Filamin-mediated linkages between transmembrane receptors (TR) and the actin cytoskeleton are crucial for regulating many cytoskeleton-dependent cellular processes such as cell shape change and migration. A major TR binding site in the immunoglobulin repeat 21 (Ig21) of filamin is masked by the adjacent repeat Ig20, resulting in autoinhibition. The TR binding to this site triggers the relief of Ig20 and protein kinase A (PKA)-mediated phosphorylation of Ser-2152, thereby dynamically regulating the TR-actin linkages. A P2204L mutation in Ig20 reportedly causes frontometaphyseal dysplasia, a skeletal disorder with unknown pathogenesis. We show here that the P2204L mutation impairs a hydrophobic core of Ig20, generating a conformationally fluctuating molten globule-like state. Consequently, unlike in WT filamin, where PKA-mediated Ser-2152 phosphorylation is ligand-dependent, the P2204L mutant is readily accessible to PKA, promoting ligand-independent phosphorylation on Ser-2152. Strong TR peptide ligands from platelet GP1bα and G-protein-coupled receptor MAS effectively accommodate a 11–13-residue-long amino acid segment of a “CD” groove in their immunoglobulin domain (Ig) that can bind Ig20, but surprisingly, the capacity of these ligands to bind the P2204L mutant was much reduced despite the mutation-induced destabilization of the Ig20 structure that supposedly weakens the autoinhibition. Thermodynamic analysis indicated that compared with WT filamin, the conformationally fluctuating state of the Ig20 mutant makes Ig21 enthalpically favorable to bind ligand but with substantial entropic penalty, resulting in total higher free energy and reduced ligand affinity. Overall, our results reveal an unusual structural and thermodynamic basis for the P2204L-induced dysfunction of filamin and frontometaphyseal dysplasia disease.
kinase binding a preferential substrate, a novel paradigm in biological signaling mechanisms. The intramolecular nature of autoinhibition means that ligands that bind Ig23 tightly can displace the Ig20 segment to potently promote phosphorylation at Ser-2152. In an analogous manner, cytoplasmic segments of many GPCRs are also very tight filamin-binding partners (19). These peptide ligands are predominantly part of a conserved intracellular helix in GPCRs that could accelerate PKA-mediated Ser-2152 phosphorylation in vitro. Consistent with this prediction, it was found that GPCR agonist stimulation enhances this Ser-2152 phosphorylation, implying a close physical contact between the GPCR and filamin (19). Although numerous studies had recognized filamin as a partner of GPCRs, filamin was thought to be more of an inert scaffolding protein in these signaling complexes (20–22). Bioinformatics studies now predict that a majority of GPCRs have segments that potentially bind filamin, and such binding may play crucial role in GPCR signaling (19).

Given that filamin binds a variety of proteins regulating key cellular functions, it is not surprising that both null and specifically clustered mutations in all three filamin genes were found to lead to many disorders of the brain, heart, muscle, and skeleton (23). The location of FLNA on the X-chromosome leads to severe phenotypes or lethality in males for many of the reported mutations (24–26). The biochemical and molecular basis for the mutant phenotypes remains elusive and have only been explored in a few cases. For example, the substitutions E254K (filamin A), W148R (filamin B), and M202V (filamin B) in the second calponin homology domain of the actin-binding region (filamin A), W148R (filamin B), and M202V (filamin B) in the second calponin homology domain of the actin-binding region in filamin A all conferred gain of function properties with increased affinity for actin (27, 28). Filamin Ig23, a class A repeat (4), harbors mutations that lead to frontometaphyseal dysplasia (FMD) and periventricular nodular heterotopia due to missense and in-frame deletions (29, 30). The deletion mutants of Ig23 were shown to be defective in their rheological properties and binding to the protein FilGAP (31). However, the disease origins of L2439M mutant in Ig23 are still unclear (31). In this paper, using recombinant and purified filamin fragments, we undertook detailed structural and biochemical studies on a disease causing P2204L mutation (24) that causes FMD, a disorder involving abnormalities in skeletal development. Our analyses revealed an unusual structural and thermodynamic basis for the P2204L mutation with defects in autoinhibition, ligand binding, and Ser-2152 phosphorylation, thus providing crucial clues for understanding the molecular pathogenesis of FMD.

Results

P2204L mutant adopts a conformationally dynamic molten globule-like state

Fig. 1A displays the crystal structure (18) of filamin Ig19–21 (PDB 2J3S) containing the autoinhibitory unit Ig20–21, where the FMD disease mutation site Pro-2204 is located in a long loop of Ig20 far away from the autoinhibited interface yet buried in a hydrophobic core of the Ig20 domain involving Val-2153, Phe-2202, Phe-2228, and Val-2230. Molecular modeling indicates that although the mutation of Pro into Leu at 2204 maintains the hydrophobic feature, the long side chain of Leu would cause a minor steric clash with some of these hydrophobic residues including Phe-2202, Phe-2228, Val-2230, and also a polar residue His-2210 (Fig. 1B), thus potentially perturbing the local folding of the protein. To evaluate this prediction, we made the minimal double domain construct Ig20–21, that has no ligand binding interference with the neighboring Ig19 or Ig23. First, we compared the gel-filtration elution profiles of the purified Ig20–21 WT with that of the disease-causing mutant P2204L under identical conditions using a Superdex75 size exclusion column. As shown in Fig. 2A, the mutant eluted as a single peak, 2.7 ml, ahead of the WT, indicating a greater radius of gyration (2017) 292(20) 8390–8400 J. Biol. Chem.

Figure 1. Location and residues around Pro-2204 in filamin A structure.

A, crystal structure of autoinhibited Ig19–21 from filamin A (PDB 2J3S). The segment of Ig20 occluding Ig21 ligand binding site is shown in blue. The Pro-2204 side chain is buried in the Ig20 segment and is proximal to the Ser-2152 phosphosite. B, substitution of Pro-2204 into leucine causes some steric clash with surrounding residues (labeled) and likely destabilizes Ig20.
B) are consistent with a conformationally fluctuating partially folded intermediate or a molten globule. Despite the mutation being in Ig20, Ig21 is affected as exemplified by the significant shift of W2262 side chain amide, suggesting that the mutated Ig20 still engages with Ig21 but in a different fashion.

Perturbed intrinsic fluorescence of a protein dominated by tryptophan residues is another measure of the folded state of the protein (32–34). The Ig20–21 construct has a single tryptophan residue at position 2262, and we recorded the fluorescence emission spectra by exciting the tryptophan at 278 nm, the absorption maximum for both the WT and the P2204L mutant Ig20–21 (0.1 mM, 64 scans, red). The emission intensity for the mutant was significantly higher, and the emission maximum ($\lambda_{max}$) was red-shifted to 340 nm from 335 nm in the WT (Fig. 2C). This tryptophan is located in
Ig21 and is >20 Å from the P2204L mutation site. This result provides strong evidence that the mutant has undergone a substantial global structural transition as opposed to a local change around the site of the mutation. To rule out the contribution of phenylalanine and tyrosine residues to the emission spectra, we recorded the fluorescence emission by exciting the protein at 295 nm, and this experiment too resulted in similar red shifted emission for the P2204L mutant (supplemental Fig. S1), furthering the notion of a molten globule.

To further gain insight into the altered folding state of the mutant, we performed 8-anilino-1-naphthalenesulfonic acid (ANS) binding experiment. The enhanced fluorescence from ANS binding to protein is a widely used sensitive marker for differentiating molten globule from folded/unfolded states of proteins (35, 36). ANS does not bind appreciably to native or totally unfolded proteins but binds well to partially folded/molten globule state with hydrophobic patches exposed (35, 36). We probed ANS binding to Ig20–21 WT and the P2204L mutant using excitation at 380 nm and recording the emission from 400–600 nm using a 50 molar excess of ANS (5 µM protein and 250 µM ANS). Fluorescence enhancement was minimal in the WT when compared with the ANS only buffer control (Fig. 2D). The mutant though had much higher fluorescent intensity, and the emission max was blue-shifted to 502 nm (Fig. 2D). This feature is consistent with a molten globule state as documented previously (35). Note that control ANS experiments in denaturant, 6 M guanidine hydrochloride, leads to a loss of these emergent fluorescent features leading to uniform spectral outputs (supplemental Fig. 2). Overall, all the four above experiments, size exclusion, HSQC-NMR, intrinsic tryptophan fluorescence, and extrinsic ANS fluorescence, together provide strong evidence that the mutant P2204L Ig20–21 is likely a molten globule.

**P2204L mutant is readily phosphorylated at Ser-2152 without ligand**

Because previous studies showed that ligand binding to Ig21 relieves Ig20 causing a global conformational change and enhanced PKA-mediated phosphorylation on Ig20 Ser-2152 (17), we first wondered how the P2204L-induced destabilization of Ig20 would impact on the Ser-2152 phosphorylation. As done previously (17), we used the dimeric Ig16–24 construct encompassing the entire rod 2 domain of filamin A to test the phosphorylation effect. Interestingly, unlike WT, which had minimal phosphorylation without ligand binding due to the autoinhibitory interface, the mutant Ig16–24 was readily phosphorylated in the absence of ligand (Fig. 3, compare lanes 1–3 with lanes 7–9). As demonstrated earlier (19), the tight binding MAS GPCR peptide enhanced Ser-2152 phosphorylation of Ig16–24 (Fig. 3, compare lanes 1–3 with lanes 4–6), but the MAS peptide led to saturation of phosphorylation at a much earlier time point in the P2204L mutant (Fig. 3, compare P2204L lanes 10–12 with lanes 1–9). The PKA reaction at the 1 h time point without the added MAS peptide was subjected to quantitative mass spectroscopy by protease digestions to conform the results from Western blotting. Table 1 summarizes the results from mass spectrometry and confirms that the P2204L mutant is at least four times (24% versus 96%) more susceptible to PKA phosphorylation than the WT Ig16–24 in the absence of MAS ligand. The Thr-2336 residue in Ig22 serves as an internal control, and this low-level phosphorylation does not change between the WT and the mutant P2204L filamin A Ig16–24 (Table 1). Theunchanging Thr-2336 phosphorylation also hints that the P2204L mutant leads to local and limited changes in Ig20–21 and does not lead to global changes in filamin structure. These data indicate that the mutation-induced dynamic and partially unfolded conformation of Ig20 in Ig16–24 became readily accessible to PKA for catalysis unlike the Ig20 in WT Ig16–24.

**P2204L mutant has impaired/reduced capacity to bind ligand**

Next, we wondered how ligands would bind to the P2204L mutant. Although the mutation site is not directly involved in the autoinhibitory interface (see Fig. 1), the mutation-induced formation of the partially folded intermediate of Ig20 should in principle destabilize its engagement with Ig21, including the autoinhibitory interface. The tightest ligand (4) for filamin repeats is GP1bα, and this peptide caused precipitation of the Ig20–21 WT protein and hence could not be used for comparative studies. We, therefore, used the MAS peptide for examination, which has slightly reduced affinity as compared with GP1bα (19) but did not lead to protein loss by precipitation. Surprisingly, although the MAS peptide led to widespread changes in the WT Ig20–21 spectrum characteristic of tight binding and relief of the autoinhibition (Fig. 4A), the P2204L mutant showed no signal narrowing/recovery of the spectrum upon the addition of the strong ligand (Fig. 4B). If MAS effectively displaced Ig20 to bind Ig21, we would have at least observed recovering signals of Ig21 bound to MAS despite the molten globule state of Ig20. Some broadened peaks are visible, such as the characteristic Ser-2279 that is near the ligand binding groove of Ig21. They are shifted upon the addition of the strong ligand (Fig. 4B). As demonstrated earlier (19), the tight binding MAS GPCR peptide enhanced Ser-2152 phosphorylation of Ig16–24 (Fig. 3, compare lanes 1–3 with lanes 4–6), but the MAS peptide led to saturation of phosphorylation at an earlier time point in the P2204L mutant (Fig. 3, compare P2204L lanes 10–12 with lanes 1–9). The PKA reaction at the 1 h time point without the added MAS peptide was subjected to quantitative mass spectroscopy by protease digestions to conform the results from Western blotting. Table 1 summarizes the results from mass spectrometry and confirms that the P2204L mutant is at least four times (24% versus 96%) more susceptible to PKA phosphorylation than the WT Ig16–24 in the absence of MAS ligand. The Thr-2336 residue in Ig22 serves as an internal control, and this low-level phosphorylation does not change between the WT and the mutant P2204L filamin A Ig16–24 (Table 1). Theunchanging Thr-2336 phosphorylation also hints that the P2204L mutant leads to local and limited changes in Ig20–21 and does not lead to global changes in filamin structure. These data indicate that the mutation-induced dynamic and partially unfolded conformation of Ig20 in Ig16–24 became readily accessible to PKA for catalysis unlike the Ig20 in WT Ig16–24.

![Figure 3. Elevated susceptibility of the P2204L mutant to PKA-mediated phosphorylation in the dimeric Ig16–24 C-terminal filamin A. Notice that the mutant phosphorylation is the ligand (MAS)-independent contrasting to the ligand-dependent phosphorylation of the WT.](image)

| MAS peptide | - | - | + | + | + | + | + | + | + | + | + |
|-------------|---|---|---|---|---|---|---|---|---|---|---|
| Ig16-24WT   | + | + | + | + | + | - | - | - | - | - | - |
| Ig16-24P2204L| - | - | - | - | - | + | + | + | + | + | + |

**Figure 3. Elevated susceptibility of the P2204L mutant to PKA-mediated phosphorylation in the dimeric Ig16–24 C-terminal filamin A. Notice that the mutant phosphorylation is the ligand (MAS)-independent contrasting to the ligand-dependent phosphorylation of the WT.**
and MAS (Fig. 5B) were enthalpically favorable (exothermic with a large negative enthalpy confirming our earlier results), the ligand binding to autoinhibited Ig20–21 was enthalpically unfavorable (endothermic) and entropy-driven (Fig. 5, C and D, and Table 2). Autoinhibition led to a reduction in the affinity of ~8 times for GP1bα and ~140 times for the MAS peptide (Table 2) and reversed the energetics of binding. Contrasting to the WT Ig20–21, P2204L destabilized Ig20, which made GP1bα binding enthalpically favorable (exothermic) but with substantial entropic penalty likely due to the fluctuating nature of the Ig20 mutant before ligand binding (much smaller entropic change than that of WT; see Table 2). As a net result, the total free energy of the GP1bα binding for the mutant is higher than the WT, which led the reduced ligand binding affinity for the mutant (Fig. 5E and Table 2), which is consistent with the NMR data in Fig. 4B. However, an accurate affinity estimate was not possible with this weak ITC data.

**Discussion**

As a major cytoskeletal protein, filamin has been extensively studied for its binding to target proteins. Filamin has two characteristic binding features; one is actin binding via the calponin homology domains at the N terminus and the other is binding to cytoplasmic regions of multiple transmembrane receptors, notably integrins and GPCRs, via seven homologous class A Ig repeats (repeat 4, 9, 12, 17, 19, 21, and 23) (4). Many disease mutations have been found in filamin, and a majority of them occur in these two types of binding regions or regions surrounding them. However, only a few very limited mutations, which are unrelated to the autoinhibition, have been biochemically and biophysically studied. The mutations W148R and M202V in filamin B that cause autosomal dominant atelosteoo-

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**Table 1**

Degree of phosphorylation in the Ig16–24 WT and mutant P2204L proteins

Peak area is the quantitation of elution peaks of the peptides from liquid chromatography entering the mass spectrometer after protease digestion of Ig16–24. The phosphorylated protein samples used were from the 1 h time point of the WT and mutant protein without added ligand (Fig. 3).

| Site  | Sequence                  | Peak area (PA) of the peptide | Ratio of PA mod/unmod |
|-------|---------------------------|-------------------------------|-----------------------|
| S2152 | APSVANVGSCLKL             | 323455221                     | -                     |
|       | AAPPVANVGSCLKL            | 50215208                      | 0.15                  |
|       | RAPSVANVGSCLKL            | 279191930                     | -                     |
|       | RAPPSVANVGSCLKL           | 145104532                     | 0.52                  |
|       | % S2152 Phosphorylation   |                               | 24%                   |
| T2356 | RLTVSSQEGLK               | 2037867418                    | -                     |
|       | RLPTVSSQEGLK              | 88835595                      | 0.044                 |
|       | % T2356 Phosphorylation   |                               | 4%                    |

| Site  | Sequence                  | Peak area | PA (mod)/PA (unmod) |
|-------|---------------------------|-----------|--------------------|
| S2152 | APSVANVGSCLKL             | 21617728  | -                  |
|       | AAPPVANVGSCLKL            | 138045044 | 6.4                |
|       | RAPSVANVGSCLKL            | 20461657  | -                  |
|       | RAPPSVANVGSCLKL           | 470350690 | 23.0               |
|       | RRAPPSVANVGSCLKLQMDoTKQVTSPSGK | 17297044 | -                  |
|       | RAPSVANVGSCLKLKPEISIQDMoTAQVTSPSGK | 2436887 | -                  |
|       | RAPPSVANVGSCLKLKPEISIQDMoTAQVTSPSGK | 38381829 | 15.8               |
|       | % S2152 Phosphorylation   |           | 96%                |
| T2356 | RLTVSSQEGLK               | 1085463085| -                  |
|       | RLPTVSSQEGLK              | 41871331  | 0.039              |
|       | % T2356 Phosphorylation   |           | 4%                 |
genesis were shown to be located in the CH2 domain of the actin-binding site of filamin B, which lead to tighter actin binding and hence the likely gain of function phenotype (27). The G2593E (Ig24) mutant in filamin A, which causes a mild form of periventricular heterotopia, was indicated to arise from filamin dimerization defect (25). Here we have undertaken the first

Figure 4. Comparison of ligand binding to WT and mutant filamin. A. $^{15}$N HSQC of the WT Ig20–21 in the free form (0.2 mM WT protein, 8 scans, black) and with the bound MAS peptide (0.2 mM protein with 0.4 mM MAS peptide, 8 scans, red). B. $^{15}$N HSQC of the mutant P2204L Ig2021 in the free form (0.1 mM P2204L mutant, 64 scans, black) and with the bound MAS peptide (0.1 mM protein with 1.2 mM MAS peptide, 64 scans, red). Both proteins bind the MAS peptide as seen by the appearance of multiple new peaks, but the mutant protein has much broadened signals and does not recover the features of single conformational class upon ligand binding. Some peaks in \( \geq 9 \) ppm range are marked by arrows to show that the protein has folded features but under dynamic intermediate exchange (molten globule). Such molten globule still binds to ligand as shown by characteristic shift of Ser-2279 in up-field, but the shift is less than that of WT (Fig. 4A), suggesting weaker binding.
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Table 2
Comparison of the thermodynamic parameters (kcal/mol) of Ig21 and Ig20–21 binding to peptides

| Experiment       | $N$ | $K_D$ | $\Delta G$ | $\Delta H$ | $\Delta S$ | $T\Delta S$ |
|------------------|-----|-------|------------|------------|------------|-------------|
|                  |     | kcal/mol | kcal/mol | kcal/mol | kcal/mol | kcal/mol |
| Ig21 + GP1ba     | 0.94| 203 ± 32 nM | -9.279 | -9.279 | -411 | -411 |
| Ig20–21 + GP1ba  | 0.89| 1.68 ± 0.58 μM | -8.011 | -8.011 | 411 | 411 |
| Ig20–21(P2204L) + GP1ba | 0.76| 5.62 ± 1.75 μM | -7.283 | -7.283 | 411 | 411 |
| Ig21 + MAS       | 0.86| 411 ± 69 nM | -8.853 | -8.853 | 411 | 411 |
| Ig20–21 + MAS    | 0.79| 61 ± 6.7 μM | -5.859 | -5.859 | 69 | 69 |
| Ig20–21(P2204L) + MAS | NA| NA | NA | NA | NA | NA |

$N$ is the number of binding sites per protein molecule; $K_D$ is the dissociation constant; $\Delta G$ is the total change in free energy; $\Delta H$ is the change in enthalpy; $\Delta S$ is the change in entropy; $T$ is the absolute temperature. All numbers use the Gibbs free energy equation $\Delta G = \Delta H - T\Delta S$. NA, $K_D$ not accurately analyzable due to very weak binding.

detailed structural and biochemical analyses of frontometaphyseal dysplasia mutation P2204L in Ig repeat 20 of filamin A. The mutation site is proximal to the phosphorylation site Ser-2152. On the other hand, although Ig20 is involved in autoinhibition, the mutation site is quite distant from the autoinhibitory interface (Fig. 1A). The binding of ligand migfilin or GP1β to Ig21 makes the protein more flexible and adopt a distinct conformation (17, 37). Our observations were interesting as the mutation affected both filamin autoinhibition and phosphorylation in an opposite and uncoupled manner, unlike the WT filamin.

Our molecular modeling analysis indicated that P2204L might induce a hydrophobic core collapse due to the steric clash of the Leu side chain with the hydrophobic core residues and thus may lead to the formation of a partially folded intermediate at Ig20. Due to the packing of Ig20 with Ig21, as in the autoinhibited conformation, this mutation in Ig20 perturbed the overall structural integrity of Ig20–21, as implicated by the substantially broadened NMR signals (Fig. 2B) and increased hydrodynamic volume (Fig. 2A). The results hinted that the destabilizing mutation likely induced partial unfolding of Ig20–21, resulting in a molten globule-like state. Although the broadened NMR peaks may alternatively reflect interchanging fully folded and totally unfolded states on the millisecond time scale, this possibility was eliminated by our subsequent ANS-binding experiments. ANS is known not to bind well to either fully folded or totally unfolded protein (35, 36), and this was reflected in our studies (Fig. 2D and supplemental Fig. S2) with the WT Ig20–21. By contrast, the P2204L mutant in phosphate buffer bound ANS robustly (Fig. 2D). If the mutant were to only sample folded and unfolded states in the millisecond timescale, ANS would not bind to the protein. Consistently the red shift of the intrinsic tryptophan fluorescence in the mutant clearly hints that the mutant is in a transition between folded and unfolded states (32–34). Thus, intrinsic tryptophan and extrinsic ANS fluorescence experiments gave further evidence that the conjoined domains 20–21 is likely a molten globule(Fig. 2, C and D) in the mutant filamin.

Unlike WT filamin, which requires the ligand binding to produce a conformationally unrestricted Ig20 to access PKA for phosphorylation catalysis, the mutant itself was readily phosphorylated without ligand, apparently due to the conformational plasticity of the mutant. Such altered capacity of the mutant for phosphorylation likely alters filamin function during cell adhesion and migration and relates to the pathogenic mechanism leading to FMD. Because of the destabilization of Ig20 by P2204L, which would loosen/disturb the Ig20 contact with Ig21, we then thought that ligand binding to the mutant would be enhanced due to the potentially weakened autoinhibition. However, to our surprise, ITC studies demonstrated that the overall $K_D$ of the mutant to ligand was reduced by >3 fold as compared with the WT filamin (Table 2). Consistently, the potent MAS ligand was unable to recover the broadened NMR signals especially for Ig21, indicating the reduced capacity of MAS to relieve the autoinhibition of the filamin mutant. Detailed thermodynamic analysis revealed that although the mutation did make autoinhibited filamin enthalpically unfavorable (exothermic) for ligand binding (with the GP1β peptide, contrasting to the WT filamin, which was enthalpically unfavorable, i.e. endothermic, see Table 2), the mutant became more entropically unfavorable for ligand binding due to the mutation-induced conformational and structural liability. This is likely the reason for broadening/disappearance of the Ig21 peaks from the HSQC spectra for the mutant Ig20–21. By contrast, our earlier results with a splice form of filamin, where the autoinhibition segment is completely missing, revealed no signal broadening for Ig21 (38, 39). This Ig20 mutant lacks 42 amino acids from Ig20 including the β-strand that blocks Ig21 CD groove (38). Thus, through entropic penalty, the P2204L mutant strengthens the autoinhibition from a thermodynamic point of view. This is clear from the ITC profile of the mutant as it binds GP1β with 3 times weaker than the WT (K_D = 5.6 μM versus 1.68 μM). The same trend is true for MAS ligand, which binds to Ig20–21 at 61 μM, but more weakly to the mutant P2204L (K_D unanalyzable due to the weak affinity, likely >200 μM). Given the negligible affinity of the MAS GPCR peptide to the P2204L mutant, other cellular ligands like integrins and migfilin binding to this FMD mutant at Ig21 will be insignificant. This weakened binding of the conjoined repeats to ligands is likely to upset mechanotransduction properties of the entire filamin molecule. This may weaken integrin-extracellular matrix adhesion strength as shown in a recent fibroblast-specific filamin knock-out study (40).

In summary, unlike WT filamin, in which the phosphorylation at Ser-2152 is strongly enhanced with the release of the autoinhibition by ligand binding, the Pro-2204 mutant is readily phosphorylated without ligand, whereas the autoinhibition

Figure 5. Isothermal titration calorimetry profiles of ligand binding to WT and mutant. A, Ig21 binding to GP1β peptide (exothermic). B, Ig21 binding to MAS peptide (exothermic). C, Ig20–21 binding GP1β peptide (endothermic). D, Ig20–21 binding to MAS peptide (endothermic). E, P2204L Ig20–21 binding to GP1β peptide (exothermic). F, P2204L Ig20–21 binding to MAS peptide. See Table 2 for details of the energetics.
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is “strengthened,” as compared with WT filamin; hence, a weakening of ligand binding in the mutant. Using combined NMR and ITC approaches, we obtained structural and thermodynamic insight into these unusual defects. Mindful of the observation that clustered mutations in the actin binding domain and in repeats 9–11 and 15–16 also produce FMD (30), this study may serve as a foundational step for elucidating the fundamental mechanisms leading to a range of filamin-related skeletal diseases. In particular, the altered capacity of filamin autoinhibition by the mutation may affect the strength and dynamics of the extracellular matrix–actin linkage in relation to cell stiffness, adhesion strength (40), etc., which are directly involved in muscle/bone development and thus contribute to the FMD.

Experimental procedures

Proteins and peptides

Ig repeats 20–21 (Gly-2136–Ser-2329) of human filamin A (uniprot; P21333) was generated by truncating the larger Ig16–24 construct using the QuikChange lightning mutagenesis kit, and the P2204L mutant within Ig20–21 and Ig 16–24 was generated using the QuikChange lightning mutagenesis kit (catalogue #210518) form Agilent Technologies. All constructs were DNA sequenced and had no untoward mutations. Proteins were expressed as glutathione S-transferase fusions and purified as described earlier (4). Very tight Ig21-binding peptides derived from human platelet protein GP1b (uniprot; P07359, FLRGLTFRSSLFLWVRPNGRV) and human MAS, a constitutively active GPCR (uniprot; P04201, KKKRFKESLKVVLTRAFK) were synthesized by the Biotechnology core of the Lerner research Institute at the Cleveland Clinic.

Molecular modeling

The A subunit in the Ig19–21 filamin A crystal structure (PDB 2J3S) was used to assess the effect of the P2204L mutation. The mutagenesis tool of the PyMOL (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC) graphics system was used to change the proline to a leucine, and the clashes generated by the side chain of introduced leucine were noted.

In vitro PKA phosphorylation assay

The kinase phosphorylation assay using 10 μM Ig16–24 WT and the P2204L mutant Ig16–24 as substrates was performed in buffer containing 50 mM Tris (pH 7.5), 10 mM MgCl2, 1 mM DTT, and 500 μM ATP. 50 μM MAS peptide was used to release autoinhibition. For each 100-μM reaction, 1000 units of murine PKA (New England BioLabs) was used. Filamin Ser-2152 phosphorylation was detected by Western blotting using polyclonal phospho-filamin A antibody #4761 from Cell Signaling Technology (Danvers, MA).

Mass spectrometry

The 1-h assay product from the PKA phosphorylation assay was used to assess and compare the degree of Ser-2152 phosphorylation in the Ig16–24 wild type and P2204L mutant filamin C terminus. Bands were excised from Coomassie-stained gels, and this protein was reduced, alkylated, and digested with trypsin. The digests were analyzed by capillary column LC-tandem MS, and the collision-induced dissociation spectra were searched against filamin sequences. The WT and mutated peptide fragments was precisely identified in the samples. Chromatograms were plotted for the unmodified and modified Ser-2152- and Thr-2336-containing peptides, and the peak area for each of these peptides was determined. These peak areas were used to calculate both the peak area ratio (modified/unmodified) and the % phosphorylation.

Size exclusion chromatography

100 μl of purified recombinant Ig20–21 WT and P2204L mutant at a concentration of 5 mg/ml was subjected to size exclusion chromatography using a Superdex S75 1660 column (GE Healthcare) on an AKTA protein purification system from GE Healthcare under identical buffer (25 mM sodium phosphate (pH 6.4), 5 mM NaCl, and 1 mM DTT) and flow conditions (1 ml/min).

Nuclear magnetic resonance

15N-Labeled proteins were subjected to HSQC-NMR using a cryo-cooled Bruker 600-MHz machine at a concentration of 0.1 mM or 0.2 mM. The protein was extensively buffered with 25 mM sodium phosphate (pH 6.4), 5 mM NaCl, and 1 mM DTT. The MAS peptide was added at 2 times in excess of the protein for the WT Ig20–21, and 8 scans were recorded. For the P2204L mutant the MAS peptide was added 12 times in excess of the protein. Spectra were recorded in identical conditions. The pH of the protein samples overlaid were within 0.03 units, and the spectra were recorded at 30°C. Spectra were processed and analyzed using nmrPipe and NMRView (41).

For ANS binding to Ig20–21 and intrinsic fluorescence, ANS (#10417) was purchased from Sigma and was of >97% purity. Fluorescence measurements was performed at room temperature for ANS binding, and native fluorescence was done on a Hitachi F-2500 fluorescence spectrophotometer in the same buffer conditions used for size exclusion chromatography and HSQC-NMR. The excitation and emission slit widths were 10 nm. A 50x excess of ANS over protein (WT Ig20–21 or P2204L Ig20–21) was used (5 μM: 250 μM) in the measurements. Excitation was induced at 380 nm, and fluorescence was recorded between 400 and 600 nm. Control experiments were performed with completely unfolded protein in 6 M guanidine hydrochloride in identical buffer conditions to rule out nonspecific effects. Native tryptophan fluorescence emission was measured between 290 and 400 nm by exciting the protein (5 μM in conditions identical to ANS binding) at 278 nm. The wild type and the mutant Ig20–21 had the same absorption maximum. The results were confirmed by experiments using 295-nm excitation as well. All the fluorescence spectra were recorded at room temperature.

Isothermal calorimetry

iTC200 calorimeter from Malvern instruments was used for measuring the affinities of peptide binding to Ig repeats of filamin. The buffer and temperature conditions were precisely
matched to those used in the HSQC NMR experiments. The syringe concentration of the peptide was 1 mM, and the Ig repeat concentration in the cell chamber was 50 μM. Ligand was injected at 1-μl increments at a stirring speed of 750 rpm. Binding affinities were calculated using the associated origin software.

Author contributions—S. S. I. conceived the mutants and designed and conducted most of the experiments, analyzed the results, and wrote the paper. K. D. made the mutants, purified the recombinant proteins, and conducted some experiments. S. P. R. provided disease expertise and suggested experiments. J. Q. designed the experiments and supervised the project. S. S. I. and J. Q. wrote the paper.

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