Interaction of filamin A with the integrin β_7 cytoplasmic domain: role of alternative splicing and phosphorylation

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Abstract Integrin–filamin binding plays an important role in adhesion-mediated control of the actin cytoskeleton. Here, using the interaction between recombinant fragments from the C-terminus of filamin A and the cytoplasmic tail of integrin β_7 as a model, we report a negative regulatory role for filamin alternative splicing. Splice variant forms of filamin A lacking a 41-amino acid segment interacted more strongly than full-length fragments. In addition, we provide evidence that phosphorylation of the splice variant region is unlikely to represent the mechanism by which binding is reduced.

Keywords: Integrin; Filamin; Splice variant; Cytoskeleton; Cell migration

1. Introduction

Integrins are heterodimeric receptors involved in cell–cell and cell–extracellular matrix interactions [1]. Integrins spatially compartmentalize signalling molecules [2] and link the extracellular matrix to the intracellular actin cytoskeleton, thereby regulating cell polarity and motility [3]. Many different intracellular proteins have been shown to interact with integrin cytoplasmic domains [4–6], including signalling adaptors and cytoskeletal proteins.

A key integrin-binding protein is filamin [7]. Filamin is a 280-kDa rod-shaped protein, composed of an N-terminal actin-binding domain (ABD), 24 homologous ~100 amino acid repeating modules, and two flexible hinge regions (between repeats 15–16 and 23–24) (Fig. 1A). Filamin forms parallel homodimers via the C-terminal 24th repeats, with the dimer adopting a V-like shape, as determined by electron microscopy [8]. The repeats are primarily composed of β-strands, although structural information for higher-order filamin proteins is lacking. Filamin crosslinks actin, forming either loose microfilament networks, or tight actin bundles, depending on the ratio of actin:filamin present [9,10]. In addition, filamin interacts with a large number of cytosolic proteins and may play important roles in the recruitment of such signalling factors to sites of adhesion. There are three filamin isoforms expressed in cells, termed filamin-A, -B and -C. Filamin-A and -B are ubiquitously expressed, whereas expression of filamin-C is restricted to skeletal and cardiac muscle [7].

Filamin has been shown to interact with the β cytoplasmic domain of several integrins, including β_1, β_2, β_3 and β_7 [11–14]. Binding of filamin to β integrin tails has been shown to be important in the regulation of cell migration, as the β_7 integrin tail, which binds strongly to filamin and plays a key role in a variety of leukocyte trafficking events, supported low levels of cell migration, whereas β_1 tails that bind weakly to filamin (β_{1A} and β_{1D}) supported higher levels of cell migration [11].

Repeats 19–24 of filamin mediate the interaction with the integrin β_1 tail [12,15]. A splice variant within this region has been discovered in which 41-amino acids are removed from a site between repeats 19 and 20: removal of this segment increased filamin affinity for the β_1 tail in vitro and as a consequence stimulated myoblast differentiation [15].

In view of the potential link between filamin binding and regulation of integrin-mediated migration, we have investigated the interaction of filamin splice variants with the integrin β_7 tail, using the interaction between a recombinant fragment from the C-terminus of filamin and the cytoplasmic tail of integrin β_7 as a model system. We show that filamin splice variants interact more strongly with the β_7 tail than wild-type fragments, indicating that the 41 amino acid splice region acts as a negative regulator of integrin binding. We also show that phosphorylation of the filamin splice region does not likely represent the mechanism by which integrin binding is reduced, suggesting that the splicing has a structural effect on the integrin-binding site within the filamin.

2. Materials and methods

2.1. Production of integrin tail model peptides

Full-length cDNA encoding integrin α_4 and β_7 were kindly donated by Dr. Christelle Perros-Huguet (Pfizer Ltd., Sandwich, Kent, UK). The integrin tails were expressed as 6× His-tagged proteins, with a coiled-coil region inserted onto the N-terminus of the protein (sequence GGCGGAQLKKKLQALKKKNAQLKWKLQALKKKLAQ, from [16]). Due to the insertion of a Cys residue at the N-terminus of the coiled-coil, the integrin tails form homodimeric structures (data not shown).

To produce the coiled-coil, overlapping PCR was performed, inserting a 5′ *Bam*HI and 3′ *Eco*RI restriction site. After digestion with

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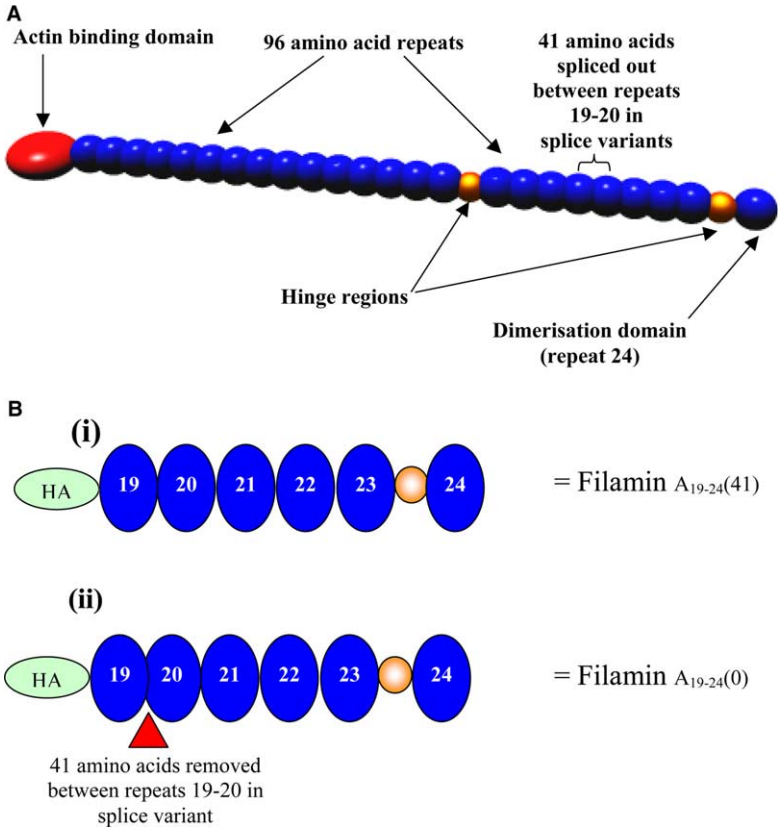

Fig. 1. Predicted structure of filamin. (A) Schematic structure of filamin. Although structural information is lacking for mammalian filamins, predictions have been made based on amino acid sequence. There is predicted to be an N-terminal ABD (red), followed by 24 repeating units of ~96 amino acids (blue), predicted to form β-sheet structure. Two hinge regions (gold) are inserted between repeats 15–16 and 23–24. Parallel dimers are formed by non-covalent interactions mediated via repeat 24. (B) Schematic representation of C-terminal fragments of filamin used. Both constructs were tagged with HA at their N-termini (green). The splice variant of filamin A has 41-amino acids removed from between repeating units 19 and 20. Filamin repeats are shown in blue, with the hinge region shown in orange.

*Bam*HI–*Eco*RI, the coiled-coil was ligated into equivalently digested pHis, a modified version of pET-15 (Novagen, Madison, WI, USA), encoding an N-terminal 6× His tag [17].

To clone the cytoplasmic domain regions of integrin α_4 and β_7, the tail regions (K968-D999 for α_4, R728-L779 for β_7) were amplified by PCR, inserting a 5′ *Mfe*I and 3′ *Eco*RI restriction site. The product was digested with *Mfe*I–*Eco*RI and ligated into *Eco*RI-digested pHis containing the coiled-coil construct. Correct ligation destroys the 5′ *Mfe*I restriction site, resulting in a 5′ *Bam*HI–3′ *Eco*RI fragment encoding the coiled-coil upstream from the integrin cytoplasmic domain. Correct insertion and sequence of the constructs was verified by DNA sequencing.

To express the integrin cytoplasmic tail proteins, JM109 (DE3) bacterial cells were transformed with integrin tail DNA, grown with shaking at 37 °C, and protein expression induced by addition of 1 mM IPTG. After 4 h, cells were harvested by centrifugation and resuspended in 40 ml/l binding buffer (0.5 M NaCl and 20 mM Tris–HCl, pH 7.9). Bacteria were lysed on ice by 6× 30 s sonication at 20% amplitude using a stepped microtip and a Vibra Cell sonicator (Sonics and Materials Inc., Newtown, CT, USA). The majority of integrin tail protein expressed was found to be insoluble (data not shown), hence lysates were centrifuged at 40 000×*g* for 20 min to isolate inclusion bodies. Pellets were resuspended in binding buffer plus 5 mM imidazole and 6 M urea, and incubated on ice for 30 min. Cellular debris was removed by centrifugation at 40 000×*g* for 20 min, and integrin tail purified from dissolved inclusion body lysate by nickel affinity chromatography, using Nickel–NTA^{TM} agarose (Qiagen Ltd., Crawley, UK). Integrin peptides were then further purified by RP-HPLC, using a C18 column (Phenomenex, Macclesfield, UK) and an AKTA purifier system (Amersham Pharmacia, Buckinghamshire, UK). Peak protein fractions were lyophilized by rotary evaporation under vacuum, resuspended in 20 mM PIPES, pH 6.8, 50 mM NaCl, and stored at −80 °C until use.

2.2. Affinity chromatography pull-down assay

COS-1 cells were transfected with filamin constructs encoding the C-terminal repeats 19–24, tagged at their N-terminus with an HA tag [15] (Fig. 1) using Lipofectamine 2000^{TM} (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Cells were lysed 48 h post-transfection in lysis buffer [buffer A (20 mM PIPES, pH 6.8 and 50 mM NaCl) plus 150 mM sucrose, 3 mM $MgCl_2$, 50 mM NaF, 40 mM Na pyrophosphate, 1% (w/v) Triton X-100, 1 mM $NaVO_3$, 1 mM PMSF, 20 μg/ml aprotinin and 5 μg/ml leupeptin] to a cell number of 1×10^7/ml. Integrin cytoplasmic domains were loaded onto nickel–agarose beads by addition of 20 μg of tail protein to 800 μl buffer A plus 200 μl of 100 mM NaOAc, pH 3.5, containing 20 μl of Nickel–NTA^{TM} agarose, for each experiment [13]. The mixture was rotated for 2 h at 4 °C and the beads were washed twice with buffer A before use in experiments.

50 μl of cell lysate was added to 450 μl of buffer A (plus 1 mM PMSF, 20 μg/ml aprotinin and 5 μg/ml leupeptin), together with the integrin tail-loaded nickel–agarose beads, and rotated overnight at 4 °C. Beads were washed five times with buffer A and boiled in 40 μl reducing Laemmli SDS sample buffer to release any bound proteins. 20 μl of the sample was resolved by SDS–PAGE, proteins transferred to nitrocellulose membrane, and HA-filamin detected by Western blot using an antibody directed against the HA tag (sc-805, Santa Cruz Biotechnology Inc., Santa Cruz, CA). The remaining 20 μl of the sample was resolved by reducing SDS–PAGE and Coomassie blue stained, to check integrin tail load in each experiment. 12.5 μl of cell lysate was loaded and subjected to Western blot using the anti-HA antibody to ensure equal filamin protein load in each experiment.

Where stated, protein bands were quantified by densitometry, using a GS-700 imaging densitometer and Molecular Analyst software (BioRad, Hertfordshire, UK). Results from at least three experiments were used to obtain average values. Statistical analyses of the results were performed using a paired Student's *t*-test.

2.3. GST-filamin expression and protein kinase A assay

Repeats 19–24 of filamin A(41) or A(0) were amplified by PCR and ligated into the GST-encoding vector pGEX 4-T-1 (Amersham Biosciences, Buckinghamshire, UK) using a 5′ *Eco*RI and 3′ *Xho*I restriction site. Proteins were expressed by transformation into JM109 bacteria, incubation at 37 °C with shaking, and induction with 1 mM IPTG. After induction, cells were incubated at 30 °C for 4 h before harvesting and resuspended in 40 ml/l binding buffer. Cells were lysed by sonication as above and GST-filamin protein purified using glutathione agarose affinity chromatography. Doses of GST-filamin protein were added to kinase buffer (25 mM HEPES, pH 7.4, 25 mM β-glycerophosphate and 3 mM $MgCl_2$) containing 1 mM ATP, 1 mM DTT, 5 μCi [γ^{32}P]ATP (Perkin–Elmer Life and Analytical Sciences, Buckinghamshire, UK), and 1 U protein kinase A (PKA, Sigma, Poole, Dorset, UK). Sterile dH_2O was added up to 20 μl and samples incubated at 30 °C for 90 min. Samples were then resolved by reducing SDS–PAGE and gels Coomassie blue, stained to reveal protein bands. ^{32}P-labelled proteins were visualized by autoradiography via exposure to photographic film and processing by an automatic film processor (Kodak, Rochester, NY).

2.4. Binding to GST Fusion Proteins

Pull-down assays have been performed as described previously [15]. Briefly, bacterially expressed glutathione *S*-transferase fused to either β_{1A}, β_{1D}, and β_7 cytoplasmic domains were purified. Subsequently, 50 μg purified fusion proteins were bound to 15 μl glutathione–Sepharose-4B beads and were incubated overnight at 4 °C with total cell lysates of DEAE-dextran transfected COS-7 cells expressing HA-tagged truncated filamin proteins. After washing with lysis buffer (10 mM can, pH 6.8, 50 mM NaCl, 150 mM sucrose, 0.5% Triton X-100, and 1 mM EDTA, and protease inhibitors), beads were centrifuged through a sucrose cushion (10 mM can, pH 6.8, 50 mM NaCl, 800 mM sucrose and 1 mM $MgCl_2$) and proteins were resolved by SDS–PAGE. HA-tagged filamin fusion proteins were visualized and quantified by immunoblotting using anti-HA antibody (12CA5) and enhanced chemofluorescence (Amersham). GST-fusion protein loading of the beads was checked by Coomassie brilliant blue staining.

3. Results and discussion

3.1. Binding of filamin fragments to the integrin β_7 tail

It has been shown previously that sites in the C-terminus of filamin mediate binding to the integrin β_1 tail [12,15]. To determine whether similar sites are involved in the interaction with the integrin β_7 tail, a C-terminal fragment of filamin A, encoding repeating units 19–24 of the protein and the 41-amino acid splice segment [Fln A_{19-24}(41)], was expressed in COS-1 cells (Fig. 1B(i)). Following cell lysis, the binding of the recombinant filamin fragment to a protein mimic of the β_7 tail was assessed using an affinity chromatography pull-down assay.

Fln A_{19-24}(41) was found to bind to the β_7 tail, but not to a protein mimic of the integrin α_4 tail (Fig. 2A), suggesting that, as for the β_1 integrin tail, sites in the C-terminus of filamin are involved in binding to the integrin β_7 tail.

It has recently been reported that a splice variant fragment of filamin, in which the 41-amino acid segment between repeating units 19 and 20 was removed, interacted more strongly with the β_1 tail than did a fragment containing the spliced region [15]. To determine the role of alternative splicing in regulating filamin–β_7 tail interactions, COS-1 cells were transfected with an HA-tagged construct lacking the 41-amino acid segment [Fln A_{19-24}(0); Fig. 1B(ii)], and binding to the β_7 tail assessed using the affinity chromatography assay employed above.

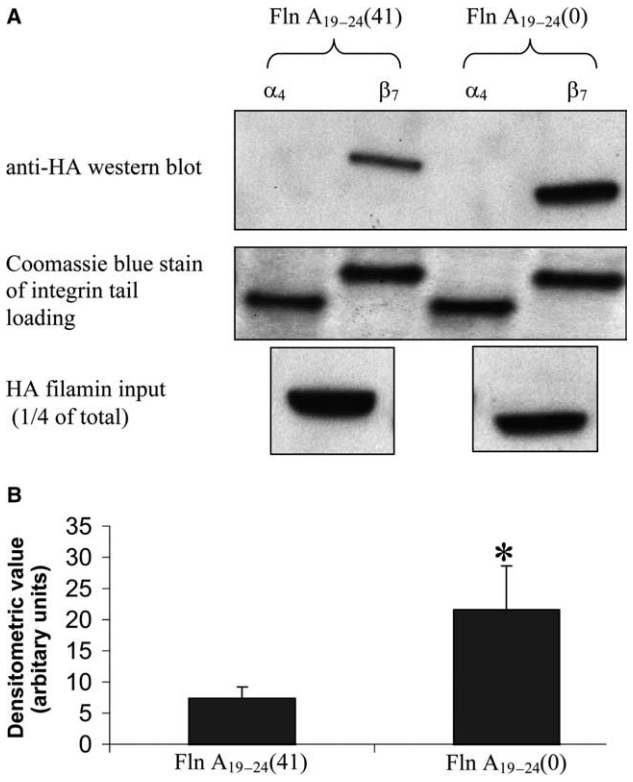

Fig. 2. Binding of mammalian expressed HA-Fln A_{19-24}(41) and Fln A_{19-24}(0) to integrin cytoplasmic domains. (A) Affinity chromatography assay, assessing binding of HA-filamin transfected COS-1 lysates to integrin tails. (B) Densitometry values for binding of HA-filamin molecules to the β_7 tail (average of three experiments, error bars are S.E.M.; *, significant $P < 0.05$ using paired Student's *t*-test).

Binding of splice variant Fln A_{19-24}(0) to the integrin β_7 tail was apparent, but not to a mimic of the α_4 integrin tail (Fig. 2A), and it appeared to be stronger than that seen with Fln A_{19-24}(41) (Fig. 1B). Indeed, quantitation of binding using densitometry showed that binding of the splice variant Fln A_{19-24}(0) was threefold higher than that seen for Fln A_{19-24}(41) (values for Fln $A_{19-24}(41) = 7.38$, Fln $A_{19-24}(41) = 21.63$, Fig. 2B). Thus, as was observed for the β_1 tail [15], binding of Fln A_{19-24}(0) was stronger than that seen for Fln A_{19-24}(41). This suggests that there are elements present in the 41-amino acid splice variant region of filamin that act to reduce binding of filamin to integrin β tails.

As filamin has been reported to bind more strongly to β_7 than to β_1, the binding of Fln A_{19-24}(41) and Fln A_{19-24}(0) to the β_{1A}, β_{1D}, and β_7 integrin tails was compared in parallel. As shown in Fig. 3, in pull-down assays, β_7 bound higher amounts of both Fln A_{19-24}(41) and Fln A_{19-24}(0) than either of the β_1 constructs. For all integrin tails, however, binding of Fln A_{19-24}(0) was higher than that of Fln A_{19-24}(41) (Fig. 3). Thus, although different integrin cytoplasmic domains have different affinities for filamin, the effects of alternative splicing on receptor binding are qualitatively the same for different integrins. This suggests that the same mechanisms are employed to regulate filamin–integrin associations within different cell types.

3.2. Role of phosphorylation of the splice region in regulating the filamin–integrin tail interaction

There are several possible explanations for the results obtained above, including a steric interference of the splice

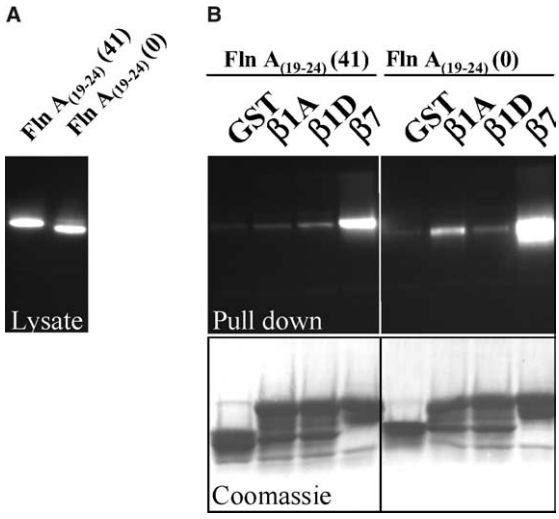

Fig. 3. Comparison of binding of mammalian expressed HA-Fln $A_{19–24}$(41) and Fln $A_{19–24}$(0) to β_1 and β_7 integrin cytoplasmic domains. (A) COS cell lysates immunoblotted with anti-HA antibody to show levels of expression of the Fln $A_{19–24}$(41) and Fln $A_{19–24}$(0) variants. (B) Pull-down assay using GST-tagged β_{1A}, β_{1D}, and β_7 integrin cytoplasmic domains. Top: immunoblot detection of bound filamin by anti-HA blotting. Bottom: Coomassie blue staining showing the amount of each integrin fusion protein used in each assay.

segment with integrin binding, a long-range effect of the splice segment on filamin conformation, and a role for the splice segment as a negative regulatory element, possibly due to post-translational modification. The amino acid sequence of Fln $A_{19–24}$(41) is shown in Fig. 4 and the 41-amino acid splice variant region is highlighted. The spliced region contains a number of serine and threonine residues that could be potentially phosphorylated in mammalian cells. It has been widely documented that filamin is a highly phosphorylated protein [7], and there are also examples where phosphorylation regulates the interaction of a protein ligand with an integrin cytoplasmic domain (for example, paxillin with α_4 [18], Shc with β_3 [19]). Hence, it is plausible that phosphorylation of the spliced region of filamin may regulate binding to the β_7 tail.

To investigate whether phosphorylation of the splice variant region was involved in the regulation of binding to the β_7 tail, potential phosphorylation sites in the splice variant region were identified using the phosphorylation prediction programmes PhosphoBase v.2.0 (http://www.cbs.dtu.dk/databases/PhosphoBase/), and NetPhos v.2.0 (http://www.cbs.dtu.dk/services/NetPhos/) (Fig. 4). PhosphoBase is a database that predicts potential phosphorylation sites based on consensus sequences in other proteins [20], whereas NetPhos is a programme based on neural networks that predicts the probability that a particular residue will be phosphorylated [21]. Fig. 4B portrays residues that have a high probability of phosphorylation and/or are contained in a consensus sequence for a common kinase.

PKA has been implicated in the regulation of integrin function by phosphorylating proteins in close proximity to the integrin cytoplasmic domains [18,22], and it is therefore a good candidate kinase for phosphorylating filamin. Indeed, there is evidence that PKA is capable of phosphorylating C-terminal regions of filamin, as shown by in vitro phosphorylation of

A

START OF REPEAT 19 (aa 2040)

```
SQSEIGDASR VRVSGQGLHE GHTFEPAEFI IDTRDAGYGG LSLSIEGPSK
VDINTEDLED GTCRVTYCPT EPGNYIINIK FADQHVHGSP FSVKVTGEGR
VKESITRRRR APSVANVGSH CDLSLKIPEI SIQDMTAQVT SPSGKTHEAE
IVEGENHTYC IRFVPAEMGT HTVSVKYKGQ HVPGSPFQFT VGPLGEGGAH
KVRAGGPGLE RAEAGVPAEF SIWTREAGAG GLAIAVEGPS KAEISFEDRK
DGSCGVAYVV QEPGDYEVSV KFNEEHIPDS PFVVPVASPS GDARRLTVSS
LQESGLKVNQ PASFAVSLNG AKGAIDAKVH SPSGALEECY VTEIDQDKYA
VRFIPRENGV YLIDVKFNGT HIPGSPFKIR VGEPGHGGDP GLVSAYGAGL
EGGVTGNPAE FVVNTSNAGA GALSVTIDGP SKVKMDCQEC PEGYRVTYTP
MAPGSYLISI KYGGPYHIGG SPFKAKVTGP RLVSNHSLHE TSSVFVDSLT
KATCAPQHGA PGPGPADASK VVAKGLGLSK AYVGQKSSFT VDCSKAGNNM
LLVGVHGPRT PCEEILVKHV GSRLYSVSYL LKDKGEYTLV VKWGHEHIPG
SPYRVVVP
```

END OF REPEAT 24 (aa 2647)

B

Residue	Predicted kinase/s (from PhosphoBase v.2.0)	Predicted score (from NetPhos v.2.0)
Serine-2131	PKC	0.987
Serine-2143	None	0.968
Threonine-2145	PKC	0.425
Serine-2152	p70S6K, CaMII, PKA	0.968
Serine-2158	CKII	0.551
Serine-2163	PKC	0.897

Fig. 4. Prediction of phosphorylated residues in the variable splice region of filamin A repeats 19–24. (A) PhosphoBase v.2.0 was used to predict potential kinase phosphorylation sites (http://www.cbs.dtu.dk/databases/PhosphoBase/) and NetPhos v.2.0 was used to predict the probability that these sites were phosphorylated (http://www.cbs.dtu.dk/services/NetPhos/). *Abbreviations*: PKA, protein kinase A; PKC, protein kinase C; CaMII, calmodulin-dependent protein kinase II; CKII, casein kinase II; p70S6K, S6 protein kinase).

filamin peptides present in bacterial lysate [23]. Only one residue, Ser-2152, is predicted to be phosphorylated by PKA in the splice variant region with a high probability ($P = 0.968$, Fig. 4B). Interestingly, recent work has shown that phosphorylation of filamin on S2152 by the kinase p21 activated kinase-1 is important in regulating cell migration [24], a cellular process also shown to be regulated by the filamin–β_7 interaction [11], and that S2152 is phosphorylated by PKA [23]. Hence, as S2152 can be phosphorylated in vivo, and this phosphorylation is functionally important, this residue was chosen for further study.

To test whether phosphorylation of Ser-2152 was important in regulating the filamin A–β_7 tail interaction, Ser-2152 was mutated to alanine (S2152A) and aspartate (S2152D). Mutation to alanine renders the residues non-phosphorylatable, whereas mutation to aspartate was intended to mimic a constitutively expressed phosphate group. As a control mutation, Ser-2131 [a potential protein kinase C (PKC) phosphorylation site (see Fig. 4)] was also mutated to Ala and Asp.

If phosphorylation of Ser-2152 were important in negatively regulating filamin binding to the integrin β_7 tail, one would expect that mutation to alanine (resulting in removal of the inhibitory phosphorylation site) would cause an increase in β_7 tail binding to Fln $A_{19–24}$(41) protein. Mutation to aspartate would result in constant occupation of the inhibitory phosphorylation site, hence a decrease in binding to the β_7 tail would be expected, compared to the wild-type Fln $A_{19–24}$(41) protein.

3.3. Phosphorylation of filamin A repeats 19–24 by PKA

To test whether there is indeed a PKA phosphorylation site in the spliced region of the Fln $A_{19–24}$(41) construct, and to test whether this site is S2152, an in vitro PKA assay was performed, using bacterially expressed GST-Fln $A_{19–24}$(41) (WT, control S2131A and S2152A) and GST-Fln $A_{19–24}$(0) as substrates.

For wild-type and potential PKC site mutant (S2131A) GST-Fln $A_{19–24}$(41), a strong, dose-dependent incorporation of [γ^{32}P] ATP was apparent, whereas only a weak incorporation was seen for the spliced GST-Fln $A_{19–24}$(0) (Fig. 5). Mutation of S2152 to Ala in Fln $A_{19–24}$(41) reduced the signal to levels seen for the splice variant GST-Fln $A_{19–24}$(0) (Fig. 5). This finding indicates that the major PKA phosphorylation site in repeats 19–24 of filamin A is S2152, situated within the splice variant region.

3.4. Effect of potential phosphorylation site mutants on binding to the β_7 tail

To test whether mutation of the potential PKA (and PKC) phosphorylation sites in the splice variant region of filamin A affects binding to the β_7 tail, affinity chromatography assays, using Fln transfected COS-1 cell lysates, were performed. As shown earlier, none of the mammalian HA-Fln constructs bound to the α_4 tail (Fig. 6). Wild-type Fln $A_{19–24}$(41) bound with weaker affinity to the β_7 tail than the splice variant form Fln $A_{19–24}$(0) (Fig. 6). All mutations of S2131 and S2152 had negligible effects on the binding of Fln to the β_7 tail versus wild-type (Fig. 6). These data indicate that phosphorylation of S2131 or S2152 does not play a role in regulating the interaction of filamin with the β_7 integrin tail.

Thus, in summary, the major finding in this report is that inclusion of the 41-amino acid splice segment in filamin-A reduces binding to the integrin β_7 cytoplasmic tail. There are several additional explanations for the suppression of integrin binding. First, the splice region may mask a binding site for the integrin tail or alter the conformation of the integrin binding site so as to lower the affinity of the interaction, as previously suggested by van der Flier and Sonnerberg [7]. It is likely that a definitive resolution of this possibility will require structure determination of the C-terminal region of filamin. Second, phosphorylation may be important in regulating the filamin–β_7 tail interaction, but our studies may not have identified the correct regulatory residues in the molecule. For example, residues outside the spliced region could potentially be phosphorylated and exert a long distance effect. In the Fln $A_{19–24}$(41) construct, there are 29 potential phosphorylation sites outside the splice variant region. There is a precedent for long distance conformational regulation of binding to integrin tails, as activation signals unmask the binding site for the β_3 tail in the head region of talin [25]. Third, other factors may bind to the spliced region of filamin and alter its binding properties. A plethora of intracellular proteins can bind to

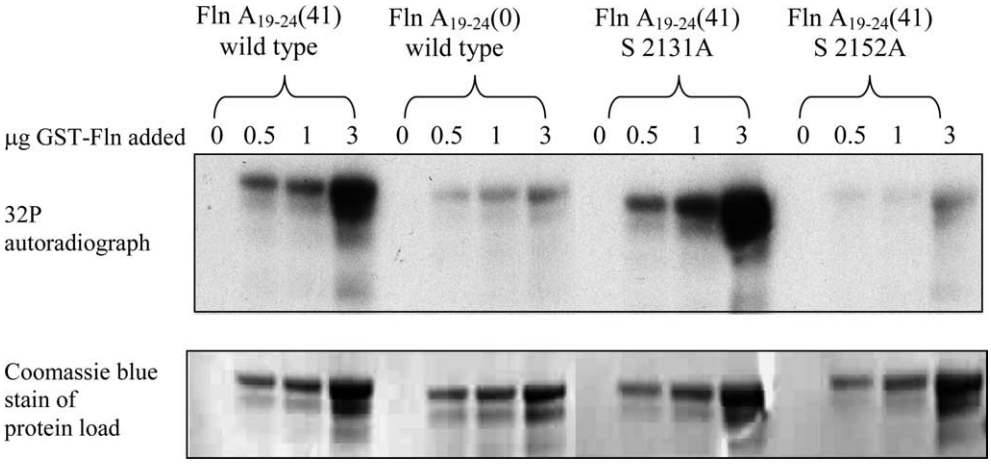

Fig. 5. PKA assay using wild-type and mutant GST-filamin as substrate. PKA was added to doses of wild-type and mutant GST-filamin in kinase buffer plus 5 μCi [γ^{32}P]ATP. Protein was resolved by reducing SDS–PAGE and stained with Coomassie blue. ^{32}P-labelled proteins were visualized by autoradiography.

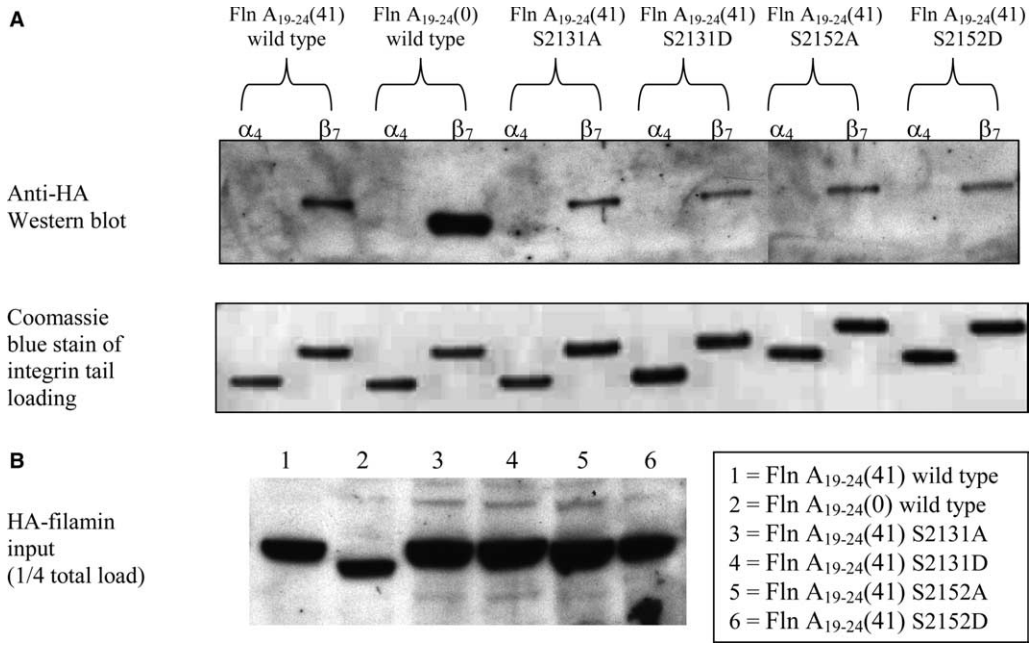

Fig. 6. Binding of wild-type and mutant GST-filamin to integrin cytoplasmic domains. (A) HA-filamin transfected COS-1cell lysates were incubated with nickel charged beads loaded with α_4 or β_7 tail. Bound protein was eluted from the beads, resolved by reducing SDS–PAGE, and Western blotted for anti-HA to detect bound filamin protein. (B) Lysates (1/4 of total input in A) were resolved by SDS–PAGE, transferred to nitrocellulose membrane, and Western blotted with an anti-HA antibody in order to check that expression of each of the HA-filamins was approximately equal.

filamin, many of which do so via the C-terminal repeats [7,10]. It is possible that a binding protein expressed in the COS-1 system binds to Fln $A_{19–24}(41)$ directly to the splice variant region, causing a reduction in binding to the β_7 tail compared to the Fln $A_{19–24}(0)$ that lacks the splice variant. Alternatively, a protein may bind to a site distal to the splice region, but induce conformational changes (involving this region) that result in reduced β_7 tail binding. Further investigation of the mechanisms underlying the role of alternative splicing in regulating integrin–filamin binding are likely to provide useful insights into the cellular signalling mechanisms employed to control migration.

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