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CRP2 is an autonomous actin-binding protein

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Abstract Cysteine-rich proteins (CRPs) have been shown to be involved in cell differentiation, transcriptional regulation and the organisation of the actin cytoskeleton. Thus far, the latter function has been inferred solely from the in vitro interaction of CRP1, CRP2, and CRP3 with α-actinin and zyxin. We show here that purified, recombinant CRP2 binds directly to F-actin in vitro in co-sedimentation assays. Using a green fluorescent protein (GFP)-tagged construct of CRP2 we analysed its localisation and dynamics in A7r5 rat smooth muscle cells. CRP2 was associated with the actin cytoskeleton and decorated actin stress fibres in a continuous fashion, unlike the periodic labelling pattern observed for α-actinin and zyxin, which also accumulate in focal adhesions. Using live video fluorescence microscopy we observed the behaviour of GFP-CRP2 during the dynamic rearrangement of the actin cytoskeleton in phorbol 12,13-dibutyrate-treated A7r5 cells. In contrast to the actin-binding proteins SM22α and α-actinin, GFP-CRP2 did not translocate into the podosomes induced by this treatment, but remained preferentially bound to the stress fibres, suggesting an actin filament-stabilising role for CRP2. When fused to the mitochondrial targeting sequence from the *Listeria* protein ActA, GFP-CRP2 was almost completely localised to mitochondria, but no significant recruitment of either α-actinin or zyxin could be observed. Taken together, our results demonstrate that CRP2 can bind to F-actin directly and that the association with the actin cytoskeleton is independent of α-actinin or zyxin localisation in the cell, thus questioning the role of CRP2 as a regulator of α-actinin function in vivo.

Key words: Cysteine-rich protein; Actin binding; Green fluorescent protein; α-Actinin; Zyxin; Cytoskeleton

1. Introduction

Cysteine-rich proteins (CRPs) are characterised by the presence of two LIM domains linked to short glyceine-rich repeats. The first member of the CRP protein family (CRP1) was identified by Liebhaber et al., [1] as a widely expressed and highly conserved CRP with an unusual zinc-finger motif. To date, four CRP family members (CRP1, CRP2, CRP3, and TLP) have been identified in vertebrates and several CRP-like LIM domain proteins have been discovered in arthropods, protozoas and plants (reviewed in [2]). These four CRP family members share high sequence homologies but differ in their expression patterns. The CRP family proteins display a dual localisation in the cell and are found both in the nucleus and along cytoskeletal elements in the cytoplasm [3–8]. CRP1, 2, and 3 localise to the cell nucleus as well as to the cytoplasm, where these proteins are associated with the actin cytoskeleton [7,8], and all three molecules contain a potential nuclear targeting sequence (KKYGPK) at amino acid residues 64–69. The dual localisation of CRPs implies different roles of the proteins in the different cellular compartments. In the nucleus, CRPs are implicated in differentiation processes by acting as cofactors of gene expression [9,10]. Together with the serum response factor (SRF) and GATA proteins, CRP2 strongly activated the promoters of smooth muscle specific genes, such as smooth muscle α-actin, h1calponin and SM22, causing the conversion of pluripotent 10T1/2 fibroblasts into smooth muscle cells. CRP2 was thus proposed to act as a bridging molecule, with its N-terminal LIM domain associating with SRF and its C-terminal LIM domain binding to GATA4 [10].

Whereas the functions of CRPs inside the nucleus have been well documented, their role in the cytoplasm remains largely unclear. CRPs have been shown to bind α-actinin [5,8] and zyxin [8,11] in vitro. These proteins have been implicated in the control of actin assembly and organisation, suggesting a role for CRPs in the organisation of the actin cytoskeleton. The actin association of CRP proteins has therefore been speculated to result from α-actinin binding [5]. Recently however, two proteins sharing sequence homologies to vertebrate CRPs, LimC and LimD, have been identified in *Dictyostelium discoidum* and have been shown to interact directly with F-actin in vitro [12].

The aim of this study was to shed light on the role of CRPs in the cytoplasm and to analyse the potential actin binding of mammalian CRP2 in greater detail.

2. Materials and methods

2.1. Constructs

Full length CRP2 was obtained from IMAGE clone 1923078. All CRP2 constructs were generated by polymerase chain reaction (PCR) using this clone as a template.

Standard protocols were used for all cDNA manipulations [13]. For recombinant expression, CRP2 cDNA was cloned into the vector pMW 172 [14] via *Bam*HI and *Eco*RI. PCR primers: FWD: 5′-GAG AGA ATT CTG GAT CCT TTA TGC CTG TCT GGG GTG GTG G-3′; REV: 5′-GAG AGA ATT CCT CGA GTT ACT GAG CAT GAA CAA GGG CCC-3′. The myc-tagged CRP2 construct was created using FWD: 5′-GAG AGA ATT CTG GAT CCT TTA TGC CTG TCT GGG GTG GTG G-3′; REV: 5′-GAG AGA ATT CCT CGA GTT ACT GAG CAT GAA CAA GGG CCC-3′; the resulting cDNA was cloned into the pRK5myc vector

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Abbreviations: GFP, green fluorescent protein; CRP, cysteine-rich protein

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[15], using *Bam*HI and *Eco*RI restriction sites. Green fluorescent protein (GFP)-CRP2 was generated from myc-CRP2, cloning the *Bam*HI–*Eco*RI cut insert into *Bgl*II–*Eco*RI-digested pEGFPC1 (Clontech, Heidelberg, Germany). GFP-CRP2-mito was created in two steps: first a CRP2 cDNA was generated using FWD: 5′-GAG AGA ATT CTG GAT CCT TTA TGC CTG TCT GGG GTG GTG G-3′ and REV: 5′-GAG AGA ATT CCT GAG CAT GCA CAA GCG CCC-3′, and cloned into pEGFPC1 via *Bgl*II and *Eco*RI. Second, the cDNA of the mitochondrially targeting sequence of ActA [16] was generated by PCR using the GFP-FPPPmito construct (kind gift of J. Wehland) as template; FWD: 5′-GAG AGA ATT CAC GTT AAT CCT TGC AAT GTT AGC-3′; REV: 5′-GAG AGG ATC CTT AGT TGT TTT TTC TTA ATT G-3′. Resulting cDNA was cloned in via *Eco*RI–*Bam*HI. The dsRed-Sm22 construct was a kind gift of Dominique Brandt (University of Hannover, Germany) and the α-actinin-GFP construct was a kind gift of J. Wehland (GBF, Braunschweig, Germany).

2.2. Expression and purification of recombinant protein

E. coli strain BL21 was used to express CRP2 protein. Expression was induced by 1 mM of IPTG for 4 h. Cells were harvested, resuspended in 20 ml of an imidazole buffer (5 mM imidazole, 30 mM KCl, 1 mM $MgCl_2$, 0.15 mM DTE, pH 7.0) and lysed by passing the suspension through a French press (SLM Aminco, USA) at 1000 bar. Cell debris was removed by centrifugation in a SS-34 rotor at 25 000 × *g* for 15 min in a Sorvall RC-5 refrigerated centrifuge. Supernatant was immediately applied onto a SP-Sepharose cation exchanger column (Amersham, Vienna, Austria) and bound proteins were eluted with a linear gradient ranging from 0 to 1 M NaCl. Fractions containing substantial amounts of CRP2 were pooled.

Glutathione-*S*-transferase (GST)-fused CRP2 was expressed in BL21 and purified via a GSTrap FF 5 ml column (Amersham, Vienna, Austria) according to the manufacturer's instructions.

2.3. Cell culture, transfection and fluorescence microscopy

A7r5 rat smooth muscle cells were grown in low glucose (1000 mg l^{-1}) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS; PAA, Linz, Austria), penicillin/streptomycin (Gibco, Austria) at 37°C and 5% CO_2. B16F1 mouse melanoma cells were maintained in high glucose (4500 mg l^{-1}) DMEM (Sigma-Aldrich, Vienna, Austria) supplemented with 10% FCS, penicillin/streptomycin at 37°C and 5% CO_2. For transient expression, cells were grown in 60 mm plastic culture dishes and transfected using Superfect (Qiagen, Hilden, Germany), essentially as described elsewhere [17]. Cells were replated onto 15 mm coverslips 12–16 h after transfection. Forty-eight hours later cells were prepared for microscopy. For immunofluorescence microscopy, cells were washed twice in PBS (138 mM NaCl, 26 mM KCl, 84 mM Na_2HPO_4, 14 mM KH_2PO_4, pH 7.4), permeabilised for 3 min in 3.7% paraformaldehyde (PFA)/0.3% Triton X-100 in PBS and fixed in 3.7% PFA (Merck, Darmstadt, Germany). Antibodies: anti-myc (Clontech, Heidelberg, Germany); anti-α-actinin IgM (Sigma, Vienna, Austria); anti-zyxin (kind gift of J. Wehland, Braunschweig, Germany). Alexa568-labelled secondary antibodies (Molecular Probes, Leiden, The Netherlands) were used.

Fluorescent images were recorded on a Zeiss Axioscope equipped with an Axiocam driven by the manufacturer's software package (all from Zeiss, Vienna, Austria) using a 63× oil immersion lens.

2.4. Live cell video microscopy

Cells were mounted in an open heating chamber (Warner Instruments, Reading, UK) at 37°C on a Zeiss Axiovert S100TV inverted microscope (Zeiss, Vienna, Austria) equipped for epifluorescence and phase contrast microscopy, using a 63×/NA 1.4 plan apochromatic objective. Data were acquired with a back-illuminated, cooled CCD (charged coupled device) camera (Princeton Research Instruments, NJ, USA) and with computer-controlled shutters (Optilas, Neufahrn, Germany) in the transmitted and epifluorescence light paths to minimise radiation damage to cells. The camera controller was driven by IPLab software (Visitron Systems, Eichenau, Germany).

2.5. Actin-binding assays

Pig stomach smooth muscle muscle actin was prepared from acetone powder according to [18]. Co-sedimentation assays were performed in a buffer containing 2 mM Tris, 0.1 mM $CaCl_2$, 0.2 mM ATP, 0.1 mM DTE, 100 mM KCl, 2 mM $MgCl_2$, pH 8.0. Proteins were mixed, incubated at 25°C for 30 min and pelleted at 100 000 × *g* for 30 min using an air-driven ultracentrifuge (Beckman Instruments). Supernatant and pellet were separated and the pellet was resuspended in PBS in the starting volume.

2.6. GST in vitro pull down assays

Glutathione-bound Sepharose beads (Amersham, Vienna, Austria) were bound to either GSTrap FF purified GST-CRP2 or equally obtained GST by overnight incubation of 500 µl beads in 5 ml protein solution (3 mg ml^{-1}) at 4°C under rotation. Thirty µl of the respective beads was then incubated with α-actinin (0.3 mg ml^{-1}) in a Tris buffer (20 mM Tris, 5% glycerol, 0.1% BSA, 50 or 140 mM NaCl) for 2 h at 4°C under rotation. Beads were washed 5× in the respective buffer without α-actinin, resuspended in 30 µl sodium dodecyl sulfate (SDS) mix and heated to 100°C for 1 min. Resulting samples were subjected to Western blotting.

2.7. Electrophoresis, Western blotting and immunoprecipitation

Analytical SDS–polyacrylamide gel electrophoresis (PAGE) on 8–22% gradient polyacrylamide mini-slab gels and Western blotting onto nitrocellulose (Amersham, Vienna, Austria) were performed as described elsewhere [19]. Transferred proteins were visualised using horseradish peroxidase-coupled secondary antibodies and the ECL chemiluminescence detection system (Amersham, Vienna, Austria). Immunoprecipitations were performed from cell lysates of B16F1 cells expressing GFP-tagged α-actinin and myc-tagged CRP2 essentially as described earlier [25] with modifications in the buffer (20 mM Tris pH 7.0, 140 mM NaCl, 5 mM KCl, 0.5% Triton X-100, 5% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 0.67 µg ml^{-1} pepstatin, 1.67 µg ml^{-1} leupeptin). Tag specific antibodies were obtained from Clontech (Heidelberg, Germany).

2.8. Reagents

Phorbol 12,13-dibutyrate (PDBu) was purchased from Sigma (Vienna, Austria).

3. Results and discussion

Fig. 1 shows the CRP2 mutants constructed for this study. For in vitro actin-binding assays we constructed cDNA plasmid for expression and purification of recombinant bacterial CRP2 and coding exclusively for the CRP2 sequence, in addition to a fusion mutant (GST-CRP2) for in vitro pull down assays. Further, we generated GFP-CRP2 and myc-CRP2 constructs, and the mutant GFP-CRP2-mito, in which the mitochondrial targeting sequence of ActA (*Listeria monocyto-*

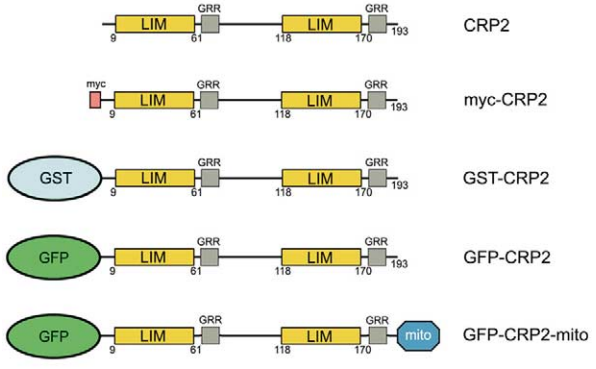

Fig. 1. CRP2 constructs used in this study. CRP2 contains two LIM domains followed by short glycine-rich repeats (GRR). Myc and GFP moieties were fused to the N-terminal end of the CRP molecule. For GFP-CRP2-mito, the mitochondrial targeting sequence of ActA (*L. monocytogenes*) was fused to the C-terminus of GFP-CRP2.

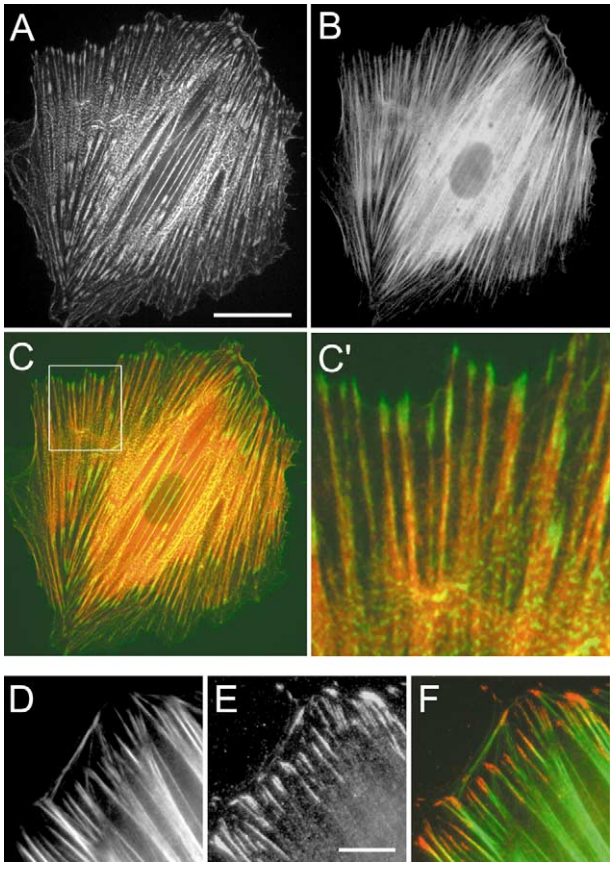

Fig. 2. Subcellular localisation of CRP2 in A7r5 cells. In contrast to the homogeneous decoration of actin filaments by myc-CRP2 (B), α-actinin-GFP localised periodically along the actin stress fibres and was strongly enriched at focal adhesions (A). Image C′ shows a detail of the merged image in C, visualising the only partially overlapping patterns of α-actinin-GFP and myc-CRP2 along the actin cytoskeleton. Cells were double-transfected with α-actinin-GFP (A) and myc-CRP2 (B), fixed and stained with anti-myc antibody and Alexa568-conjugated secondary antibody. Scale bar, 20 μm. D–F: Zyxin was found associated with focal adhesions, whereas GFP-CRP2 localised exclusively along the actin stress fibres. A7r5 cells expressing GFP-CRP2 were fixed and stained with an anti-zyxin antibody and an Alexa568-conjugated secondary antibody. Scale bar, 5 μm.

genes) was fused to the C-terminus of GFP-CRP2, for expression in mammalian cells.

When myc-CRP2 was transfected into cultured A7r5 vascular smooth muscle cells it localised to the stress fibre bundles continuously. In contrast to α-actinin-GFP, which localised periodically along the actin stress fibres as well as in focal adhesions (Fig. 2A–C). GFP-tagged CRP2 localised identically with myc-CRP2, also with a conspicuous absence from the stress fibre ends, where zyxin preferentially was found (Fig. 2D–F).

This difference in localisation between these proteins, formerly identified as binding partners, raised the concern that N-terminal fusion of CRP2 may interfere with its interaction with α-actinin. To test this possibility we employed pull down in vitro assays with CRP2 fused to GST at the N-terminus. As seen in Fig. 3 α-actinin readily co-sedimented with GST-CRP2 bound to glutathione beads (lanes 1 and 2), but not with GST alone (lanes 3 and 4). Notably, the interaction with α-actinin was significantly reduced under physiological salt conditions (140 mM NaCl) as compared to the low salt conditions in 50 mM NaCl, suggesting that the interaction could be of ionic nature. Taken together, the N-terminal fusion of GFP to CRP appears not to affect the interaction with α-actinin and should thus not influence the subcellular localisation of the protein. However, the observed in vitro interaction between GST-CRP2 and α-actinin could not be confirmed by co-immunoprecipitation experiments using ectopically expressed GFP-tagged α-actinin and myc-tagged CRP2 under physiological conditions (data not shown).

These results suggested that the association of CRP2 with the actin cytoskeleton may be independent of the interaction with the two binding partners α-actinin and zyxin. To further investigate this possibility we employed high speed actin co-sedimentation assays. Purified recombinant CRP2 was centrifuged at $100\,000 \times g$ in the presence or absence of pure smooth muscle actin, and supernatant and pellet were analysed on SDS–PAGE gels. As shown in Fig. 4, CRP2 was found enriched in the pellet together with F-actin, whereas CRP2 alone did not sediment. This experiment clearly demonstrated that CRP2 binds directly to F-actin in vitro in the absence of other actin-binding proteins.

It thus seems that zyxin and α-actinin are dispensable for the recruitment of CRP2 to the actin cytoskeleton. However, one cannot exclude a possible influence of the localisation of CRP2 on the binding characteristics of α-actinin and zyxin. To address this question we fused the mitochondrial targeting sequence of the *Listeria* protein ActA to GFP-CRP2 to induce subcellular mislocalisation. The fusion caused an almost com-

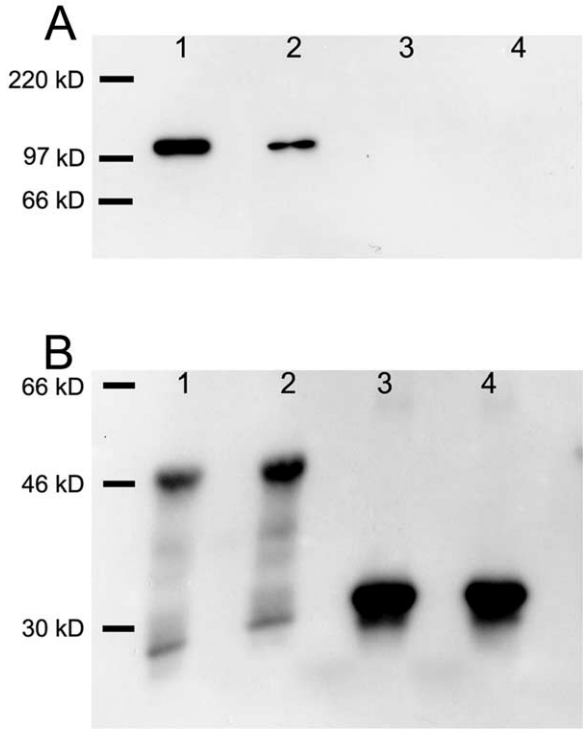

Fig. 3. In vitro pull down assay with α-actinin and GST-CRP2. GST-CRP2 (lanes 1 and 2), but not GST alone (lanes 3 and 4), co-precipitates α-actinin. The interaction is sensitive to increasing ionic concentrations. Lanes 1 and 3: 50 mM NaCl; lanes 2 and 4: 140 mM NaCl. Western blot probed with anti-α-actinin (A) or anti-GST (B) antibody. For Western blot B, samples from A were diluted 1:10.

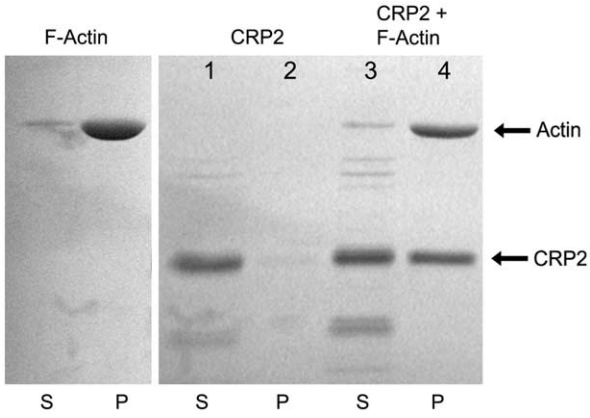

Fig. 4. Co-sedimentation of F-actin and CRP2. Purified, recombinant CRP2 (2 mg ml^{-1}) was incubated with (lanes 3 and 4) or without (lanes 1 and 2) F-actin (2 mg ml^{-1}) and centrifuged at 100 000 × *g*. Subsequently, supernatants (S) and pellets (P) were separated and subjected to PAGE. CRP2 bound to F-actin as indicated by its presence in the actin pellet after centrifugation (lane 4).

plete alteration in the localisation of transiently transfected CRP2 in A7r5 cells, and the GFP signal was now concentrated exclusively at the mitochondrial surface. However, this mislocalisation of CRP2 did not impact on the localisation of α-actinin, which showed its normal, actin- and focal adhesion-associated subcellular distribution (Fig. 5). Likewise, the localisation of zyxin was unchanged in GFP-CRP2-mito-expressing cells (not shown). Taken together, these data support the autonomous, and α-actinin and zyxin-independent actin binding and cytoskeleton associating properties of CRP2.

If CRP2 is not involved in regulating the function of cytoskeleton organising components as a 'secondary modulating component', what other physiological role could then be ascribed to cytoplasmic CRP2? It has been shown recently that treatment of A7r5 cells with the phorbol ester PDBu induces the rapid remodeling of the actin cytoskeleton and the formation of podosomes at the cell periphery [20,21], and that different actin-binding proteins segregated differentially during this process [22]. For example, SM22 and α-actinin were rapidly translocated to podosomes, whereas overexpression of calponin stabilised actin filaments in living cells and inhibited podosome induction in response to PDBu [22]. In GFP-CRP2-transfected cells treated with 1 μM PDBu for 20 min,

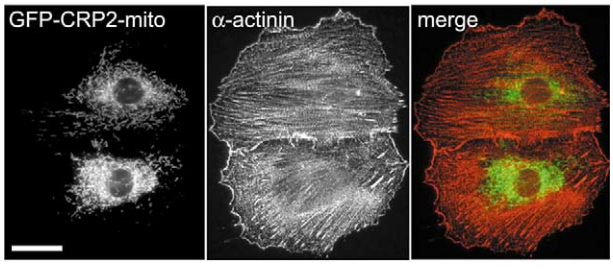

Fig. 5. GFP-CRP2-mito does not recruit endogenous α-actinin. GFP-CRP2 fused to the mitochondrial targeting sequence of ActA localises to the mitochondrial surface. The subcellular distribution of α-actinin at actin fibres and in focal adhesion, however, is unaffected. A7r5 cells transfected with GFP-CRP2-mito were fixed, and stained with an anti-α-actinin antibody and Alexa568-conjugated secondary antibody. Scale bar, 20 μm.

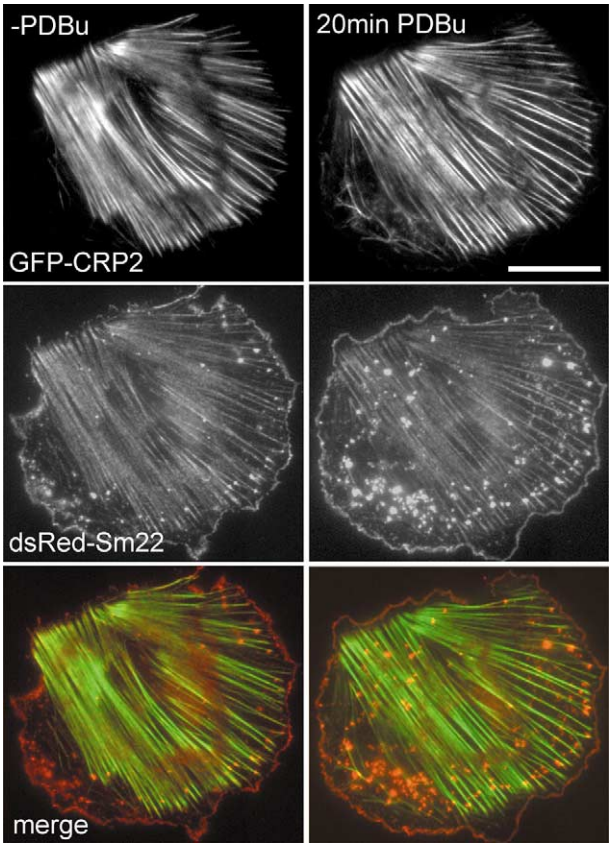

Fig. 6. CRP2 during F-actin remodelling. Treatment of A7r5 cells with 1 μM PDBu causes the rapid rearrangement of the actin cytoskeleton and leads to the formation of podosomes at the cell periphery. Selected frames of a movie sequence of an A7r5 cell expressing GFP-CRP2 and dsRed-SM22 are shown. While dsRed-SM22 is rapidly translocated and incorporated into newly forming podosomes, GFP-CRP2 remains bound to the actin stress fibres. Scale bar, 20 μm.

the molecule remained associated with actin filament bundles despite the formation of peripheral podosomes (Fig. 6). The turnover of the actin cytoskeleton appeared delayed and translocation of detectable amounts of GFP-CRP2 to podosomes was only seen after prolonged exposure to PDBu by which time most of the stress fibres had disassembled (not shown).

In summary, our results identify mammalian CRP2 as an F-actin-binding molecule which can associate autonomously with the actin cytoskeleton. This finding is well supported by a recent report from the Noegel lab, who identified direct actin-binding capacity in several CRP orthologs from *Dictyostelium discoideum* [12]. In light of these results the role of CRP2 as a potential regulator of α-actinin (and zyxin?) function in vivo should be reconsidered.

4. Conclusion and hypothesis

CRP2 functions and localises independently of α-actinin and zyxin, and may not play a significant role in the regulation of the cellular function of these cytoskeletal components. CRP2 binds directly to actin and, similar to zyxin, another LIM domain-containing protein may shuttle from the cytoplasm to the nucleus. There, CRP2 can act as a cofactor of SRF, and is involved in enhancing the expression of smooth

muscle specific gene products like calponin, SM22α or smooth muscle α-actin. Results from the Treisman lab [23,24] suggest that SRF activation requires decreased G-actin levels and that SRF-controlled expression of muscle specific cytoskeletal components may be a method to relay morphological changes in the cell. The data argue for a mechanism capable of sensing the status of actin polymerisation in the cytoplasm, and responding by activation of SRF-dependent gene expression. It is tempting to speculate that CRP2 could be a part of such a putative 'sensor' complex that is sequestered on actin and has the ability to translocate from the cytoplasm to the nucleus, to signal increased activity of SRF. Future studies should address the potential involvement of CRP molecules in regulating SRF in response to alterations in the balance between G- and F-actin in the cell.

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