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Rhombohedral crystals of the human vinculin head domain in complex with a vinculin-binding site of talin

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Intermolecular interactions between the cytoskeletal proteins talin and vinculin are required for outside-in integrin signaling triggered by the formation of focal adhesions. Talin possesses three non-contiguous vinculin-binding sites (VBS); binding of one of these motifs, VBS3, provokes dramatic alterations in the structure of the vinculin's head (Vh) domain and this activates vinculin (Izard *et al.*, 2004). To address the role of talin's other VBSs in vinculin activation, talin VBS1 (human residues 607–636) was crystallized in complex with the Vh (human residues 1–258) domain. Rhombohedral crystals of human Vh–VBS1 were obtained. The crystals belong to space group $R32$, with unit-cell parameters a = 88.7 Å, α = 105.5° and diffract to 2.4 Å on a third-generation synchrotron source. The packing density for one heterodimer in the asymmetric unit is 3.04 Å^3 Da^{-1}, with a solvent content of 0.59.

1. Introduction

Focal adhesions form when cells come in contact with components of the extracellular matrix (ECM). Formation of these complexes relies on the association of the ectodomains of integrin receptors with ligands of the ECM and these interactions are unique in that they initiate both inside-out and outside-in signaling (Schwartz & Ginsberg, 2002; Kim *et al.*, 2003; Ridley *et al.*, 2003). A common denominator of integrin signaling is rapid changes and re-organization of the actin cytoskeleton and this requires the cytoskeletal proteins talin and vinculin (Xu *et al.*, 1998; Priddle *et al.*, 1998). Talin directly interacts with an NP*x*Y/F motif present in the cytoplasmic tails of β-integrin subunits of these receptors and this association is mediated by talin's FERM (4.1, ezrin, radixin and moesin) motif, which is present in its N-terminal head domain (Calderwood *et al.*, 2002; Garcia-Alvarez *et al.*, 2003). In turn, talin binds to vinculin through three non-contiguous vinculin-binding sites (VBS1, VBS2 and VBS3) present in its central rod domain (Bass *et al.*, 1999, 2002) and, once activated, vinculin binds to other key regulators of the cytoskeleton, including paxillin, α-actinin and vinexin, as well as to F-actin, and the latter interaction effectively joins the 'bridge' linking integrin receptors with the actin cytoskeleton (Critchley, 2000).

Exactly how signals transmitted during outside-in integrin signaling provoke such rapid changes in the actin cytoskeleton has remained a mystery. The conventional model has posited that activation of vinculin is a proximal event in the signaling cascade and that this is triggered by the binding of acidic phospholipids such as phosphatidylinositol 4,5-bisphosphate (PI4,5P$_2$) to vinculin's tail (Vt) domain (human vinculin residues 879–1066). The binding of PI4,5P$_2$ to Vt has then been speculated to sever the intramolecular association of Vt with vinculin's head (Vh) domain (human vinculin residues 1–258; Winkler *et al.*, 1996; Gilmore & Burridge, 1996; Bakolitsa *et al.*, 1999; Miller *et al.*, 2001), allowing Vt and Vh to interact with their binding partners, including the binding of paxillin and F-actin to Vt, and of talin and α-actinin to Vh (Johnson & Craig, 1994, 1995; McGregor *et al.*, 1994; Gilmore & Burridge, 1996).

Recent findings now challenge this model as they point towards talin, rather than vinculin, playing a proximal role in directing outside-in integrin signaling. Firstly, the binding of talin's FERM domain to integrin receptors directly activates both inside-out and outside-in signaling (Calderwood *et al.*, 2002; Garcia-Alvarez *et al.*, 2003; Tadokoro *et al.*, 2003). Furthermore, talin also binds to and activates phosphatidylinositol phosphate kinase-1γ (Di Paolo *et al.*, 2002; Ling *et al.*, 2002; Barsukov *et al.*, 2003), which generates PI4,5P$_2$, and the binding of PI4,5P$_2$ to talin's head domain facilitates its interaction with β-integrin receptor cytoplasmic tails (Isenberg & Goldmann, 1998; Martel *et al.*, 2001), effectively amplifying the response. Finally, structural and biochemical studies have now revealed that the binding of one of talin's VBSs to Vh, VBS3 (talin residues 1944–1969), provokes dramatic distortions in the structure of the N-terminal helical bundle of Vh, by a process termed

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helical bundle conversion, and that these alternations abolish the binding site for Vt. Furthermore, talin VBS3 can effectively displace Vt from pre-existing Vh–Vt complexes, supporting the model in which talin activates vinculin in focal adhesions (Izard *et al.*, 2004).

Talin harbors three non-contiguous high-affinity VBSs for vinculin; exactly why this is the case is unclear. All of talin's VBSs can bind to Vh (Bass *et al.*, 1999) and can act as effective competitive inhibitors of Vt binding to Vh (Bass *et al.*, 2002). We reasoned that human talin VBS1 (residues 607–635) would bind to a similar region of Vh and provoke structural alterations in Vh structure similar to those observed in the Vh–VBS3 crystal structure. Here, the crystallization of the human Vh–VBS1 complex is described, with the crystals having sufficient quality for determination of its structure.

2. Methods and results

Human Vh was expressed and purified as described by Izard *et al.* (2004). The purified protein was dialyzed into 10 m*M* Tris–HCl pH 7.6 containing 0.1 m*M* EDTA and 1 m*M* DTT, concentrated to 15 mg ml^{-1}, aliquoted and stored at 253 K. Human talin VBS1 (residues 607–635) was synthesized and HPLC-purified in our in-house facility. VBS1 was resuspended in water and added to Vh at a ratio of 2 mol VBS1 to 1 mol Vh.

Initial crystallization conditions for Vh–VBS3 were identified using the microbatch system at the Hauptman–Woodward Institute (Buffalo, NY, USA). Cubic Vh–VBS3 crystals appeared under oil within 1 d by equilibrating 2.4 mol of VBS3 per mole of Vh against a reservoir consisting of 2% 2-methyl-2,4-pentanediol (MPD), 100 m*M* citric acid pH 4.0 and 100 m*M* $CdCl_2$ (Izard *et al.*, 2004). We used these Vh–VBS3 crystallization conditions as a starting point to obtain our initial Vh–VBS1 crystals. These crystals appeared at room temperature using the hanging-drop vapor-diffusion technique (VDX plate; Hampton Research) with drops containing 1 µl reservoir solution and 1 µl protein solution. However, Vh–VBS1 crystals only appeared when using a higher percentage of MPD. The dimensions of the crystals were further improved by increasing the concentration of citric acid and replacing the additive. The best crystals were obtained from 5% MPD, 150 m*M* citric acid pH 4.0 and 150 m*M* γ-buterolactone. These crystals appeared within 5 d and continued to grow for up to two weeks (Fig. 1). Vh–VBS1 crystals were cryoprotected in Paratone oil and mounted in a nylon loop of 0.2 mm diameter.

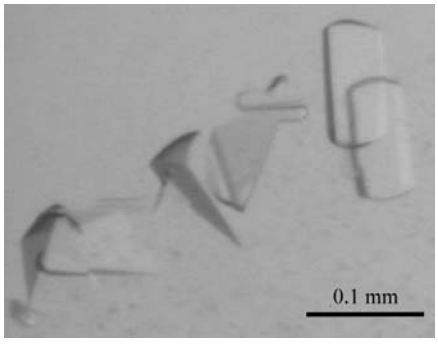

Figure 1
Crystals of the human Vh–VBS1 complex. Crystals grew to 0.15 × 0.06 × 0.06 mm within two weeks.

Table 1
Data-reduction statistics of the Vh–VBS1 data set.

Total measurements	280945
Unique reflections	15061
Redundancy	18
$F^2 > 3\sigma(F^2)$ (50–2.42 Å) (%)	75.3
$F^2 > 3\sigma(F^2)$ (2.51–2.42 Å) (%)	33.3
Average $F^2/\sigma(F^2)$	8.3
Average $F^2/\sigma(F^2)$ (2.51–2.42 Å)	3.0

Resolution range (Å)	$R_{\rm merge}$†	Completeness (%)
50.00–5.21	0.100	98.9
5.21–4.14	0.135	99.9
4.14–3.61	0.210	100.0
3.61–3.28	0.359	100.0
3.28–3.05	0.412	100.0
3.05–2.87	0.411	100.0
2.87–2.73	0.412	99.9
2.73–2.61	0.444	99.9
2.61–2.51	0.478	96.5
2.51–2.42	0.485	80.8
50.00–2.42	0.179	97.6

† $\sum_{\rm unique\ reflections}(\sum_{i=1}^{N}|I_i-\overline{I}|)/\sum_{\rm unique\ reflections}(\sum_{i=1}^{N}I_i)$.

X-ray data were collected at 100 K at the Advanced Photon Source (SBC-CAT ID beamline) using a 3 × 3 mosaic CCD detector (Westbrook & Naday, 1997). The high-resolution and low-resolution data were collected separately on the same crystals with exposure times of 20 and 1 s per degree, respectively. The data were measured at a wavelength of 0.97889 Å and a crystal-to-detector distance of 250 mm and processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Data statistics are provided in Table 1.

The results of autoindexing with *DENZO* (Otwinowski & Minor, 1997) are consistent with the crystals having a primitive rhombohedral lattice, with unit-cell parameters a = 88.7 Å, α = 105.5° in the rhombohedral setting (equivalent to $a = b$ = 141.1, c = 104.9 Å in the hexagonal setting). Analysis of the simulated $hk0$ precession photographs showed 6*mm* symmetry with the mirror planes every 60° about c^*, indicating the presence of twofold axes perpendicular to and related by a threefold axis. Moreover, since the $R_{\rm merge}$ ($\sum|I-\overline{I}|/\sum I$) for data reduced in $R32$ was not of lesser quality than for data reduced in $R3$, the human Vh–VBS1 crystals belong to space group $R32$. An assumption of one heterodimer per asymmetric unit leads to an acceptable packing density, $V_{\rm M}$ (Matthews, 1968), of 3.04 Å^3 Da^{-1}, corresponding to a solvent content of about 0.59.

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