The Adaptor Protein CD2AP Is a Coordinator of Neurotrophin Signaling-Mediated Axon Arbor Plasticity

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Growth of intact axons of noninjured neurons, often termed collateral sprouting, contributes to both adaptive and pathological plasticity in the adult nervous system, but the intracellular factors controlling this growth are largely unknown. An automated functional assay of genes regulated in sensory neurons from the rat in vivo spared dermatome model of collateral sprouting identified the adaptor protein CD2-associated protein (CD2AP; human CMS) as a positive regulator of axon growth. In non-neuronal cells, CD2AP, like other adaptor proteins, functions to selectively control the spatial/temporal assembly of multiprotein complexes that transmit intracellular signals. Although CD2AP polymorphisms are associated with increased risk of late-onset Alzheimer’s disease, its role in axon growth is unknown. Assessments of neurite arbor structure in vitro revealed CD2AP overexpression, and siRNA-mediated knockdown, modulated (1) neurite length, (2) neurite complexity, and (3) growth cone filopodia number, in accordance with CD2AP expression levels. We show, for the first time, that CD2AP forms a novel multiprotein complex with the NGF receptor TrkA and the PI3K regulatory subunit p85, with the degree of TrkA:p85 association positively regulated by CD2AP levels. CD2AP also regulates NGF signaling through AKT, but not ERK, and regulates long-range signaling through TrkA/H11001/RAB5/H11001 signaling endosomes. CD2AP mRNA and protein levels were increased in neurons during collateral sprouting but decreased following injury, suggesting that, although typically considered together, these two adult axonal growth processes are fundamentally different. These data position CD2AP as a major intracellular signaling molecule coordinat- ing NGF signaling to regulate collateral sprouting and structural plasticity of intact adult axons.

Key words: collateral sprouting; nerve growth factor; neural plasticity; signalosome; spared dermatome; transcriptomics

Significance Statement

Growth of noninjured axons in the adult nervous system contributes to adaptive and maladaptive plasticity, and dysfunction of this process may contribute to neurologic pathologies. Functional screening of genes regulated during growth of noninjured axons revealed CD2AP as a positive regulator of axon outgrowth. A novel association of CD2AP with TrkA and p85 suggests a distinct intracellular signaling pathway regulating growth of noninjured axons. This may also represent a novel mechanism of generating specificity in multifunctional NGF signaling. Divergent regulation of CD2AP in different axon growth conditions suggests that separate mechanisms exist for different modes of axon growth. CD2AP is the first signaling molecule associated with adult sensory axonal collateral sprouting, and this association may offer new insights for NGF/TrkA-related Alzheimer’s disease mechanisms.
Introduction

Plasticity of cellular structure is a fundamental biological process. In neurons, structural plasticity occurs in the dendrites, dendritic spines, axons, and axon terminal arbors. Significant advances have been made in understanding the mechanisms underly- ing structural plasticity of dendrites and dendritic spines, and identifying adaptive/homeostatic neuronal processes and disease states in which these mechanisms are involved (e.g., Tavosanis, 2012). On the axonal side, much effort has been directed toward understanding the mechanisms regulating the guidance/pathfinding of axons during development or during regeneration (or failure thereof) of damaged axons in the adult (Fagoe et al., 2014; Mar et al., 2014; e.g., Eva and Fawcett, 2014; Doron-Mandel et al., 2015), but the neuron-intrinsic signaling components that regulate structural plasticity of noninjured axon arbors in the adult nervous system remain undefined.

Structural rearrangement of axonal arbors is required for diverse adaptive functions both within the adult nervous system and in its interaction with the periphery (Diamond et al., 1992; Kawabuchi et al., 1995; Hsieh et al., 1996; Botchkarev et al., 1997; Li et al., 1997; Hsieh and Lin, 1999; Selim et al., 2007; Calinescu et al., 2011; e.g., Yasuda et al., 2011; Ibrahim et al., 2013; Gonzalez-Freire et al., 2014). These same processes contribute to pathologies, such as Alzheimer’s disease (Wu et al., 2014), sudden cardiac death (for review, see Vaseghi and Shvikum, 2008), autonomic dysreflexia after spinal cord injury (Krenz et al., 1999; Marsh et al., 2002; Wanigasekara and Keast, 2006), and exacerbation of epilepsy (Noebels, 1984; Davenport et al., 1990; Van der Zee et al., 1995; Adams et al., 1997; Scharfman et al., 1999; Pitkänen and Sutula, 2002; Sierra et al., 2015). Hyperinnervation of visceral tissue and bone is a characteristic of some of the most agonizing forms of pain (Woolf, 1996; Jimenez-Andrade et al., 2010, 2011; Bloom et al., 2011; López-Álvarez et al., 2015). In addition, although long-distance regeneration of damaged axons rarely occurs after brain or spinal cord injuries or stroke, there is often some degree of spontaneous recovery (e.g., Fawcett et al., 2007), which in part is mediated by short-distance restructuring of CNS axons (e.g., Rosenzweig et al., 2010). Identifying the mechanisms controlling axon plasticity of noninjured neurons could lead to new approaches to enhance recovery from disease and disability.

Although neural activity and extracellular trophic factors are necessary, sufficient, and/or modular for axonal plasticity, the intracellular pathways transducing these factors and the cellular processes involved are largely unknown. We used the spared dermatome model of sensory neuron collateral sprouting (Diamond et al., 1992) to identify genes related to axonal plasticity of noninjured neurons in vivo. In this model, de- nerved skin increases its production of NGF and axons in the adjacent dermatome grow to reinervate the denervated region in an NGF-dependent manner. Importantly, this model stimulates sensory neurons to undergo axonal arbor remodeling without their injury/disruption, so it can be used to study the molecular mechanisms of axon arbor dynamics independent of injury-induced intracellular signaling.

Analysis of transcriptomic profiles of DRGs in the spared dermatome model published in a separate manuscript (Harrison et al., 2015) revealed upregulation of the adaptor/scaffolding protein CD2-associated protein (CD2AP; CMS in human) (Kirsch et al., 1999). Adapter proteins selectively control the spatial and temporal assembly of multiprotein intracellular signaling complexes. Although not well studied in the nervous system, CD2AP has a regulatory role in trophic factor-dependent survival of some neurons (Tsuji and Pierchala, 2008). In other tissues, CD2AP coordinates actin dynamics, trophic factor receptor internalization, signal transduction, and endosomal sorting (Table 1) (Dustin et al., 1998; Cormont et al., 2003; Huber et al., 2003; Lynch et al., 2003; Kobayashi et al., 2004; Welsch et al., 2005; Tossidou et al., 2007; Johnson et al., 2008; Tsuji and Pierchala, 2008; Yaddanapudi et al., 2011; Tang and Briere, 2013; Zhao et al., 2013), all processes that are predicted to occur during expansion of axon arbors. In the spared dermatome model of plasticity, NGF currently is the only known regulatory factor (Diamond et al., 1992). Although various models of axonal arbor plasticity involve trophic factors (e.g., Scharfman et al., 1999; Zhou et al., 2004; Chen et al., 2006; Iarikov et al., 2007; Prince et al., 2009), the signaling can differ by condition and cell type, and it is often unclear how any specificity arises. We sought to determine whether CD2AP might act to influence NGF:TrkA signaling and axon arbor dynamics.

Materials and Methods

Materials. All chemicals and reagents were obtained from Sigma-Aldrich unless otherwise stated. Ultra-pure biochemistry-grade chemicals were used whenever possible.

Animals. All animal procedures were approved by the Institutional Animal Care and Use Committee of the State University of New York at Stony Brook and the University of Louisville consistent with federal regulations and guidelines. All rats were female Sprague Dawley 160–200 g. Mice were C57Bl/6 male 20–30 g.

Assessment of transcription profiles of genes regulated in DRG during sensory arbor expansion. It is currently unclear the degree to which axonal regeneration after nerve injury and arbor expansion of noninjured neurons share intracellular mechanisms. It was therefore desirable to use a model enriched for noninjured adult neurons undergoing axon growth, and depleted of neurons undergoing any other type of axon growth (e.g., injury-induced regeneration, integration of newly generated neurons, etc.). The thoracic and upper lumbar segments provide an advantageous model to study plasticity of noninjured afferents because their nerves have no plexus (Ygge, 1984; Baron et al., 1995). The spared dermatome model of cutaneous afferent sprouting applied to this region reduces the presence of injured neurons in the DRG, which might otherwise dilute the arbor expansion-associated transcript pool with injury/regeneration-associated transcripts. In this model, adapted from (Diamond et al., 1992), one nerve is spared and the two adjacent nerves on either side are transected (Fig. 1A–D). The axons of the interposed spared nerve subsequently undergo terminal arbor expansion into the adjacent skin that had lost its innervation (denervated dermatomes).

Details of the procedures used to generate the transcriptomic profile from microarray assessments, and the microarray data, are published previously (Harrison et al., 2015). The procedures used for the molecular assessments presented here are identical to those used for the microarray study (Harrison et al., 2015). Briefly, adult Sprague Dawley rats (160–200 g) were anesthetized with 60 mg/kg pentobarbital. All left-side thoracic
ninth through 13th dorsal and lateral cutaneous nerves, except T11 (i.e., T9, T10, T12, T13 D/LCnn) were cut and then ligated with fine suture to prevent regeneration. The skin wound (made to the right of midline so as not to injure axons in the left-side T11 skin) (Hill et al., 2010) was then closed with 4 – 0 suture and surgical staples. The T11 dermatome was unperturbed and bordered by adjacent denervated skin, and no damage was done to any of the spinal roots. Sensory arbors expand from intact dermatomes into the denervated skin to restore sensation (Fig. 1C,D).

To define the innervated and denervated areas of skin, we used the cutaneous trunci muscle reflex (CTMR) (Petruska et al., 2014) as was done previously (Diamond et al., 1992). The CTMR is a puckering of the back skin in response to noxious stimulation (e.g., heat or pinch) and can be used to report on the sensory innervation status of the skin. The area of innervated and denervated skin was mapped acutely after spared dermatome surgery; and from this baseline, we measured the extent of arbor expansion over time (Fig. 1E). After 7 or 14 d, time points corresponding approximately to the initiation (7 d) and maintenance (14 d) phases of sensory arbor expansion (Diamond et al., 1992), animals were killed by overdose of pentobarbital anesthesia and exsanguinated by intracardial perfusion of cold PBS. DRGs were then retrieved and processed for mi-
croarray assessment as described previously (Harrison et al., 2015). The three groups were naive (n = 4), 7 d after spared-dermatome surgery (n = 8), and 14 d after spared-dermatome surgery (n = 10). Neither analyses of raw microarray image data (Harrison et al., 2015) nor the molecular data presented here were adjusted for possible influence of estrous cycle.

**DRG CD2AP expression assay.** Using an additional set of animals which underwent the same procedures using the same protocols as those listed above, individual DRGs were processed in tandem for RNA and protein assays as follows:

- qPCR. DRGs were extracted from exsanguinated rats perfused with 60 ml 30% RNAlater (QIAGEN) in PBS. The spared DCn was then traced back to the noninjured/sprouting DRG for dissection and stored in 100% RNAlater. Individual DRGs were then homogenized and RNA extracted as per Hill et al. (2010) and the column flow-through preserved for protein analysis (see below). Amplicons for SYBR Green qPCR detection were designed as previously described (Harrison et al., 2014). RNA quality control, reverse transcription, and qPCR were performed according to Hill et al. (2010). Briefly, 250 ng of RNA input was used to generate cDNA using Quantitect RT mastermix (QIAGEN) that contains a mixture of oligo(dT) and random primers to reduce 3' bias. PCRs were performed with 5 ng input cDNA in triplicate using SYBR Green master mix (Roche) and a Corbett Rotorgene thermocycler (QIAGEN).

**Protein assay.** Protein was precipitated from column flow-through using 5 volumes of cold acetone overnight at −20°C. Precipitates were then centrifuged at 12,000 × g for 10 min, the supernatant removed and pellets air dried. Pellets were resuspended with 50 µl chroatic lysis buffer (7 M urea, 2 M thiourea, 1% NP-40) for Western blot analysis.

**Western blot analysis.** Western blot procedures were performed as previously described (Harrison et al., 2008), with the following modifications: 10 µg of DTT-reduced protein/lane was separated in 4%–12% gradient PAGE gels (Invitrogen). Protein was then transferred onto nitrocellulose membranes, before blocking for 1 h in antibody dilution buffer (ABD-PBS containing 0.05% Tween 20 and 5% nonfat milk) before primary antibody incubation at 4°C for 18 h in ABD at the following concentrations: rabbit anti-CD2AP (1:20,000, gift from Andrey Shaw laboratory) (Grunkemeyer et al., 2005), mouse anti-GAPDH (Millipore) (1:2000), rabbit anti-TrkA (Millipore) (1:500), rabbit anti-p85 (1:1000), rabbit anti-phospho-ERK (1:1000), rabbit anti-phospho-AKT (1:1000), rabbit anti-total-ERK (1:1000), and rabbit anti-total-AKT (1:1000), all from Cell Signaling Technology. Membranes were then washed (PBST) before incubation with HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) diluted 1:5000 in ABD at 20°C for 2 h.

Protein was detected with enhanced chemiluminescence and radiographic film according to standard protocols. Radiographs were scanned at 600 dpi for densitometric analysis using ImageJ software. Band densities were measured using imageJ software for background removal (median method) and deconvolution.

**Neurite outgrowth assay: cerebellar granule neurons (CGNs) on inhibitory substrate.** To elucidate transcripts with neurite arbor growth-promoting activity, genes with known intracellular signaling activity were selected from the microarray screen (Harrison et al., 2015). Expression plasmids encoding candidate genes were purchased from the public clone collection (Open Biosystems). Plasmids were electroporated into postnatal CGNs for neurite growth assay on inhibitory substrate (Harrison et al., 2008). Cells were transfected with 0.5 µg of plasmid DNA and 2.5 µl of Lipofectamine-2000 (Invitrogen) per milliliters of culture medium according to the manufacturer’s instructions for 18 h in growth media.

**Analysis of CD2AP expression during PNS and CNS development.** Postnatal day 1, 7, 14, 21, and 35 rats pups (n = 4 each time) were anesthetized, decapitated, and their DRGs and brains removed before rapid freezing by 60% RNAlater. Individual DRGs were then homogenized and RNA extracted as per Hill et al. (2010) and the column flow-through preserved for protein analysis (see below). Amplicons for SYBR Green qPCR detection were designed as previously described (Harrison et al., 2014). RNA quality control, reverse transcription, and qPCR were performed according to Hill et al. (2010). Briefly, 250 ng of RNA input was used to generate cDNA using Quantitect RT mastermix (QIAGEN) that contains a mixture of oligo(dT) and random primers to reduce 3' bias. PCRs were performed with 5 ng input cDNA in triplicate using SYBR Green master mix (Roche) and a Corbett Rotorgene thermocycler (QIAGEN).

**Analysis of CD2AP expression during PNS injury and regeneration.** The nerve crush model was used to induce a robust injury and regeneration response in L4 and L5 DRG. To ensure that the maximum number of L4/L5 DRG neurons were injured and to prevent arbor expansion of any noninjured neurons in the same ganglia (i.e., to provide a “pure” population of injured neurons), the lumbar plexus was crushed just central to the lumbar sympathetic T8 ganglia and L1 dorsal cutaneous nerves (DCNs) were cut and ligated to prevent their regrowth, and the skin closed with surgical staples. The innervated/dermatomized border was determined acutely using the CTMR (Petruska et al., 2014) measured under light pentobarbitals anesthetia (40–50 mg/kg) and clearly marked with permanent ink. Sensory arbor expansion then proceeded from the flanking DCNs (T9 and L2) into the denervated zone for 3 weeks. The migrated border of innervation was again determined before death using the CTMR and clearly marked with permanent ink, thus defining a region of skin that had been denervated but had regained innervation by axonal arbor expansion.

For imaging, an Olympus confocal microscope equipped with a 40× water objective was used to acquire 1024 × 1024 xy slices at 1 µm intervals through the z-plane. Images were then processed in Amira (FEI) software for background removal (median method) and deconvolution.

**PCR12 culture, plasmid/siRNA lipofection and NGF-induced differentiation.** PC12 cells were maintained in RPMI-1640 media supplemented with 10% heat-inactivated horse serum and 5% FBS in suspension.
medium. For siRNA lipofection, 2 μl of DharmaFECT-1 transfection reagent was used with 1.25 μl of 20 μM ON-TARGETplus SMARTpool CD2AP siRNA per milliliters of culture medium according to the manufacturer’s instructions for 18 h in growth medium.

To differentiate PC12 cells, growth media were replaced with RPMI-1640 media supplemented with 1% HHS and 25 μM mouse maxillary gland 2.5% NGF (Sigma) for 48 h.

Coimmunoprecipitation assays. Following transfection with/without NGF stimulation as indicated, growth media were gently removed and plates were snap cooled on ice for 15 s before gentle washing with cold PBS and then scraped into 1.5 ml centrifuge tubes with 1 ml of cold PBS and the cells sedimented at 400 × g for 4 min at 4°C. Cells were then lysed using coimmunoprecipitation buffer (25 mM Tris, pH 8.5, 150 mM NaCl, 1 mM EGTA, 1 mM NaF, 1 mM NaVO₃, 3 mM beta-glycerophosphate, 1% NP40, and 5% glycerol, supplemented with complete protease inhibitor tablets, Roche) for 10 min at 4°C with constant rotation. Lysate was then spun twice at 12,000 × g for 10 min and the pellet discarded to ensure complete removal of insoluble debris. Protein concentration was then determined using the Bradford method according to the kit manufacturer’s instructions (Bio-Rad).

A total of 25 μl of washed protein A/G agarose bead sediment (Pierce); 200 μg of 1 μg/μl protein lysate in coimmunoprecipitation buffer was added, mixed gently, and the required antibody added (CD2AP 1:5000, p85 1:50, TrkA 1:100) before incubation at 4°C for 2 h with constant rotation. Following incubation, spin columns were spun at 200 × g for 30 s and the unbound fraction removed and snap-frozen for analysis. Beads were washed 4 times with 400 μl of coimmunoprecipitation buffer for 1 min at 4°C with constant rotation. Columns were then spun at 200 × g for 30 s and the flow-through discarded. Protein was then eluted by addition of 50 μl Laemmli sample buffer (5% SDS, 0.5 M DTT, 0.04% bromophenol blue, and 0.5 M Tris-HCl, pH 6.8) and heating to 95°C for 5 min before spinning at 10,000 × g for 2 min. Precipitates were analyzed by Western blot as above.

Neurite outgrowth assay-sholl analysis of PC12 cells. PC12 cells were transfected with CD2AP-GFP plasmids or GFP control plasmids and differentiated and fixed using CB, 4% PFA and stained using the immunostaining protocol as above. The following primary antibodies were used: rabbit anti-CD2AP (Andrey Shaw laboratory; 1:10,000), goat anti- TrkA (R&D Systems; 1:100), mouse anti-Rab7, mouse anti-RAB5, and mouse anti-PAKT1 1:50 (all from Cell Signaling Technology). Appropriate secondary antibodies were used conjugated to either Alexa-488, Cy3, or Alexa-647 as indicated.

Growth cones were imaged as described above. Z-stacks were then denoised and deconvolved using Amira (FEI) software and colocalization quantified using the Colocalization Threshold plugin of ImageJ that employs Costes’ method (Costes et al., 2004) for unbiased analysis. Mean colocalization coefficients (Persons’, M1 and M2) per condition were analyzed using ANOVA followed by Fisher’s LSD post hoc t tests.

Results
CD2AP is identified as upregulated during axon growth of noninjured neurons

Arbor expansion was assessed using the CTRM (Petruska et al., 2014) to define the border between innervated skin (T11 dermatome) and denervated skin (Diamond et al., 1992). Arbor expansion restored sensation to the entire denervated area by 28 d (Fig. 1E). Arbor expansion was first significantly detectable at ∼10 d when the sensory innervation border had expanded ∼1 mm (repeated-measures ANOVA, n = 4, post hoc HSD, p = 0.009) (Fig. 1E). We therefore designated the 7 d time point (expansion not significantly increased, p = 0.143) as reflective of an initiation phase. Reinnervation was essentially complete by CTRM testing by 28 d. Therefore, the half-latency from surgery to full-reinnervation (14 d) was considered to reflect the maintenance phase of arbor expansion. To determine the profile of transcriptional response during arbor expansion, microarray expression profiles were generated from DRGs harvested from rats at either 7 d (initiation) or 14 d (maintenance) after surgery. As described in detail previously (Harrison et al., 2015), a threshold of a mean fold change of at least 1.2 with a p value of <0.05 (determined by Limma) revealed 917 differentially expressed probe sets at day 7 versus naive and 1920 differentially expressed probe sets at day 14 versus naive.

For discovery of intracellular arbor expansion–regulating signaling molecules, the largest gene expression changes were considered regardless of what was already known about those genes. One of the expression differences of largest magnitude was CD2A2-associated protein (CD2AP; mean fold change = 3.2, p = 0.021), antibody incubation, filamentous actin (F-actin) was stained using biotin-conjugated phalloidin (Invitrogen) diluted 1:10 in PBS for 20 min at room temperature, washed 3 times for 1 min with PBS and then incubated with Pacific Blue-conjugated streptavidin (Invitrogen; diluted 1:25) in PBS for 20 min at room temperature, washed 3 times for 5 min with PBS before mounting as above.

High-resolution confocal stacks were taken of at least 10 random growth cones from 3 independent coverslips, with a 100× NA1.3 objective using an Olympus confocal microscope at the Nyquist sampling limit. The microscope operator was blinded to experimental conditions by sample coding. To further reduce selection bias, image stacks of solitary cones were collected from fields in a line across the coverslip, viewed at 100×. Image stacks were denoised using the medium local intensity method, and the background subtracted and the image deconvolved using Amira (FEI) software. Maximum intensity projections of each processed stack were then used for analysis using the following method: Using ImageJ software (Schneider et al., 2012), a 20 μm line was traced centered at growth cone tips, 1 μm from the extent of tubulin signals. Line histogram plots of F-actin signals were then used to count the number of filopodia per μm based on the line drawn outside the tubulin-positive border. Mean data per condition were analyzed using ANOVA followed by Fisher’s LSD post hoc t tests.

Endosome imaging and colocalization analysis. PC12 cells were transfected, differentiated, and fixed using 4% PFA in cytoskeletal buffer (see above) without Triton and stained using the immunostaining protocol as above. The following primary antibodies were used: rabbit anti-CD2AP (Andrey Shaw laboratory; 1:10,000), goat anti-TrkA (R&D Systems; 1:100), mouse anti-Rab7, mouse anti-RAB5, and mouse anti-PAKT1 1:50 (all from Cell Signaling Technology). Appropriate secondary antibodies were used conjugated to either Alexa-488, Cy3, or Alexa-647 as indicated.

Growth cones were imaged as described above. Z-stacks were then denoised and deconvolved using Amira (FEI) software and colocalization quantified using the Colocalization Threshold plugin of ImageJ that employs Costes’ method (Costes et al., 2004) for unbiased analysis. Mean colocalization coefficients (Persons’, M1 and M2) per condition were analyzed using ANOVA followed by Fisher’s LSD post hoc t tests.
CD2AP is a cytosolic adaptor protein that serves as a docking site for protein complexes involved in growth factor signaling (Kirsch et al., 1999; Grunkemeyer et al., 2005) and are highly expressed in DRG neurons during development (Lehtonen et al., 2008) (Fig. 2B). Our Western blot assessments supported these previous observations, demonstrating the presence of CD2AP protein in adult naive rodent cerebellum, cerebral cortex, liver, and kidney (Fig. 3A), and demonstrated the utility of the antibody. The Allen Brain Atlas also indicates that CD2AP mRNA is present in adult rat brain in specific regions, notably regions with high constitutive plasticity, such as hippocampus, cortex, and cerebellum (Fig. 3B, C). Immunofluorescence assessment of tissue sections demonstrated CD2AP protein in cerebellar Purkinje cells (Fig. 3D–F) and in the DRG where it appeared to be concentrated predominantly (but not exclusively) in small and medium neurons, and some axons (Fig. 3G). CD2AP protein was also observed in naive adult hairy skin where, although it was observed predominantly in epidermal cells and blood vessel endothelia, it is also observed in axons projecting into the epidermis (Fig. 4). Thus, our protein expression studies show that CD2AP is expressed in many cell types, including in neurons.

**In vitro analyses reveal a role for CD2AP in axon growth**

To determine the relative axon growth-regulating ability of CD2AP versus other genes identified from our microarray analysis (Harrison et al., 2015), we used an established in vitro system with automated analysis of neurite outgrowth from postnatal CGNs seeded on an inhibitory substrate (Hutson et al., 2011). We overexpressed individual target genes as C-terminal GFP-fusion proteins. Overexpression of two plasmids, CD2AP-GFP and KLF7, significantly (ANOVA with post hoc t test) increased average neurite length (Fig. 5A; CD2AP-GFP mean fold change = 1.43 vs GFP control, \( p = 0.023 \); KLF7 mean fold change = 1.61 vs empty vector control, \( p = 3.19 \times 10^{-7} \)). From the microarray data presented in a separate manuscript (Harrison et al., 2015), KLF7 was determined to be significantly upregulated at 14 d (mean fold change = 1.41, \( p = 0.0041 \)). That CD2AP performed similarly to KLF7 is especially encouraging because KLF7 is often used as a positive control for increasing neurite outgrowth and is regulated by NGF in DRG neurons (Lei et al., 2005; Blackmore et al., 2012). Conversely, shRNA-mediated reduction of CD2AP expression resulted in significantly decreased neurite length in accord with the degree of reduction of protein (Fig. 5B) (e.g., shRNA4 = −1.50 mean fold change vs scrambled sequence control, \( p = 0.0033 \)).

Recruitment of intracellular signaling complexes by the CD2AP scaffold is induced by action of multiple growth factors...
CD2AP is expressed in neurons. A, Western blot assessment of CD2AP protein in tissue lysates. CB, Cerebellum; CX, cortex; Liv, liver; Kid, kidney. Allen Brain Atlas demonstrates CD2AP mRNA in cerebellum (B) and hippocampus (C). D–F, CD2AP protein expression in rat cerebellum. Note high expression levels in neurons. G, CD2AP protein in DRG. Note elevated levels of punctate staining in small diameter soma (closed arrowheads) and axons (open arrowheads).

CD2AP is expressed in axons in superficial skin. Whole naive adult mouse dorsal skin was immunostained for CD2AP (green) and neuron-specific tubulin (TUBB3, magenta). Skin was cleared using organic solvent to allow confocal imaging throughout the tissue in whole mount. A–C, Maximum-intensity projection of confocal slices acquired from 10 µm through 30 µm into the tissue from the epidermal surface. A subpopulation of epidermal axons are CD2AP-positive (filled arrowheads). D–F, Maximum-intensity projection of slices from 40 µm through 80 µm from the epidermal surface (therefore dermis). CD2AP is largely undetectable in axons in the dermis (open arrowheads). In merged images: b, Blood vessel; e, epidermis; h, hair follicle.
on different cell types, including EGF and VEGF acting on kidney podocytes and by GDNF acting on cultured sympathetic neurons (Table 1). Ideally, the involvement of CD2AP in arbor expansion-related processes would include assessment in the whole animal where the multitude of neuron–cell interactions is orchestrated. However, germline deletion of CD2AP expression causes critical kidney abnormalities and mice die at an early age (Kim et al., 2003), and conditional and/or inducible knock-out animals are not currently available. Also, we observed that CD2AP expression is decreased by direct axonal injury (Fig. 2A) and also by tissue disruption during culturing of adult neurons (data not shown), precluding the use of adult neuron in vitro preparations. CD2AP was robustly expressed in cultured PC12 cells, and its expression was significantly upregulated (mean fold change = 1.90, n = 5, t test, p = 0.008) by NGF treatment (Fig. 6A), a trophic factor known to induce PC12 cells to extend neurites (Greene and Tischler, 1976). Similarly, sensory axon arborization in vivo is mediated at least partially through NGF signaling (Diamond et al., 1992). We therefore opted to use PC12 cells for mechanistic studies. In agreement with studies performed in CGNs, overexpression of CD2AP significantly increased mean neurite length (mean fold change = 1.32) and complexity in PC12 cells as assessed by Sholl analysis (Fig. 6B–E). These functional studies conducted in vitro supported a role for CD2AP in axon elongation and collateral sprouting.

**CD2AP positively regulates the number of filopodia on PC12 growth cones**

In a variety of cell types, CD2AP locates to structures near the cell membrane where it couples growth factor signaling with the local actin cytoskeleton (Table 1). We therefore sought to determine whether CD2AP plays a similar role in neurons. To determine the subcellular distribution of CD2AP in NGF-stimulated PC12 cells, PC12 cells were triple-stained with phalloidin (which binds to F-actin), and with antisera against CD2AP and tubulin. CD2AP immunostaining was punctate in the soma and neurites, and accumulated at F-actin/tubulin neurite tips, branch points, and swellings (Fig. 7). These observations are similar to those in other cells where CD2AP concentrates into punctae in the cytosol (Cormont et al., 2003) where it regulates actin dynamics during membrane ruffle and lamellipodia formation (Zhao et al., 2013). In PC12 cell homogenates, we determined that endogenous CD2AP protein levels were increased by NGF treatment (Fig. 6A) and that this increase continued and was maintained for at least 72 h with continued NGF treatment (data not shown). We therefore examined the effects of CD2AP concentration on the actin structures in growth cones. PC12 growth cones have multiple filopodia composed of F-actin. Overexpression of a GFP-CD2AP fusion protein caused an ~50% decrease in the number of filopodia (Fisher’s LSD: p = 0.0012), whereas downregulation of CD2AP with siRNA caused a ~50% decrease (p = 0.011) (Fig. 8). Thus, CD2AP is localized at key F-actin structures in neurites and growth cones, CD2AP protein levels are positively regulated by NGF, and in turn positively regulate filopodia formation in PC12 cells.

**CD2AP forms a novel multiprotein complex with TrkA and p85, and is a positive regulator of AKT phosphorylation**

Having established a link between CD2AP concentration and the structure of the actin cytoskeleton in growth cones, we next sought to characterize the involved signaling pathways. NGF is necessary for axonal arbor expansion of at least some neurons in the spared dermatome model of collateral sprouting (Diamond...
CD2AP locates to the growing tips of PC12 neurites. PC12 cells were treated with NGF for 48 h, fixed, and costained to visualize the following: A, CD2AP (green); B, α-tubulin (red); and D, E, F-actin (using phalloidin; blue). CD2AP locates to tubulin+/F-actin+ neurite tips (F, G). *CD2AP-positive neurite tip (growth cone).

PC12 cells and the levels of TrkA were measured. In homogenates from PC12 cells not treated with NGF, CD2AP co-precipitated with low but detectable levels of TrkA. TrkA was detected in CD2AP precipitates from homogenized PC12 cells, which had been treated with NGF for 5 min or 24 h (Fig. 9F). Together, these data demonstrate a novel interaction of CD2AP with TrkA that is rapidly induced by NGF and treated for at least 24 h of continued exposure to NGF.

Having established that a novel interaction occurs between CD2AP and TrkA during NGF-induced signaling, we next sought to determine whether CD2AP might play a role in specifying downstream signaling. CD2AP has been shown to acutely regulate (for up to 30 min) two signaling cascades downstream of growth factor receptors: the PI3K/AKT and MAPK/ERK pathways (Table 1). Interestingly, similar to the temporally biphasic responses known for NGF-induced effects (Petruska and Mendell, 2004), these intracellular signaling cascades are known to be either transient or sustained, with the sustained response phase displaying different characteristics than the transient response. In cases where a sustained response is induced, there is often a physical translocation of signaling components to the soma and often to the nucleus to drive transcriptional responses, including those affecting neurite outgrowth (Marshall, 1995; Limpert et al., 2007). However, a role for CD2AP in chronic signaling, particularly long-distance retrograde signaling, has to our knowledge not been established. Because this form of long-distance sustained signaling could be expected in our model of axonal arbor expansion, we sought to determine whether CD2AP levels could regulate phosphorylation (activation) of PI3K/AKT and MAPK/ERK pathways and localization of signaling complex components following 24 h of NGF stimulation. Reduction of CD2AP protein concentration by siRNA transfection significantly reduced the levels of NGF-induced phospho-AKT by a mean of 2.85-fold (p = 0.049), whereas ERK phosphorylation was not significantly affected (Fig. 10 A, B), suggesting that CD2AP coordinates responses specifically through this signaling cascade/mode in NGF-stimulated PC12 cells.

Having observed that CD2AP regulated signaling via AKT but not via MAPK in NGF-stimulated PC12 cells, we sought to identify possible mechanisms by which NGF signaling might lead to CD2AP-regulated activation of AKT. A principal activator of AKT is PI3K (through 3-phosphoinositide-dependent protein kinase 1). PI3K is the PI3K regulatory subunit of PI3K binds directly to the cytosolic domain of growth factor receptors via phosphotyrosine-SH2-domain interactions (Domchek et al., 1992), resulting in PI3K activation. PI3K then phosphorylates 3-phosphoinositide-dependent protein kinase 1, which in turn phosphorylates and activates AKT. Also, p85 directly interacts with the N-terminal SH3-domain-containing region of CD2AP (Huber et al., 2003). We therefore hy-
pothesized that, following NGF stimulation, CD2AP positively regulates the AKT pathway by interacting with p85 and recruiting p85 to TrkA in a multiprotein complex. We tested this by altering the levels of CD2AP in NGF-stimulated PC12 cells and measuring the levels of p85 and TrkA in immunoprecipitated protein complexes. In accord with our hypothesis, siRNA-mediated reduction of CD2AP concentration resulted in a qualitative reduction in levels of p85 in TrkA-containing protein complexes (Fig. 10C). As an indication of the specificity of the interaction, the same effect was observed when assessing the level of TrkA in p85-containing complexes. As a further measure of this interaction, we quantified the spatial colocalization of p85 and TrkA in PC12 cell neurites. Confocal microscopy revealed that siRNA-mediated reduction of CD2AP concentration resulted in a significant reduction in colocalization of p85 and TrkA in growth cones by mean 18% (p = 0.034) (Fig. 10D–P). In addition, overexpression of CD2AP via transfection with a plasmid for GFP-CD2AP fusion protein resulted in a significantly increased colocalization of p85 and TrkA by mean 45%. These data suggest that CD2AP scaffold protein supports the recruitment of p85 to the TrkA receptor in neurites upon NGF stimulation.

Figure 8. CD2AP is a positive regulator of the number of filopodia on growth cones. PC12 cells were transfected with GFP (A–C), GFP-CD2AP fusion protein (D–F), control siRNA (G–I), or CD2AP siRNA (J–L) for 24 h before NGF treatment for 48 h. Cells were fixed and stained to visualize F-actin (using phalloidin; red; C, F, I, L), α-tubulin (blue; B, E, H, K), and either GFP (green; A, D) or CD2AP (green; G, J). Growth cones were imaged by confocal microscopy using 100× objective with 5× digital zoom. Images are representative deconvolved maximum-intensity projections. Line histograms were generated for lines exactly 20 μm in length applied exactly 1 μm outside of the tubulin-positive boundary (gray lines). Registration marks (yellow dots) indicate how the histogram relates to the line (x axes = distance, y axes = log of actin fluorescence intensity). M, Mean number of filopodia per condition. *Significant difference (t test). Error bars indicate SEM.
CD2AP is associated with RAB5-bound endosomes and positively regulates TrkA location to endosomes

Addressing mechanisms of long-range signaling is especially pertinent to sensory neurons whose fibers can reach meters in length in large animals. Biochemical signaling over long distances requires the signaling complex to be highly compartmentalized, beginning at cell membrane lipid rafts, before being packaged into concentrated foci for transport, usually in signaling endosomes (SEs). CD2AP has been observed associated with endosomes in non-neural cells (Table 1), and our microscopy data indicate a subcellular distribution of CD2AP in DRG neurons and axons (Fig. 3G) and in PC12 cells (Fig. 7) that could reasonably include endosomes. We therefore sought to determine whether CD2AP was associated with SEs present in growth cones. Consistent with observations in other cell types (Table 1), CD2AP colocalizes with early endosomes, here labeled with RAB5, in PC12 cell growth cones (representative growth cone shown in Fig. 11A–C). Long-range NGF signaling in sensory neurons is regulated, at least in part, by retrograde transport of NGF bound to TrkA in RAB5-decorated endosomes (Deinhardt et al., 2006). Given our observation of CD2AP colocalization with RAB5, we also sought to determine whether CD2AP levels might affect TrkA compartmentalization in endosomes. Following transfection of NGF-stimulated PC12 cells with CD2AP siRNA, TrkA:RAB5 colocalization was significantly (p = 0.021) reduced by mean 21% (Fig. 11D–H). Following transfection of NGF-stimulated PC12 cells with GFP-CD2AP, TrkA:RAB5 colocalization was increased (p = 0.043) by mean 20% (Fig. 11D–H). Together, these data suggest that CD2AP is a positive regulator of TrkA endocytosis and translocation into endosomes.

Considered together, our observations suggest that CD2AP acts as a positive regulator of NGF signaling through endocytosis of TrkA and subsequent activation of the AKT pathway. It was reasonable to consider that, during axonal arbor expansion into denervated skin, CD2AP protein may be involved in NGF signaling in axons innervating the target tissue. We therefore examined skin that had been denervated and reinnervated by collateral sprouting of noninjured axons, stained for CD2AP and neuron-specific tubulin (TUBB3). In support of this consideration, CD2AP was present in axons that had reinnervated formerly denervated skin (Fig. 12).

Discussion

Despite its fundamental importance for adult nervous system homeostasis, response to injury, and disease etiology, the molecular control of axonal structural plasticity in adult noninjured neurons is largely unknown. Screening revealed candidate genes, including some known to play a role in regulating intracellular signaling. These are of particular interest because NGF is the only known signaling factor clearly regulating collateral sprouting in the spared dermatome model (e.g., Diamond et al., 1992; but see Maysinger et al., 2008), but NGF has roles in stimulating numerous forms of plasticity (Petruska and Mendell, 2004). Understanding the principles at work in the various forms of NGF-induced plasticity and realizing the therapeutic potential of manipulating these forms of plasticity requires a dissection of which specific pathways are involved with each. Because there are many downstream effects of NGF signaling, it is likely that there are factors that specify which subsets of the numerous pathways are activated. For example, NGF rapidly induces sensitization of sensory neurons (e.g., Cheng and Ji, 2008), but sensitization may not occur during NGF-dependent collateral sprouting (Pertens et al., 1999).

Our transcriptomic screen (Harrison et al., 2015) revealed CD2AP as highly regulated during collateral sprouting. CD2AP associates with multiple growth factor receptors and directs downstream signaling (Table 1). Here we demonstrate a novel interaction with the NGF receptor, TrkA.

Given the clear transcriptional response induced in the DRG during arbor expansion in the spared dermatome model (Harrison et al., 2015), which is dependent at least in part on NGF, it is reasonable to consider a role for long-distance retrograde signaling mechanisms involving NGF:TrkA:CD2AP. CD2AP links receptor signaling components with the endocytosis machinery in non-neuronal cells (Lynch et al., 2003). We observed that CD2AP colocalizes with the early endosomes in PC12 cells and that increasing levels of CD2AP concomitantly increases the concentration of TrkA associated with these endosomes. Upon NGF:TrkA

stimulation of PC12 cells, supporting the hypothesis that CD2AP is a positive regulator of the AKT pathway during neurite structural plasticity.
binding and endocytosis, TrkA-positive endosomes have multiple fates. They may be recycled to the lysosome for degradation or sent back to the membrane for continued local signaling. In the latter, receptor complex turnover by endocytosis can result in local elevations of signaling cascades within growth-structures, thereby enhancing outgrowth (e.g., Georgieva et al., 2011).

Additionally, endosomes can function as mobile rafts from which signaling continues (SEs) during long-range retrograde transport to the soma, and potentially into the nucleus (for review, see Matusica and Coulson, 2014). Our initial qualitative observations of CD2AP puncta in cell bodies and processes suggested that CD2AP may be associated with endosomes in neurons, perhaps even SEs. In PC12 cells and in neurons, a pool of TrkA-containing SEs destined for long-range signaling are initially RAB5-decorated and sorted to RAB7-positive late endosomes for retrograde transport (Matusica and Coulson, 2014). CD2AP may act to influence this sorting through its interactions with RAB GTP-ases (e.g., Rab4) (Cormont et al., 2003), a large family of multifunctional proteins involved in cytoskeleton-associated membrane plasticity (Stenmark, 2009). We demonstrate a novel association between CD2AP and early endosomes as defined by RAB5. Previous studies report no colocalization of CD2AP with RAB5 in non-neuronal cells (Welsch et al., 2005), suggesting a neuron-specific (or PC12 cell-specific) role for CD2AP.

Figure 10. CD2AP is a positive regulator of AKT phosphorylation and increases p85 recruitment to TrkA complexes. A, Western blot assay of protein samples from NGF-treated PC12 cells assessed for phospho-AKT (pAKT), total-AKT (tAKT), phospho-ERK (pERK), total-ERK (tERK), and loading control (GAPDH) following transfection with scramble control (Sc) or CD2AP siRNA. B, Densitometric quantification of pAKT and pERK, relative to tAKT and tERK, respectively. C, Western blot assay of p85 and TrkA immunoprecipitates following transfection with scramble control (Sc) or CD2AP (C) siRNA. CD2AP, TrkA, and p85 protein concentrations are shown in input (In) and bound fractions. D, Quantification of TrkA:p85 colocalization in growth cones following transfection with GFP (E–G), GFP-CD2AP (H–J), scramble control (K–M), or CD2AP siRNA (N–P). Representative images from the 3D colocalization analyses are shown for each condition. *Statistically significant changes compared with the respective control (ANOVA, followed by t test compared with control).
with RAB5-decorated endosomes. Our data, in conjunction with the literature, suggest that CD2AP may participate in directing intracellular signaling local to NGF:TrkA-binding, and also the sustained long-distance signaling mechanism, at least in PC12 cells, where CD2AP is a positive regulator of NGF signaling (Fig. 13).

Although it remains to be determined whether other trophic factors can regulate axonal arbor expansion in the spared dermome model, it is known that NGF is required, but the intracellular mechanisms leading to axon growth are unclear. We have determined that CD2AP is a positive regulator of NGF-stimulated PI3K/AKT signaling and associates with complexes containing the NGF receptor TrkA, and the p85 regulatory subunit of PI3K. Previous work shows that N-terminal SH3 domains of CD2AP can form complexes with p85 (Huber et al., 2003).
TrkA also forms complexes with p85, but to our knowledge the nature of the p85:TrkA interaction is not well defined and may occur via an undefined adaptor protein (Vanhaesebroeck and Waterfield, 1999). Following NGF stimulation, p85 association with TrkA is regulated by levels of CD2AP (Fig. 10), suggesting that CD2AP could be an unidentified adaptor mediating p85:TrkA interaction (Fig. 13).

CD2AP regulates both AKT and MAPK/ERK signaling in kidney podocytes (Table 1). Interestingly, we did not observe any regulatory effects of CD2AP levels on ERK signaling after 24 h of NGF stimulation. In many settings, the NGF-induced ERK and AKT cascades serve nonredundant functions. For example, in cultured DRG neurons, RAF overexpression (stimulating ERK) leads to production of elongated thin neurites, whereas AKT activity increases axon branching (Markus et al., 2002). In our model, NGF signaling may be directed by CD2AP to activate the AKT pathway that may drive axon branch formation during axon expansion. Accordingly, NGF promotes filopodia formation and axonal branching in a PI3K-dependent manner (Ketschek and Gallo, 2010) by inducing local actin nucleation by the Arp2/3 complex (Spillane et al., 2011). Independently, CD2AP has been shown to directly bind Arp2/3 to positively regulate actin nucleation and concomitant actin dynamics (Table 1). Also, CD2AP directly binds to Septin-7 and is a positive regulator of its assembly into filaments (Wasik et al., 2012), a process known to trigger filopodia formation during axonal branching (Hu et al., 2012). Our data therefore suggest a role for CD2AP in driving trophic-factor-mediated actin dynamics, possibly via the AKT pathway, during axonal plasticity (Fig. 13).

AKT phosphorylates a diverse array of substrates, and the vast majority of evidence strongly suggests that it is a positive regulator of neurite growth (Read and Gorman, 2009; Miyamoto et al., 2013). It could occur, for example, by negative regulation of GSK3β or activation of mTOR (for review, see Read and Gorman, 2009). However, the downstream components linking AKT to axon growth are not well defined and may be context dependent. Interestingly, the only intracellular molecule identified as having a role in NGF-dependent collateral sprouting in the spared dermatome model, the intermediate filament protein peryrin (Belecky-Adams et al., 2003), is itself an AKT substrate (Konishi et al., 2007).

Targeting signaling regulators, such as CD2AP, may enable a more refined approach to therapies involving growth factor signaling than bulk approaches, such as antibody-based ligand sequestration. This strategy could provide much needed therapeutic interventions for the many disease states known to involve axonal plasticity: sensory neuropathies (Khan and Smith, 2015), autonomic dysreflexia after spinal cord injury (Rabchevsky, 2006), neuropathic pain (Khan and Smith, 2015), sudden cardiac death (Ieda and Fukuda, 2009), etc. CD2AP mutations, including one inducing an amino acid change in its coiled-coil domain (Vardarajan et al., 2015), are associated with late-onset Alzheimer’s disease (Hollingworth et al., 2011; Naj et al., 2011). CD2AP can affect Aβ protein in neuroblastoma cells (Liao et al., 2015) and tau toxicity in Drosophila eyes (Shulman et al., 2014). This is particularly compelling considering that dysfunction of NGF signaling, axonal transport, and neural plasticity (Schindowski et al., 2008; Houeland et al., 2010; Niewiadomska et al., 2011) all contribute strongly to Alzheimer’s disease. As succinctly stated by Cattaneo and Calissano (2012), “... a new NGF hypothesis can be built, with neurotrophic deficits of various types representing an upstream driver of the core AD triad pathology... therapies aimed at reestablishing a correct homeostatic balance...” Interestingly, and perhaps very importantly, we observe that CD2AP is upregulated in DRGs during axonal sprouting (spared dermatome model) but downregulated during axonal regeneration. This difference highlights an important fact: the degree of similarity/difference in the intracellular molecular control of regeneration of injured axons and the collateral sprouting of non-injured axons remains an open question. This is understandable given the fact that genes regulating collateral sprouting are undoubtedly represented (although perhaps less prominently) in screens using models with mixed populations.

Many studies of adult axon growth regulators use cultured neurons, which are injured by the dissociation process. Although these models are enriched in that they include only injured neurons, they are limited for being in vitro. Others use nerve-injury models, predominantly sciatic nerve injury where the lumbar DRGs are then sampled for gene expression (Costigan et al., 2002; Stam et al., 2007; Mar et al., 2014). The DRGs contain both injured and uninjured neurons (e.g., neurons projecting into dorsal cutaneous nerves and the PBST muscle nerves). Still others use CNS conditions where neurons undergo axon growth but the...
injury status of the neurons is unclear (Li et al., 2010). Although successful for identifying regeneration-associated genes, mixed models are of unclear value for identifying genes regulating collateral sprouting. The magnitude of gene regulation appears much lower for collateral sprouting compared with injury/regeneration (Harrison et al., 2015). Thus, in models with mixed populations, the large changes in injured/regenerating neurons could easily overshadow changes in noninjured neurons. However, the genes expressed in those noninjured neurons would nonetheless be classified as “regeneration-associated genes” for their mere presence in the dataset. For this reason, it was vital to use a model with minimal contributions from injured/regenerating neurons as we did with the spared dermatome model, which led to our identification of CD2AP.

References


