p80 ROKα binding protein is a novel splice variant of CRMP-1 which associates with CRMP-2 and modulates RhoA-induced neuronal morphology

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Abstract Using antibody against the Rho binding domain of ROKα, two neuronal phosphoproteins of 62 and 80 kDa were co-immunoprecipitated from brain extracts. Peptide analysis revealed their identity as collapsin response mediator proteins (CRMPs); p62 was CRMP-2 whereas p80 was a novel splice form of CRMP-1 with an extended N-terminus. p80 CRMP-1 was able to complex with CRMP-2, suggesting that p80 CRMP-1 and CRMP-2 form oligomers. CRMP-2 was the major substrate of ROK. p80 CRMP-1 interacted with the kinase domain of ROKα, resulting in inhibition of the catalytic activity towards other substrates. Over-expression of p80 CRMP-1 and CRMP-2 together counteracted the effects of RhoA on neurite retraction, an effect enhanced by mutation of the ROK phosphorylation site in CRMP-2. p80 CRMP-1 and CRMP-2 may be modulators of RhoA-dependent signaling, through interaction with and regulation of ROKα.

Key words: ROKα; CRMP family; RhoA; Neuronal morphology

1. Introduction

The Rho GTPases regulate neuronal morphology by influencing the stability and assembly of the actin cytoskeleton [1,2]. They mediate signaling pathways that respond to upstream signals and studies on fibroblasts and other cells have revealed cross-talk amongst Rho GTPases, resulting in either a hierarchical linear cascade [3] or antagonistic effects of Rho and Rac activities [4]. In neuronal cells, activation of Rac/Cdc42 and antagonism of RhoA signaling appears to be implicated in neurite outgrowth [5], suggesting that counterbalancing of Rac and RhoA signaling is essential for forming complex neuronal morphology [2]. The activity of RhoA effector kinases ROKs (Rho kinases) has been shown to play a key role in regulating neurite outgrowth [5–7], probably through multiple effects on the dynamic changes in various cytoskeletal components [8]. An involvement of Rho GTPase in semaphorin 3A (Sema3A)-mediated axonal guidance and growth cone motility has also been demonstrated (see [9] for review). Rac1 was first shown to act downstream of Sema3A in dorsal root ganglion neurons where dominant negative Rac1 inhibited growth cone collapse [10]. Antibodies against a member of the collapsin response mediator proteins (CRMPs), a family of dihydropyrimidinase-related neuronal proteins involved in axonal outgrowth [11–13], can also block Sema3A-induced growth cone collapse [14], suggesting that CRMP-2 may play a role downstream of the Sema3A receptor, neuropilin 1 and its co-receptor plexin A1 [15]. Recently it has been shown that Rac1 interacts directly with the plexin B1 cytoplasmic tail [16–18] indicating that some receptor subtypes may engage directly with the Rho GTPase pathways. Furthermore, clustering of the cytoplasmic domain of the co-receptor results in RhoA activation and actin contractility [18]. *Drosophila* plexin B has also been shown to both inhibit Rac-GTP and signal through Rho [19], possibly via PDZ RhoGEFs, which interact specifically with plexin B family members [20]. We have found that CRMP-2 in combination with active Rho-GTPases can switch signaling between Rac and Rho [21]. Furthermore, CRMP-2, a ROK substrate, is phosphorylated by ROK in lipophosphatidic acid (LPA)-induced growth cone collapse, but not in response to Sema3A [22]. It appears that Sema3A impinges on Rac and Rho effects on the actin dynamics, resulting in growth cone motility and alterations in axonal guidance cues [9].

Here using ROKα antibodies we have been able to detect two CRMP family members associated with ROKα: CRMP-2, and a novel splice variant of CRMP-1 with an extended N-terminus. p80 CRMP-1 and CRMP-2 can form hetero-oligomers. The interaction of p80 CRMP-1 was mapped to the kinase domain and resulted in inhibition of the ROK activity towards other substrates. p80 CRMP-1 and CRMP-2 co-expression can counteract the effect of Rho on neurite retraction in SY5Y neuroblastoma cells, suggesting that these CRMP proteins are modulators of Rho activity in neuronal morphogenesis.

2. Materials and methods

2.1. Construction of expression vectors

Full-length p80 cDNA was obtained by first performing a PCR reaction of mouse cDNA based on the reported sequence of an

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Abbreviations: CRMP, collapsin response mediator protein; ROK, RhoA binding kinase; MLC2, myosin light chain 2; Sema3A, semaphorin 3A; LPA, lipophosphatidic acid; NGF, nerve growth factor

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EST clone (BM950288) encoding the N-terminus of an alternatively spliced form of CRMP-1 (see Fig. 1B), using two adapter primers, 5′-CTAAGCTTCCGGAGACGGGGATGG-3′ and 5′-GTCTCGAGGTCAACCGAGGCTGG-3′. The 2.1 kb PCR fragment was digested with *Hin*dIII and *Xho*I and cloned into pXJ40-FLAG and pXJ40-HA vectors for sequencing and expression studies. The 5′ sequence encoding the p80 N-terminus (amino acids 1–127) was obtained by PCR of the full-length p80 with primers 5′-CTAAGCTTCCGGAGACGGGGATGG-3′ and 5′-GGTCACTCTGGCCATTGTC-3′ and subcloning of the *Bam*HI/*Bal*I fragment into *Bam*HI/*Sma*I sites of vector pGEX-4T1. pXJ40-FLAG- and pXJ40-HA-tagged CRMP-2 and the mutant CRMP-2 (T555A), pXJ40-HA ROKα$^{1-1379}$, ROKα$^{1-543}$, ROKα$^{971-1379}$ and pGEX4T2-myosin light chain 2 (MLC2) were obtained as previously described [21,23].

2.2. Cell culture, transfection and cell staining

Neuroblastoma SY5Y cells were cultured in RPMI/MEM (1:1) medium containing 10% fetal bovine serum (FBS). Subconfluent SY5Y cells plated on coverslips for 48 h were transfected with various HA- or FLAG-tagged DNA constructs (1 μg/ml) with Effectene transfection reagent (Qiagen) according to the recommended protocol. Sixteen hours after transfection, cells were fixed with 4% paraformaldehyde and stained with a combination of various primary antibodies: anti-HA (12CA5; Boehringer Mannheim) or anti-FLAG, (M2; IBI). Stained cells were analyzed with a Bio-Rad Radiance 2000 confocal imager adapted to a Nikon microscope. COS-7 cells grown in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS were similarly transfected with various constructs. Twenty-four hours after transfection, cell extract preparation and immunoprecipitations were carried out as previously described [23]. PC12 cells grown in DMEM with 10% FBS were treated with nerve growth factor (NGF) as previously described [6].

2.3. Preparation of antibodies, protein and enzymatic analyses

Affinity-purified antibodies against different domains of ROKα were obtained as described previously [23]. Polyclonal antibodies against the unique N-terminus of p80 CRMP-1 were obtained by injecting the thrombin-cleaved polypeptide from glutathione *S*-transferase (GST) fusion protein into mice and purified through antigen affinity column for use in Western blotting, cell staining and immunoprecipitation assays. Kinase assays were carried out in buffer containing 20 mM Tris–HCl pH 7.5, 75 mM NaCl and 10 mM $MgCl_2$ as previously described [23]. Analysis of tryptic peptides and phosphopeptides was performed with a QSTAR mass spectrometer (Perkin Elmer).

3. Results

3.1. Identification of p62 and p80 ROKα binding proteins as CRMP-2 and a novel splice variant of CRMP-1 respectively

To identify and characterize potential binding proteins for ROKα, we made use of the polyclonal antibodies of ROKα in an immunoprecipitation assay. Two proteins of molecular mass 62 and 80 kDa were the major components immunoprecipitated from brain extracts with the antibody against the RhoA binding domain of ROKα (dBD-Ab; Fig. 1A [23]). p62 in particular was readily phosphorylated when the immunoprecipitated products were subject to kinase assay (Fig. 1A, left panel). The phosphorylation of p80 was much weaker than for p62. We subjected these two proteins for mass spectrometry analysis and the data indicated that the p62 protein is CRMP-2 whereas p80 is a novel splice form of CRMP-1. This was further confirmed by Western blotting with antibody for CRMP-2 and CRMP-1 (against the unique N-terminus, see Section 2) that specifically recognized the co-immunoprecipitated p62 and p80 respectively (Fig. 2A).

From the reported sequence of an EST cDNA (BM950288), we obtained the mouse cDNA encoding the full-length protein

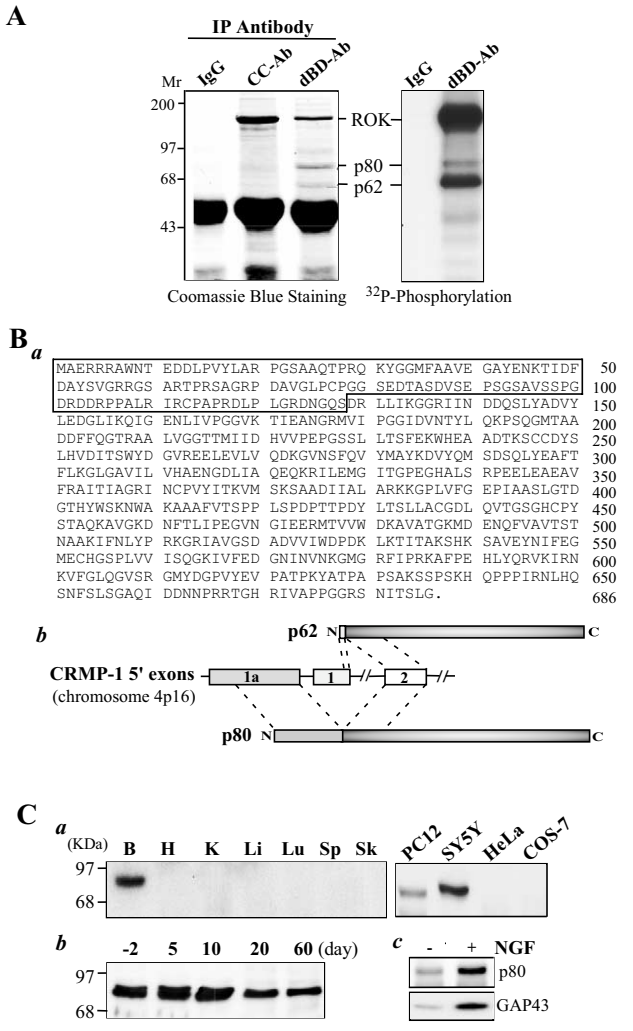

Fig. 1. Identification of p62 and p80 as major ROKα binding proteins. A: Soluble rat brain extract was subjected to immunoprecipitation by dBD-Ab and CC-Ab anti-ROKα antibodies with rabbit IgG being used as control. Immunoprecipitates were separated on SDS–PAGE and either stained with Coomassie blue (left panel) or subjected to phosphorylation assay (right panel). The major bands at 62 and 80 kDa are indicated. B: Amino acid sequence of the mouse p80 CRMP-1 derived from DNA sequence revealed a splicing event at the first exon. The spliced N-terminus of p80 CRMP-1 is boxed (a). This sequence was derived from a 5′ exon (exon 1a) from a human chromosome 4 gene (b). C: Tissue and developmental expression of p80 CRMP-1. (a) Soluble extracts (100 μg) from various tissues of adult rat or cell lines were resolved on SDS–PAGE, transferred to polyvinylidene difluoride filter and immunoblotted with anti-p80 CRMP-1 antibody. For the developmental profile (b), rat brain cytosolic extracts (100 μg) from different ages were used. In panel c, PC12 cells with and without NGF treatment (50 ng/ml) for 3 days were analyzed with either anti-p80 or anti-GAP43 antibodies.

of p80 CRMP-1 by PCR. Sequence analysis of p80 cDNA and comparison with the known p62 CRMP-1 confirm that both p80 and p62 CRMP-1 are derived from the same gene (Fig. 1B). The derived amino acid sequence of p80 CRMP-1 revealed an alternative splicing event from an upstream exon (exon 1a) to generate p80 mRNA. It has been reported previously that the N-terminus of p62 CRMP-1 was derived from exon 1 from chromosome 4p16 [24]. There is no sequence similarity of this unique N-terminal sequence of p80 to any

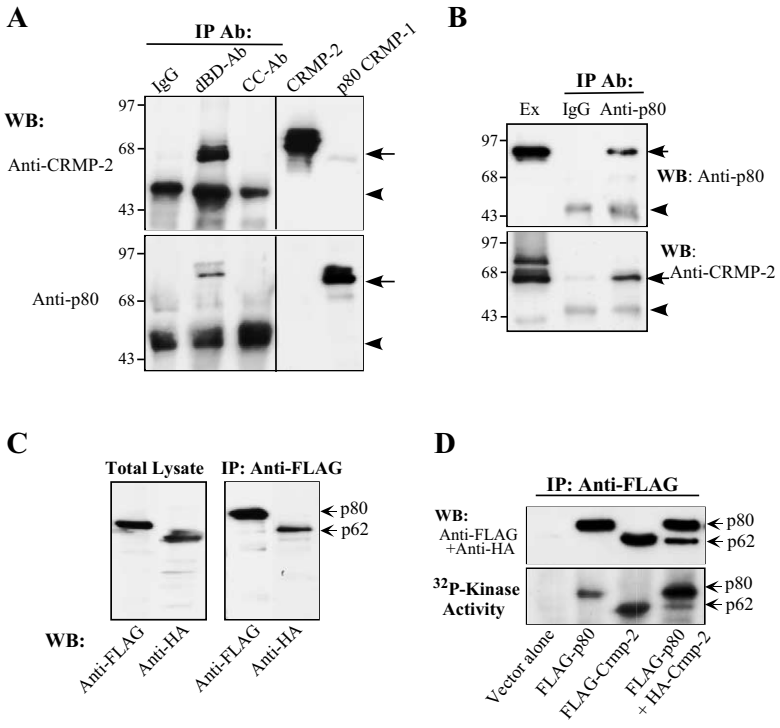

Fig. 2. p80 CRMP-1 and p62 CRMP-2 can form hetero-dimers. A: Antibodies to CRMP-2 and p80 CRMP-1 specifically recognized p62 and p80 from immunoprecipitation by dBD-Ab. Immunoprecipitates from rat brain using either dBD-Ab or CC-Ab were probed with specific antibody to CRMP-2 or p80 CRMP-1. Soluble cell extracts from COS-7 cells transfected with CRMP-2 or p80 CRMP-1 construct were used as controls (arrows). The position of IgG bands is marked by an arrowhead. B: Co-immunoprecipitation of CRMP-2 with p80 CRMP-1 antibody from rat brain extract. Soluble rat brain extract was subjected to immunoprecipitation with either p80 CRMP-1 antibody or mouse IgG as control. Immunoprecipitates were separated on SDS–PAGE and probed with anti-p80 CRMP-1 or anti-CRMP-2 antibody. Total extract (Ex) was used for comparison. C: Co-immunoprecipitation of p80 CRMP-1 with CRMP-2 in over-expressed system. FLAG-tagged p80 CRMP-1 construct was co-transfected with the HA-tagged CRMP-2 in COS-7 cells. Immunoprecipitations (IP) were carried out using anti-FLAG antibody and immunoblotted (WB) with anti-FLAG or anti-HA antibody. Total lysate is shown for comparison (left panel). D: p80 CRMP-1 and CRMP-2 are substrates of ROKα. Soluble extracts from COS-7 cells transfected with the various constructs were immunoprecipitated with anti-FLAG antibody as described above and immunoblotted (WB) with anti-FLAG and anti-HA antibodies. Kinase assay on the immunoprecipitates was carried out using the catalytic domain of ROKα and [^{32}P]ATP.

known motif in the protein database. The sequence is relatively proline-rich and may have a coiled structure at the first 50 amino acids of the N-terminus.

Like other CRMP proteins [25], the p80 CRMP-1 protein was expressed exclusively in the brain and cell lines of neuronal origin (Fig. 1C, a). In rat brain, highest expression was observed at around 5–10 days and at consistently lower levels after day 20 (Fig. 1C, b); and similar to GAP43, p80 CRMP-1 expression can be induced by NGF in PC12 cells (Fig. 1C, c).

3.2. p62 CRMP-2 and p80 CRMP-1 can form hetero-dimers

As only p62 CRMP-2 and p80 CRMP-1 were identified in ROKα immunoprecipitates by both peptide sequencing and immunoblotting analyses (Fig. 2A), it was of interest to see if these two proteins can form oligomers as has been reported for other CRMP proteins [26–28]. Both p80 CRMP-1 and CRMP-2 were co-immunoprecipitated from rat brain extract using antibody to p80 CRMP-1, indicating that these two proteins exist as a dimer or higher molecular form (Fig. 2B). Furthermore, FLAG-tagged p80 CRMP-1 also co-immunoprecipitated with HA-tagged CRMP-2, supporting the notion that these two proteins form hetero-dimers (Fig. 2C). Furthermore, the catalytic domain of ROKα could phosphorylate both immunoprecipitated p80 CRMP-1 and CRMP-2. The phosphorylation towards CRMP-2 was consistently weaker when phosphorylation was carried out with the co-precipitated p80 and CRMP-2 (Fig. 2D). It is at present not known if this is due to the lower amount of CRMP-2 co-immunoprecipitated with p80-CRMP1.

3.3. p80 CRMP-1 interacted with the ROKα kinase domain and inhibited the catalytic activity

To map the region of ROKα that interacted with p80 CRMP-1, we co-expressed the various regions of ROKα with p80 CRMP-1. Only the full length and the kinase domain were detected in CRMP-1 immunoprecipitates, but not the C-terminal region, indicating that p80 CRMP-1 interacted with the kinase domain (Fig. 3A).

Likewise, when CRMP-2 and p80 CRMP-1 were co-expressed with the ROKα kinase domain, both CRMP proteins were detected in the ROKα kinase immunoprecipitates (Fig. 3B). This gives further evidence that these CRMP proteins can interact with the kinase domain of ROKα.

Furthermore, this interaction resulted in an inhibition of the catalytic activity of ROKα, as the kinase co-precipitated with p80 CRMP-1 was relatively inactive towards the exogenous substrate MLC2 (Fig. 3C).

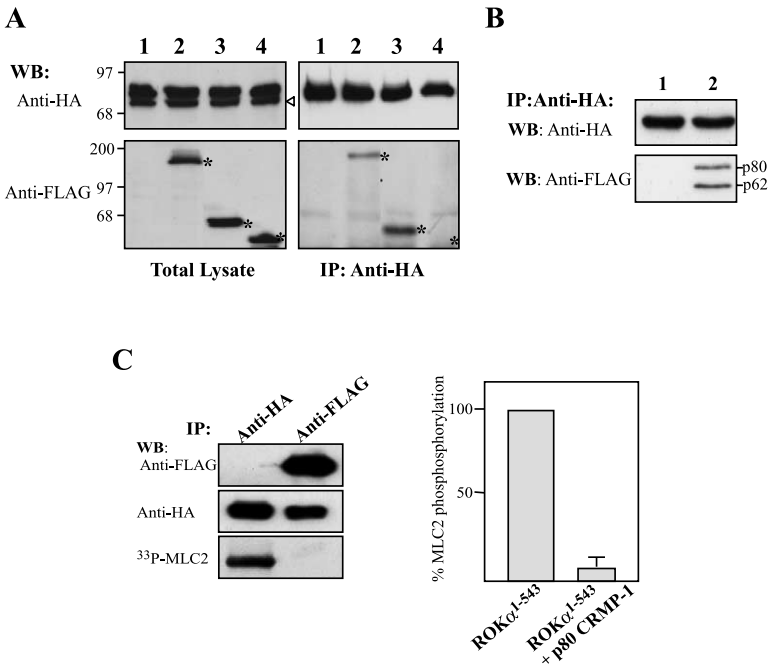

Fig. 3. p80 CRMP-1 binds ROKα at the kinase domain and inhibits the catalytic activity. A: Mapping of the kinase domain of ROKα as binding site for p80 CRMP-1. HA-tagged p80 CRMP-1 construct was transfected alone (lane 1), or co-transfected with either FLAG-tagged ROKα$^{1-1379}$ (lane 2), ROKα$^{1-543}$ (lane 3) or ROKα$^{971-1379}$ (lane 4). Immunoprecipitations were carried out with anti-HA antibody and immunoprecipitates were immunoblotted with anti-HA or anti-FLAG antibody. The arrowhead indicates the non-specific band recognized by the anti-HA antibody in total lysate and the asterisk marks the corresponding ROK protein immunoprecipitated by p80 CRMP-1. B: HA-tagged ROKα$^{1-543}$ construct was expressed alone (lane 1) or co-expressed with FLAG-tagged p80 CRMP-1 and CRMP-2 (lane 2) in COS-7 cells. Immunoprecipitations were carried out with anti-HA antibody and immunoprecipitates were immunoblotted with anti-HA (top panel for ROKα$^{1-543}$) or anti-FLAG antibody (bottom panel for p80 CRMP-1 and CRMP-2). C: Inhibition of ROKα catalytic activity upon p80 CRMP-1 binding. Soluble extract from COS-7 cells expressing HA-tagged ROKα$^{1-543}$ alone (left lane) or HA-tagged ROKα$^{1-543}$ and FLAG-tagged p80 CRMP-1 (right lane) were subjected to immunoprecipitation (IP) with either anti-HA or anti-FLAG antibody. The immunoprecipitates recovered were blotted (WB) and probed with anti-HA or anti-FLAG antibody. The kinase activities of the immunoprecipitates towards GST-MLC2 were quantified and calculated as means and S.D. of the percentage of the ROKα kinase activity obtained from immunoprecipitates of HA-ROKα$^{1-543}$ (as 100%).

3.4. p80 CRMP-1/CRMP-2 expression counteracts RhoA activity on neurite retraction in SY5Y neuroblastoma cells

Activation of Rho kinase/ROK by RhoA in neuronal cells caused neurite and cell body retraction in a variety of neuronal cells [5–8]. In neuroblastoma SY5Y cells, over-expression of RhoA also resulted in dramatic neurite and cell body retraction (Fig. 4A). The over-expression of p80 CRMP-1 and CRMP-2 together significantly counteracted this RhoA activity. Interestingly, co-expression of CRMP-2 T555A (mutated at the ROK phosphorylation site (T555) in CRMP-2 [22]) had a more potent effect on Rho inhibition (Fig. 4B). These results indicated that the two CRMP proteins have a modulatory effect on RhoA activity in neuronal cells, probably as a result of an inhibitory effect on the RhoA-dependent ROKα catalytic activity towards other substrates.

4. Discussion

Here we have demonstrated that ROKα can interact with two CRMP proteins, CRMP-2 and p80 CRMP-1, a novel splice variant. A previous study using a CRMP-2 antibody has also demonstrated the association of an unidentified 190 kDa presumptive protein kinase [29]. It is possible that this was ROKα. The effective detection with antibody recognizing the Rho binding region (dBD-Ab), but not with CC-Ab, would suggest that only ROKα in the active state could interact. The antibody dBD-Ab, but not CC-Ab, is capable of inducing conformational changes of ROKα [23]. Interestingly, CRMP-2 and p80 CRMP-1 can form hetero-oligomers and this could be the functional form that interacted with ROKα, when the kinase is in an active conformation (i.e. induced by Rho-GTP binding or by interaction with the antibody dBD-Ab).

CRMP proteins are likely to form hetero-tetrameric structures [26] and CRMP-2 has been reported to bind CRMP-4 and CRMP-5 [27,28]. However, neither CRMP-4 nor the shorter CRMP-1 isoform (p62 CRMP-1) was detectable in our ROKα immunoprecipitates from rat brain (data not shown). It has been reported that CRMP-2 was a substrate of ROK/Rho kinase, phosphorylated in LPA-induced growth cone collapse [22]. The morphological effects of CRMP-2 in association with dominant active Rac and Rho are also influenced by ROK phosphorylation [21]. We propose here that the novel p80 CRMP-1 may be a functional partner of CRMP-2. Furthermore, p80 CRMP-1 alone can bind ROKα at the kinase domain and this resulted in inhibition of the catalytic activity towards other substrates. It is possible that p80 CRMP-1 and its partner CRMP-2 play a role as direct modulators of RhoA and ROK signaling events. Over-expression of p80 CRMP-1 and CRMP-2 was effective in counteracting RhoA-induced neurite retraction in neuroblastoma SY5Y cells, indicating that these proteins can inhibit RhoA action on neuronal morphology. Interestingly, the enhanced inhibition of Rho-induced collapse elicited by the non-

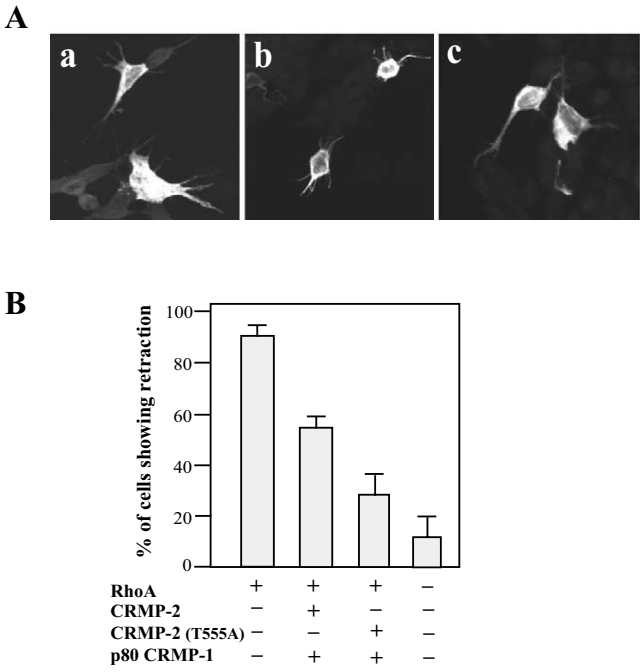

Fig. 4. Over-expression of CRMP-2 and p80 CRMP-1 counteracts the effect of RhoA-induced neurite retraction in SY5Y neuroblastoma cells. A: In (a) SY5Y cells transfected with pXJ40-FLAG p80 CRMP-1 and immunostained with anti-FLAG antibody showed normal cell morphology. In (b) and (c), cells transfected with pXJ-HA RhoA (wild-type) alone or together with pXJ40-FLAG-CRMP-2/p80 CRMP-1 were fixed and stained with mouse anti-HA/FITC anti-mouse, or co-stained with anti-HA/FITC and anti-FLAG/TRITC; only anti-HA staining is shown. B: Statistical analysis of the scores of SY5Y cells showing neurite retraction from transfection with various combinations of DNA constructs (as in A). The scores are the means and S.D. from three or four separate experiments.

phosphorylatable CRMP-2 mutant (T555A) gives further support for the phosphorylation of CRMP-2 by ROK in this regulatory event [22,23].

Over-expression of CRMP-2 in primary hippocampal neurons was recently shown to produce multiple axons [30]. This may well involve inhibition of the Rho pathway. Indeed p190 RhoGAP has been shown to participate in regulating axon branch stability [31]. The relationship of CRMP-2 to Sema3/plexin signaling is unclear. It remains to be seen if CRMP-2 requires eventual phosphorylation to free itself from the interaction with ROKα complex and release the inhibitory effect on the kinase activity, thus providing a mechanism for switching Rho GTPase activity in response to Sema3A/Rac-mediated and other guidance signals.

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