.e., repeat 21 masked by repeat 20). (ii) It has two ligand binding repeats (Ig19 and Ig21) that can be used as a proof of principle to demonstrate multiligand binding. (iii) It has a well-resolved nuclear magnetic resonance (NMR) spectrum for unambiguous NMR analysis. We expressed repeats 19−21 and, for comparison, filamin-$A_{var\text{-}1}$ repeats 19−21 also in *E. coli* using standard molecular biology protocols. Constructs were confirmed by DNA sequencing. The glutathione *S*-transferase-fused

Received: March 3, 2011
Revised: April 20, 2011
Published: April 27, 2011

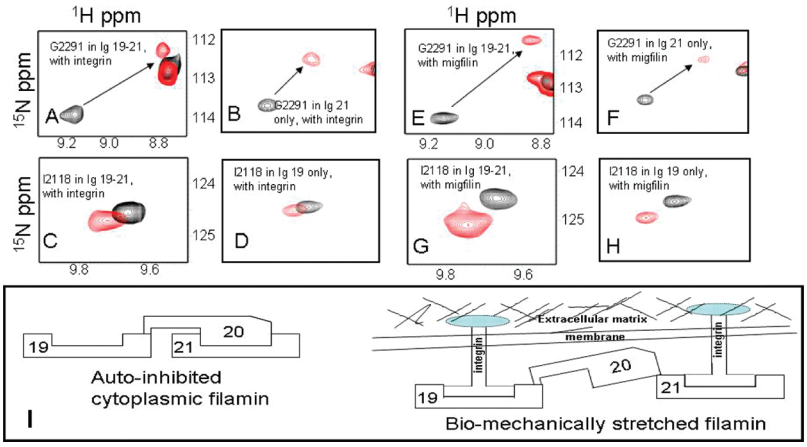

Figure 1. HSQC spectral changes of selected Ig19−21 residues (A, C, E, and G), free (black) and bound (red) to the integrin β7 peptide and migfilin peptide (protein:peptide ratio of 1:4). Panels B, D, F, and H show the chemical shift changes of the same residues in Ig21 and Ig19 only proteins (protein:peptide ratio of 1:2). All the spectra were recorded at 600 MHz and 30 °C. Full spectra are shown in panels A and B of Figure S6 of the Supporting Information.

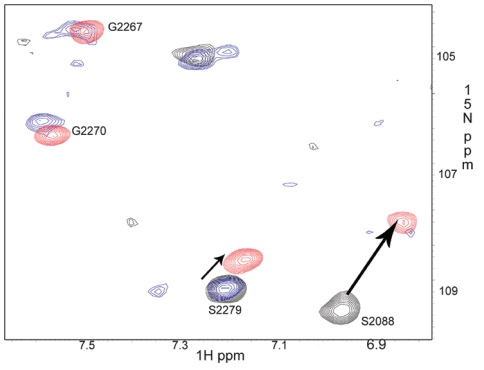

Figure 2. HSQC titration of migfilin with ^{15}N-labeled Ig19−21 shows that even at low peptide concentrations both Ig19 and Ig21 resonances are perturbed. The diagnostic G2267, G2270, S2279, and S2088 peak changes are highlighted. The protein concentration in all the experiments was 0.1 mM, and the migfilin peptide concentration was 0.005 mM (black, 20:1 protein:peptide ratio), 0.025 mM (blue, 4:1 protein:peptide ratio), or 0.4 mM (red, 1:4 protein:peptide ratio). Notice that the signal for Ig19 S2088 at a 4:1 protein:peptide ratio is broadened but appears at the 1:4 protein:peptide ratio (red). Also note that the signals for G2267 and G2270 for a 20:1 protein:peptide ratio are located elsewhere, whereas the assignments of G2267 and G2270 at 4:1 (blue) and 1:4 (red) protein:peptide ratios in Ig19−21 are unambiguous because they overlay exactly with those in the Ig21 bound to migfilin.

protein was cleaved and purified to homogeneity using standard affinity and chromatography methods described previously.[13] ^{15}N-labeled proteins for heteronuclear single-quantum coherence (HSQC) experiments were made in minimal medium supplemented with $^{15}NH_4Cl$. All proteins and peptides used in this study were dissolved in 25 mM sodium phosphate buffer, 5 mM NaCl, and 1 mM DTT (pH 6.3). The pH was matched within 0.03 unit when comparing spectra. HSQC comparison of repeats 19−21 with filamin-A_{var-1} repeats 19−21 revealed that most peaks corresponding to repeats 19 and 20 were missing or congested to the unstructured regions for the filamin-A_{var-1} 19−21 protein (Figure S2 of the Supporting Information). Filamin-A_{var-1} Ig19−21 peaks matched mostly with those of the free form of repeat 21, indicating that in this splice variant only Ig21 is folded (Figure S3 of the Supporting Information), whereas the deletion of 14 amino acids from repeat 19 and 27 amino acids from Ig20 impaired the structural integrity and unfolded the repeats. Note that this variant could bind migfilin robustly, indicating that the Ig21 binding site is intact and accessible to ligands (Figure S4 of the Supporting Information). In contrast to filamin-A_{var-1} Ig19−21, the wild-type Ig19−21 protein has peaks corresponding to Ig19 and Ig21, but a significant number of peaks did not overlap with those of free form Ig21, which is consistent with the autoinhibition of Ig21 by Ig20 (Figure S5 of the Supporting Information). Because Ig21 is autoinhibited in filamin Ig19−21, we thought that a ligand should in principle occupy the free Ig19 and cause the chemical shift changes of the residues in Ig19. However, spectral analysis based on the published chemical shifts[20] demonstrates unequivocally that both Ig19 and Ig21 were perturbed by either β7 integrin (Figure 1A,C) or migfilin (Figure 1E,G). The shifts were strikingly similar to that induced in Ig21 (Figure 1B,F) and Ig19 (Figure 1D,H) solitary repeats. This finding not only demonstrates the two-site ligand binding on Ig19−21 but also indicates the relief of the autoinhibition on Ig21. The latter has a significant implication in that filamin is likely to be in a stretched mode when engaged with integrin or migfilin (Figure 1I). Integrin β7 that binds filamin weaker than migfilin leads to some line broadening (Figure S6A of the Supporting Information), and thus, it is more notable to see the peak shifts of both Ig19 and Ig21 by migfilin (Figure S6B of the Supporting Information). More careful titration of migfilin into Ig19−21 from 4:1 to 0.05:1 peptide:protein ratios revealed definitive chemical shift changes to specific Ig19 and Ig21 residues (Figure 2). Importantly, at all concentrations, Ig19 and Ig21 appeared to bind simultaneously (Figure 2). This is evident in the spectra where the level of migfilin peptide was substantially lower than that of Ig19−21, but both Ig19 and Ig21 experienced the chemical shift changes (Figure 2). Further confirmation that both Ig19 and Ig21 can engage ligands simultaneously was obtained using isothermal titration calorimetry (ITC). Using the migfilin peptide as a ligand, we were able to show the two-site occupation of the Ig19−21 trirepeat protein. The obtained data were fit to a two-"identical site" binding model, and the affinities of the two sites were 72.9 ± 29.3 μM (Figure 3). The data could also be fit to a two-site sequential model with dissociation constants of 98 ± 29 and 112 ± 25 μM, respectively. The similar values make it clear that in this construct the occupation of Ig19 and Ig21 is almost simultaneous, corroborating our NMR

dx.doi.org/10.1021/bi2003229 |*Biochemistry* 2011, 50, 4229–4231

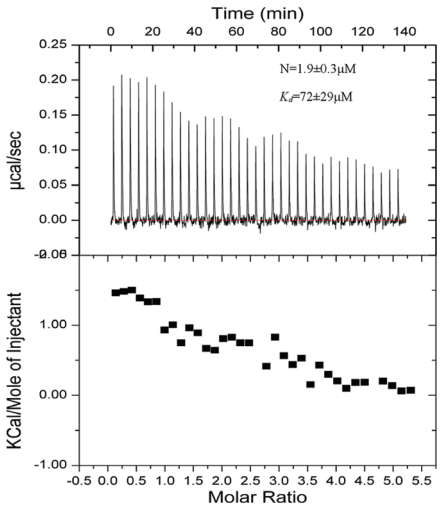

Figure 3. Isothermal calorimetric profile of migfilin binding to filamin A repeats 19−21. Note that the energetics was endothermic while the isolated Ig21 binding was exothermic. One possible explanation for this is the large-scale displacement of repeat 20 when Ig21 is occupied. The fit of the data to a model with two identical sites is shown. The data fitted well even with a two-site sequential model of binding with K_d values of 98 ± 29 and 112 ± 25 μM.

titration data (Figure 2). The combined facts argue for a dynamic equilibrium to toggle between the free and bound forms of Ig19−21, and the sequential binding (first to 19 followed by 21 or vice versa) model is not supported. Compared to the affinity of the isolated Ig21 for migfilin (2−8 μM), the Ig19−21 repeat has weaker binding presumably because of the effect of autoinhibition. Our attempts to measure the affinity of isolated Ig19 for migfilin were unsuccessful, though we see robust binding by NMR.[13] The energetics of Ig19 and Ig21 binding are therefore very different and may be a result of their varying stabilities.[21] The occupation of repeat 21 by migfilin in the Ig19−21 protein displaces Ig20, and the resulting solvent and protein rearrangements account for the differential affinities in the individual repeats compared to multiple repeats. The NMR and ITC data thus demonstrate that Ig21 can be readily unmasked by integrin β7 CT or migfilin ligands. In a natural protein, the Ig19 pocket if blocked by Ig18 will be refractory to binding. In conclusion, our results provide strong evidence that the filamin autoinhibition can be relieved by integrin or migfilin ligand itself and that filamin is engaged with the ligands via a multisite binding mechanism. At the biological level, the data imply that filamin is already stretched when engaged with integrin and migfilin and that extracellular matrix−actin traction is not necessary for relief of autoinhibition. The lack of any overt phenotype in the migfilin knockout mice is surprising given its strong affinity for filamin.[22] The function of migfilin may be developmentally compensated by other important filamin binding proteins that have not yet been discovered. The balance between the stretched and autoinhibited forms may allow the dynamic regulation of the actin−extracellular matrix linkage, thereby promoting dynamic cell adhesion processes such as cell shape change, cell migration, and survival.

■ ASSOCIATED CONTENT

Supporting Information. Details of the materials and methods, sequence comparison, and additional spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Department of Molecular Cardiology, Mail Code NB20, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Ave., Cleveland, OH 44195. Phone: (216) 444-5392. Fax: (216) 445-1466. E-mail: qinj@ccf.org.

Funding Sources

The study was supported by National Institutes of Health grants to J.Q.

■ ACKNOWLEDGMENT

We thank Jianmin Liu and Jun Yang for assistance in resonance assignments and technical insights into protein−protein interactions by nuclear magnetic resonance.

■ REFERENCES

(1) Hynes, R. O. (2002) *Cell 110*, 673–687.
(2) Shattil, S. J., Kim, C., and Ginsberg, M. H. (2010) *Nat. Rev. Mol. Cell Biol. 11*, 288–300.
(3) Moser, M., Legate, K. R., Zent, R., and Fässler, R. (2009) *Science 324*, 895–899.
(4) Qin, J., Vinogradova, O., and Plow, E. F. (2004) *PLoS Biol. 2*, 726–729.
(5) Kiema, T., Lad, Y., Jiang, P., Oxley, C. L., Baldassarre, M., Wegener, K. L., Campbell, I. D., Ylänne, J., and Calderwood, D. A. (2006) *Mol. Cell 21*, 337–347.
(6) Calderwood, D. A., Huttenlocher, A., Kiosses, W. B., Rose, D. M., Woodside, D. G., Schwartz, M. A., and Ginsberg, M. H. (2001) *Nat. Cell Biol. 3*, 1060–1068.
(7) Nakamura, F., Stossel, T. P., and Hartwig, J. H. (2011) *Cell Adhes. Migr. 5*, 160–169.
(8) Ithychanda, S. S., Das, M., Ma, Y. Q., Ding, K., Wang, X., Gupta, S., Wu, C., Plow, E. F., and Qin, J. (2009) *J. Biol. Chem. 284*, 4713–4722.
(9) Nieves, B., Jones, C. W., Ward, R., Ohta, Y., Reverte, C. G., and LaFlamme, S. E. (2010) *J. Cell Sci. 123*, 1216–1226.
(10) Tu, Y., Wu, S., Shi, X., Chen, K., and Wu, C. (2003) *Cell 113*, 37–47.
(11) Lad, Y., Jiang, P., Ruskamo, S., Harburger, D. S., Ylänne, J., Campbell, I. D., and Calderwood, D. A. (2008) *J. Biol. Chem. 283*, 35154–35163.
(12) van der Flier, A., and Sonnenberg, A. (2001) *Biochim. Biophys. Acta 1538*, 99–117.
(13) Ithychanda, S. S., Hsu, D., Li, H., Yan, L., Liu, D., Das, M., Plow, E. F., and Qin, J. (2009) *J. Biol. Chem. 284*, 35113–35121.
(14) Nakamura, F., Pudas, R., Heikkinen, O., Permi, P., Kilpeläinen, I., Munday, A. D., Hartwig, J. H., Stossel, T. P., and Ylänne, J. (2006) *Blood 107*, 1925–1932.
(15) Lad, Y., Kiema, T., Jiang, P., Pentikäinen, O. T., Coles, C. H., Campbell, I. D., Calderwood, D. A., and Ylänne, J. (2007) *EMBO J. 26*, 3993–4004.
(16) Heikkinen, O. K., Ruskamo, S., Konarev, P. V., Svergun, D. I., Iivanainen, T., Heikkinen, S. M., Permi, P., Koskela, H., Kilpeläinen, I., and Ylänne, J. (2009) *J. Biol. Chem. 284*, 25450–25458.
(17) Pentikäinen, U., and Ylänne, J. (2009) *J. Mol. Biol. 393*, 644–657.
(18) van der Flier, A., Kuikman, I., Kramer, D., Geerts, D., Kreft, M., Takafuta, T., Shapiro, S. S., and Sonnenberg, A. (2002) *J. Cell Biol. 156*, 361–376.
(19) Travis, M. A., van der Flier, A., Kammerer, R. A., Mould, A. P., Sonnenberg, A., and Humphries, M. J. (2004) *FEBS Lett. 569*, 185–190.
(20) Heikkinen, O., Permi, P., Koskela, H., Ylänne, J., and Kilpeläinen, I. (2009) *Biomol. NMR Assignments 3*, 53–56.
(21) Jiang, P., and Campbell, I. D. (2008) *Biochemistry 47*, 11055–11061.
(22) Moik, D. V., Janbandhu, V. C., and Fässler, R. (2011) *J. Cell Sci. 124*, 414–421.