Cingulin interacts with F-actin in vitro

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Abstract Cingulin, a M_r 140–160 kDa protein of the cytoplasmic plaque of epithelial tight junctions (TJ), interacts in vitro with TJ proteins and myosin. Here we investigated cingulin interaction with actin, using His-tagged, full-length *Xenopus laevis* cingulin expressed in insect cells, and glutathione *S*-transferase (GST) fusion proteins of fragments of cingulin expressed in bacteria. Purified full-length cingulin co-pelleted with F-actin after high speed centrifugation, and promoted the sedimentation of F-actin under low speed centrifugation, suggesting that cingulin is an actin-cross-linking protein. The actin interaction of GST fusion proteins containing fragments of *Xenopus* cingulin suggested that the F-actin binding site is between residues 101 and 294.

Key words: Cingulin; Actin; α-Actinin; Tight junction

1. Introduction

In vertebrate tissues, tight junctions (TJ) between polarised epithelial cells play a key role in the maintenance of epithelial polarity and in the formation of barriers between different tissue compartments. The molecular structure of TJ has been clarified in recent years, with the characterisation of specific membrane proteins which constitute the TJ strands (reviewed in [1]) and a large group of cytoplasmic plaque proteins, which are believed to form a scaffold for membrane proteins and participate in signaling events linked to cell differentiation and biogenesis of cell polarity (reviewed in [2]).

Cingulin is one of the components of the cytoplasmic plaque of TJ, and has been detected in TJ-bearing epithelial and endothelial cells [3,4]. Biochemical evidence and sequence prediction studies indicate that the cingulin molecule is a parallel dimer, each subunit consisting of a globular 'head', a coiled-coil 'rod' and a small globular 'tail' [5,6]. Cingulin forms a complex and interacts in vitro with several TJ proteins, including ZO-1, ZO-2 and ZO-3 [5]. In addition, cingulin co-purifies with actomyosin [4], and interacts in vitro with myosin [5], raising the possibility that cingulin functions by linking specific TJ proteins to the actomyosin cytoskeleton. The actomyosin cytoskeleton is associated with the cytoplasmic face of TJ, and mediates physiological TJ regulation [7]. However, the molecular details of this regulation and the identity of TJ proteins involved in an actin interaction during TJ assembly are unknown.

To further investigate the relationship of cingulin with the actin cytoskeleton, we studied the interaction of purified recombinant cingulin with F-actin in vitro. Our results show that cingulin interacts directly with F-actin, strengthening the hypothesis that cingulin may play a role in linking TJ components to the actomyosin cytoskeleton.

2. Materials and methods

2.1. Antibodies

Rabbit polyclonal anti-cingulin antiserum (C532) and secondary, alkaline phosphatase-labelled secondary antibodies were used for immunoblotting as described [5]. Rabbit anti-His antibodies (Qiagen) were used at 1:2000 dilution.

2.2. Proteins

SwissProt accession numbers for proteins used in this study were: *Xenopus laevis* cingulin (Q9PTD7), human α-actinin 2A (P35609), *Saccharomyces cerevisiae* Rap1 (P11938).

2.3. Bacterial expression and purification of proteins

Constructs for expression of cingulin glutathione *S*-transferase (GST) fusion proteins in bacteria were generated by subcloning fragments amplified by PCR into pGEX-4T1 [5]. Bacterial expression of GST fusion proteins and purification by affinity chromatography on glutathione-Sepharose beads were as described [5]. For expression of His-tagged α-actinin, BL21 codon plus (RIL, Stratagene) bacteria were transformed with plasmid α-act2a, containing full-length, N-terminal His-tagged α-actinin (2A isoform) (a gift of Dr Mathias Gautel, Max Planck Institute, Dortmund, Germany) [8]. Bacteria were induced by 0.5 mM IPTG (1 h at 37°C, 3 h at 30°C), pelleted, lysed in 50 mM Na_2HPO_4, 0.5 M NaCl, 0.05% Triton X-100, 5 mM imidazole, pH 8.1, 20 mM β-mercaptoethanol, and sonicated. The lysates were clarified by centrifugation (100 000 × *g*, 30 min) and the supernatant was loaded on a Ni-agarose superflow resin (Qiagen). Purified protein was eluted with a step of 250 mM imidazole.

2.4. Insect cell expression and purification of full-length *X. laevis* cingulin and *S. cerevisiae* Rap1

The construct for expression of full-length *Xenopus* cingulin containing a His tag at the C-terminal end (Bac-XC) was generated in pFASTBAC1 by amplifying the insert of construct XC(1–1368)-myc ([5]; D'Atri and Citi, unpublished results). Bacmid isolation and viral stock production was as described [9]. Baculovirus stock for expression of His-tagged *S. cerevisiae* Rap1, a transcription factor [10], was a gift of Dr Alessandro Bianchi (Shore laboratory, University of Geneva, Switzerland). To induce protein expression, Sf21 cells (density 0.5×10^6 cells/ml) were infected with baculovirus, incubated at 30°C for 40 h, pelleted by centrifugation at 700 × *g*, and lysed as described [5]. Lysates were clarified at 100 000 × *g* before GST pull-down assays. Recombinant His-tagged proteins (Bac-XC and Bac-Rap1) were purified from lysates of insect cells lysed in 300 mM NaCl, 50 mM NaH_2PO_4, 10 mM imidazole, pH 8.0. Lysates were clarified by centrifugation at 100 000 × *g*, and loaded onto Ni-agarose resin. Purified protein was eluted with 250 mM imidazole.

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Abbreviations: GST, glutathione *S*-transferase; TJ, tight junction

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2.5. Purification of actin

Rabbit skeletal F-actin was purified from muscle acetone powder (a gift of Dr J. Kendrick-Jones, MRC, Cambridge, UK) by extraction at 0°C with 2 mM Tris–HCl, pH 8.0, 0.5 mM ATP (G-buffer). The extract was filtered and clarified by centrifugation at 30 000×*g* for 15 min. NaCl 4 M and $MgCl_2$ 1 M were added to final concentrations of 20 mM and 0.7 mM, respectively, to induce actin polymerisation. After 2 h incubation at room temperature, F-actin was pelleted by centrifugation at 100 000×*g*, homogenised in G-buffer, and depolymerised by dialysis against G-buffer containing 0.1 mM ATP. Insoluble material was removed by centrifugation, and dithiothreitol (DTT) was added to 1 mM. The resulting G-actin was used to make fresh F-actin, by adding $MgCl_2$ to 2 mM and NaCl to 100 mM, incubating at room temperature for 2 h, and centrifuging.

2.6. High speed actin co-sedimentation assay

Freshly polymerised F-actin was homogenised in actin buffer AB1 (100 mM NaCl, 1 mM $MgCl_2$, 10 mM Tris–HCl, pH 8.0, 1 mM EGTA, 0.1 mM ATP, 0.1 mM DTT). Purified Bac-XC and Bac-Rap1 were dialysed for 12 h against AB1 and mixed with F-actin (final concentration 15 μM) at final concentrations of 18 nM, in a final total volume of 100 μl, containing 100 mM NaCl, 2 mM $MgCl_2$, 20 mM Tris–HCl, pH 8.0, 1 mM EGTA, 1 mM ATP, 1 mM DTT. After 20 min incubation at room temperature, samples were centrifuged for 20 min at 100 000×*g* at 4°C. Supernatant and pellet fractions were analysed by SDS–PAGE and immunoblotting. Bovine serum albumin (BSA) was used as a control for unspecific trapping; no significant trapping was observed.

2.7. Low speed actin co-sedimentation assay

To detect actin cross-linking activity, purified recombinant proteins (Bac-XC and α-actinin) were dialysed separately against AB1 buffer at 4°C, diluted to a final concentration of 1.5 μM and mixed with F-actin (final concentration 15 μM). After 1 h incubation at room temperature, samples were centrifuged for 20 min at 10 000×*g* [11]. Supernatant and pellet fractions were analysed by SDS–PAGE.

2.8. GST pulldown assays

GST fusion proteins (250 nmol) were incubated with glutathione-Sepharose resin, washed and resuspended in 0.5 ml lysis buffer–Triton (LBT = 150 mM NaCl, 20 mM Tris–HCl, pH 7.5, 5 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml antipain, 5 μg/ml pepstatin) containing 0.1 mM ATP and 1 mM $MgCl_2$. F-actin (350 μM in AB1 buffer) was added to the beads to a final concentration of 20 μM, and incubated for 1 h at 4°C. Beads were washed three times with wash buffer (LBT containing 0.5% Triton, plus ATP and $MgCl_2$), by centrifuging at 10 000×*g* at room temperature, and aspirating the supernatant. Proteins bound to beads were eluted by boiling in SDS sample buffer, and analysed by SDS–PAGE.

2.9. SDS–PAGE and immunoblotting

SDS–PAGE and immunoblotting were carried out as described [5], using Coomassie G250 to stain proteins. For actin pelleting assays, after centrifugation 75 μl of supernatant were collected and mixed 1:1 with SDS sample buffer. The remaining supernatant was discarded, and the pellet was solubilised for 2 h on ice in 100 μl SDS sample buffer, and diluted 1:1 with AB1 buffer. 20 μl samples were loaded on 8–10% polyacrylamide gels. For quantification, gels or blots were scanned and the intensity of the bands was measured using Biosoft Quantiscan 2.1 software. A BSA internal standard curve was used to measure protein concentration [5].

3. Results

3.1. Recombinant full-length Xenopus cingulin co-pellets with F-actin filaments

To determine whether cingulin interacts with actin, full-length, His-tagged *X. laevis* cingulin (Bac-XC) and the control His-tagged protein Rap1 were expressed in baculovirus-infected insect cells, purified from cell lysates by affinity chromatography, and incubated with purified rabbit skeletal F-actin (Fig. 1A). F-actin was sedimented by high speed centrifugation, and proteins present in the supernatant and pellet fractions were detected by immunoblot (Fig. 1B). In the absence of actin, >70% of cingulin was in the soluble supernatant fraction, whereas in the presence of F-actin, >80% was co-pelleted with actin (Fig. 1B,C). The control protein Rap1 did not show an actin-dependent shift in its solubility (Fig. 1B,C), indicating that cingulin association with actin is not the result of non-specific interaction with the His tag. Thus, full-length recombinant cingulin interacts directly with F-actin.

3.2. Recombinant full-length Xenopus cingulin shows F-actin bundling activity in vitro

To determine whether cingulin can bundle actin filaments, we used a low speed actin co-sedimentation assay, whereby pelleting of F-actin filaments is increased through cross-linking by an exogenous protein. Analysis of supernatant and pellet fractions by SDS–PAGE showed that in the absence of added proteins ~90% of F-actin was detected in the supernatant (Fig. 2A). However, when purified cingulin was added to F-actin filaments, ~54% of actin was detected in the pellet fraction (Fig. 2B). When purified α-actinin was added to F-actin filaments, ~78% of F-actin was detected in the pellet

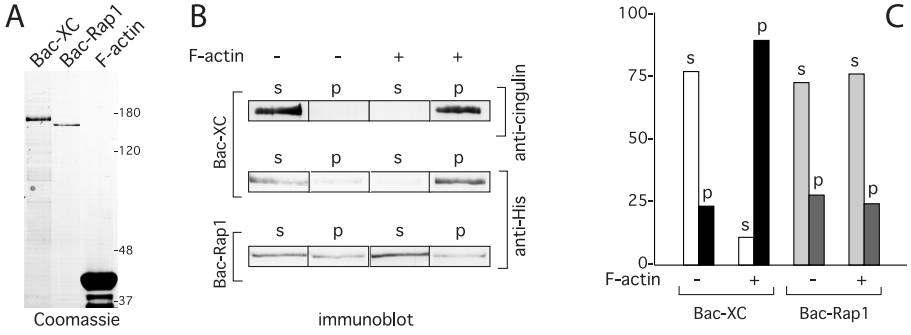

Fig. 1. Cingulin interacts directly with F-actin in vitro. A: Coomassie-stained gel lanes showing purified recombinant full-length *X. laevis* cingulin (Bac-XC), purified recombinant yeast Rap1 (Bac-Rap1), and rabbit skeletal muscle F-actin (F-actin). Numbers on the right indicate migration of prestained markers (kDa). B: Immunoblot analysis of supernatant (s) and pellet (p) fractions after high speed centrifugation of Bac-XC or Bac-Rap1 incubated without (−) or with (+) F-actin. Anti-cingulin (C532, top panel) and anti-His (middle panel) were used to label Bac-XC, whereas Bac-Rap1 was labelled using anti-His (bottom panel). C: Histogram showing the relative amounts of protein (expressed as % of total) detected in the supernatant (s) and pellet (p) fractions, after densitometric analysis of antibody-labelled bands. The results show an average of two separate experiments.

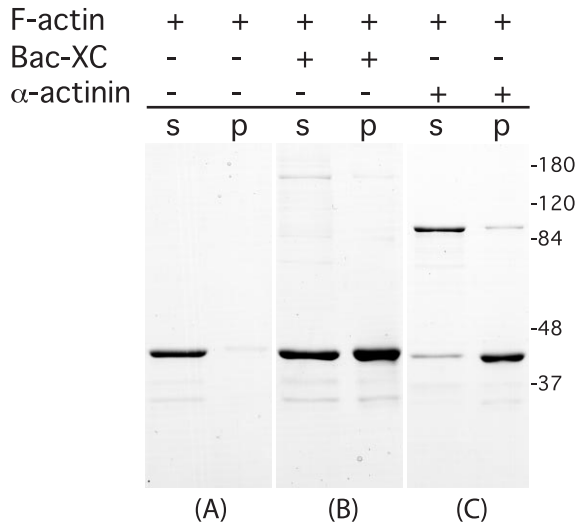

Fig. 2. Cingulin cross-links actin filaments in vitro. Coomassie-stained gel lanes showing proteins in supernatant (s) and pellet (p) fractions after low speed centrifugation of purified F-actin alone (A), or in the presence of purified Bac-XC (B) or α-actinin (C). Numbers on the right indicate migration of prestained markers (kDa). Note that F-actin sedimentation is significantly increased in the presence of cingulin or α-actinin. No cross-linking activity was detected using yeast Bac-Rap1 (not shown).

(Fig. 2C). Thus, cingulin shows F-actin cross-linking activity in vitro.

3.3. Mapping cingulin sequences involved in actin interaction

We next used in vitro GST pulldown assays to identify sequences responsible for cingulin interaction with actin. Proteins consisting of GST fused to different regions of the *Xenopus* cingulin molecule were purified on glutathione-Sepharose beads and incubated either with buffer or with F-actin. After incubation, proteins bound to the beads were analysed by SDS–PAGE (Fig. 3). F-actin was clearly pelleted by a fusion protein comprising the first 378 residues of the cingulin head (Fig. 3A). Deletion of the first 40 (Fig. 3B) or 100 (Fig. 3C) residues from the cingulin head fragment did not abolish F-actin interaction. However, when the fusion protein contained only residues 295–378, no F-actin was detected (Fig. 3D). Similarly, no F-actin was detected when we used as baits proteins comprising the first 99 (Fig. 3E) or 192 (Fig. 3F) residues of the cingulin head. F-actin interaction was restored if the fusion protein comprised residues 1–294 (Fig. 3G). F-actin was not significantly pelleted by GST or by a GST fusion protein of the C-terminal fragment of the rod (Fig. 3H,I). Since sequences within residues 101–192 may not interact with actin in the construct GST-XC(1–192) due to conformational problems or lack of accessory sequences, our analysis suggests that sequences within residues 101–294 are sufficient for an interaction of the cingulin head with F-actin. Interestingly, this sequence does not show obvious sequence homology to known actin binding proteins.

4. Discussion

Actin is a key structural and functional component of TJ. Electron microscopy shows that F-actin filaments are present in the submembrane region of TJ [12,13]. Disruption of the actin cytoskeleton by microfilament-active drugs results in perturbation of TJ barrier function [14,15] and redistribution

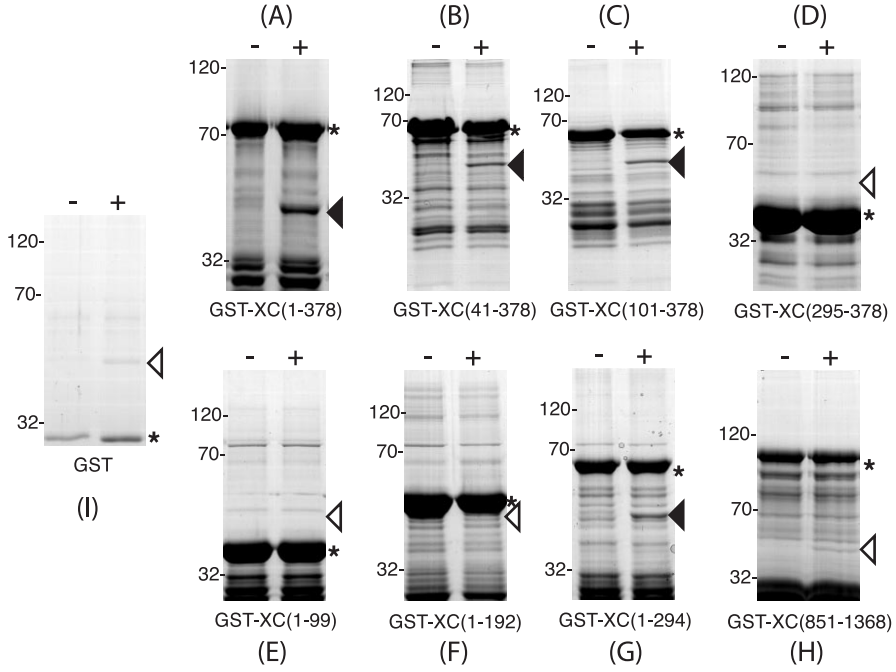

Fig. 3. F-actin interacts with specific cingulin GST fusion proteins. Coomassie-stained gel lanes showing proteins associated with glutathione-Sepharose beads conjugated to different cingulin GST fusion proteins (A–H), GST (I), and incubated with either buffer (−) or F-actin (+) (see Section 2). Below each pair of lanes is the name of the construct, indicating the cingulin residues included in the fusion protein. Numbers on the left indicate migration of prestained protein markers (kDa). Symbols on the right indicate migration of GST fusion protein (asterisks), or actin (arrowheads). Black arrowheads denote actin detected in association with GST fusion protein, white arrowheads indicate actin not detected above background. The background actin level was that observed using glutathione-Sepharose beads alone (not shown), or beads with bound GST (I).

of TJ-associated proteins [16,17]. Contraction of the perijunctional actomyosin ring occurs in parallel with myosin regulatory light chain phosphorylation and increased TJ permeability in several physiological models of TJ regulation (reviewed in [7]). Furthermore, studies in invertebrate model systems show that the cortical actin cytoskeleton is critically involved in the establishment of cell polarity (reviewed in [18]). Thus, actin filaments may be important not only in modulating TJ permeability, but also in establishing the polarised organisation of the cytoplasmic plaque of vertebrate TJ. To understand the molecular mechanisms through which the organisation and contraction of the actomyosin cytoskeleton regulate TJ assembly and permeability, it is crucial to study the interaction of TJ proteins with actin and associated proteins.

We report here that cingulin interacts directly with actin in vitro. There is evidence that other TJ proteins structurally interact with actin. A direct in vitro interaction of F-actin with TJ membrane and plaque proteins, namely occludin, ZO-1 and ZO-3, has been described [19–21]. In addition ZO-1 interacts with actin binding proteins, such as α-catenin [19] and cortactin [22]. Our observation that cingulin interacts with F-actin confirms the notion that the structural linkage between actin cytoskeleton and TJ is based on multiple interactions. So, which of the TJ proteins are the physiological partner(s) of actin during the assembly of TJ and the modulation of paracellular permeability? Although further studies in vivo are necessary to address this question, one interesting observation reported in this paper is that cingulin, unlike ZO-2, ZO-3 and occludin [21], acts as a F-actin cross-linking protein in vitro. Since in single epithelial cells cingulin is first detected in association with thick bundles of F-actin at free cell borders [23], the actin-bundling activity of cingulin might be involved in the still poorly understood mechanism that links cytoskeletal assembly, development of cell polarity and TJ assembly. In mature junctions cingulin and other actin binding TJ proteins may recruit actin filaments and transmit mechanical force generated by actomyosin contraction to the membrane domain of TJ, resulting in modulation of TJ barrier function. Stably transfected epithelial cell lines expressing cingulin and other TJ proteins will be useful to test these hypotheses.

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