Identification and characterization of splice variants of ephrin-A3 and ephrin-A5

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Abstract Ephrins and Eph receptors have been implicated to play important roles in axon guidance. A variable spacer region exists that differs significantly among distinct ephrins. An ephrin-A5 isoform has previously been isolated which lacks 27 amino acids within the spacer region. The expression and biological activities of this isoform, as well as the existence of isoforms for other ephrins that show variation within the spacer region, remain unknown. We report here a novel alternatively spliced isoform of ephrin-A3 which lacks the corresponding variable region. When compared to the longer isoforms, the shorter isoforms of both ephrin-A3 and ephrin-A5 remained less prominent in the brain during development, though their expression increased at postnatal stages. In addition, they could inhibit neurite outgrowth of dorsal root ganglia (DRG) neurons, suggesting that the corresponding variable regions were not essential for their axon guidance activities.

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Key words: Ephrin; Eph receptor; Ephrin isoform; Axon guidance

1. Introduction

Eph receptor interacting proteins (Ephrins) have been demonstrated to act as positional cues in axon guidance and embryonic segmentation [1,2]. They are membrane-bound proteins and the membrane anchorage is essential for their activities [3]. Ephrins are classified into two classes based on their mode of attachment: those that anchor to the membrane by glycosylphosphatidylinositol (GPI) linkage are classified as ephrin-A, while ephrin-B ligands contain a transmembrane domain. Both classes of ephrins mediate their activities through interactions with the Eph receptor tyrosine kinases. Binding studies reveal that ephrin-A ligands interact selectively with EphA receptors while ephrin-B ligands bind to the EphB receptors and there is little cross-reactivity between members of the different classes [4]. However, a high degree of promiscuous binding is exhibited among different members of the same class, though there are wide variations in the binding affinities [4]. The binding promiscuity suggests certain degrees of functional redundancy among different ephrins and their receptors. The repulsive guidance activity of ephrins was first demonstrated in the retinotectal system [5–7]. Subsequently, ephrins were found to be involved in guidance of other neurons in both CNS and PNS [8–13].

There are eight ephrins isolated thus far. They share 30–70% amino acid identity in their core regions, where four conserved cysteine residues are present [2]. Moreover, there is a conserved gene structure for different ephrins, i.e. the gene is encoded by four or five exons with conserved intron/exon structure [14]. However, a spacer region in the carboxyl terminal half differs significantly in both the length and amino acid composition among different ephrins [15]. Isoforms for two of the ephrins had previously been identified. An alternatively spliced isoform of ephrin-B2 was reported in which a conserved region of 31 amino acid residues including the fourth invariable cysteine was deleted [16]. On the other hand, an ephrin-A5 isoform which lacked 27 amino acid residues within the spacer region had been identified [17]. However, little was known about the expression and function of these isoforms. In addition, the existence of isoforms for other ephrins had not been reported.

Our studies aim to characterize the short isoform of ephrin-A5 and to look for isoforms for other ephrins. Using RT-PCR, we cloned a novel isoform of ephrin-A3, which lacked 26 amino acids within the spacer region. Interestingly, the deletion that gave rise to the shorter isoforms of ephrin-A3 and ephrin-A5 occurred at the corresponding region. The relative expression of the short isoforms of both ephrins increased in the brain during later stages of development. Furthermore, both isoforms inhibited neurite outgrowth of dorsal root ganglia (DRG), suggesting that the deleted variable regions are not required for their repulsive guidance activities.

2. Materials and methods

2.1. Cloning of rat ephrin-A3 and ephrin-A5, and the construction of Fc-tagged fusion proteins

The short isoform of ephrin-A3 was first identified by RT-PCR from adult rat brain RNA using a pair of primers flanking the position where deletion of ephrin-A5 occurred (5′-GTACGTGCTGTACATGG and 5′-CTAGGAGGCCAAGAACGTC). The full-length rat ephrin-A3 was subsequently amplified from adult brain cDNA using Vent polymerase by primers derived from human ephrin-A3 that contained the starting methionine and the stop codon: 5′-GGGGATGGCGGCGGCTC and 5′-CTAGGAGGCCAAGAACGTC. On the other hand, the short isoform of ephrin-A3 was made by deleting the variable region from the full-length construct, using Vent polymerase with internal primers corresponding to the deleted region, as previously described [18]. The full-length ephrin-A5 and its shorter isoform were amplified from rat astrocyte cDNA by Vent polymerase, using the primers 5′-GCTAGGCGTGATGTTGC and 5′-GCTATAATGTCAAAAGCATCGCCAG. All the full-length constructs were subcloned into the expression vector pCDNA3neo. Fusion proteins of ephrin-A3 and ephrin-A5, in which their C-termini were tagged with the Fc-epitope, were made by amplifying the corresponding fragments up to the region near their hydrophobic ends using Vent polymerase. The fragments were subsequently fused with the Fc portion of human IgG in the expression vector pMT21 as previously described [18]. The identities of all the full-length constructs were verified by DNA sequencing.

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2.2. Northern blotting and RT-PCR analysis of ephrin expression

Total RNA was collected from different tissues, and 15 mg of each was electrophoresed on a 1% agarose-formaldehyde gel and transferred onto nylon membrane. For RT-PCR, total RNA was reverse transcribed using Superscript RT (Gibco, NY, USA) and PCR was performed by primers flanking the variable regions of the two ephrins (for ephrin-A3, they were the ones used in the identification of the short isoform mentioned above; for ephrin-A5, the primers were 5′-GGACCGCTGAAGTTCTCG and 5′-GCTATAATGTCAAAAG-CATCGCCAG). Twenty-five cycles of amplification were employed (except for the expression of ephrin-A3 and ephrin-A5 in muscle and liver, in which 30 cycles were performed). The PCR products were analyzed by 1.2% agarose gel and Southern blotting. Both the Southern and Northern blots were hybridized with probes that correspond to full-length rat ephrin-A3 and ephrin-A5.

2.3. Binding assay of Fc-tagged ephrins

In our study, the interactions between the receptor EphA3 and the isoforms of ephrin-A3 and ephrin-A5 were determined. Full-length EphA3 was amplified from E16 rat brain RNA by RT-PCR using Vent polymerase and was subsequently subcloned into pCDNA3neo. To construct stable 293 fibroblast line expressing EphA3, the cells were transfected by lipofectamine using the protocol recommended by the supplier (Gibco, NY, USA), followed by selection in medium containing 600 μg/ml G418. Stable clones were isolated and their expression of EphA3 receptor was screened by staining with Cos cell supernatant containing Fc-tagged ephrin-A5, performed as previously described [4]. Fc-tagged ephrins were produced by transfecting the DNA constructs into Cos-5 cells using DEAE-dextran as previously described [18]. Three days after transfection, the conditioned medium was collected. The amount of different Fc-tagged proteins were quantified by ELISA using HRP-anti-human Fc antibody (Amersham, UK).

The binding assay was performed as previously described [4]. Briefly, EphA3-expressing 293 cells (7×10^6) were plated 2 days before assay. They were then incubated with conditioned medium that contained equalized amount of Fc-tagged ephrin-A3 or ephrin-A5 isoforms. An unrelated protein, Fc-tagged neurotrophin-7, acted as the negative control in the binding assay. After washing, the cells were incubated with anti-human antibody conjugated with alkaline phosphatase (Promega, WI, USA). The cells were then washed with the phosphatase inhibitor levamisole and the cell lysate was collected. The enzyme activity was assayed by using PNPP as the substrate and the absorbance at 405 nm was measured. Since 293 fibroblasts expressed a low level of endogenous EphA receptors, as revealed by staining with Fc-tagged ephrin-A5, the absorbance values obtained by mock-transfected 293 were subtracted for each Fc-tagged protein in order to assess their interactions with EphA3 receptors.

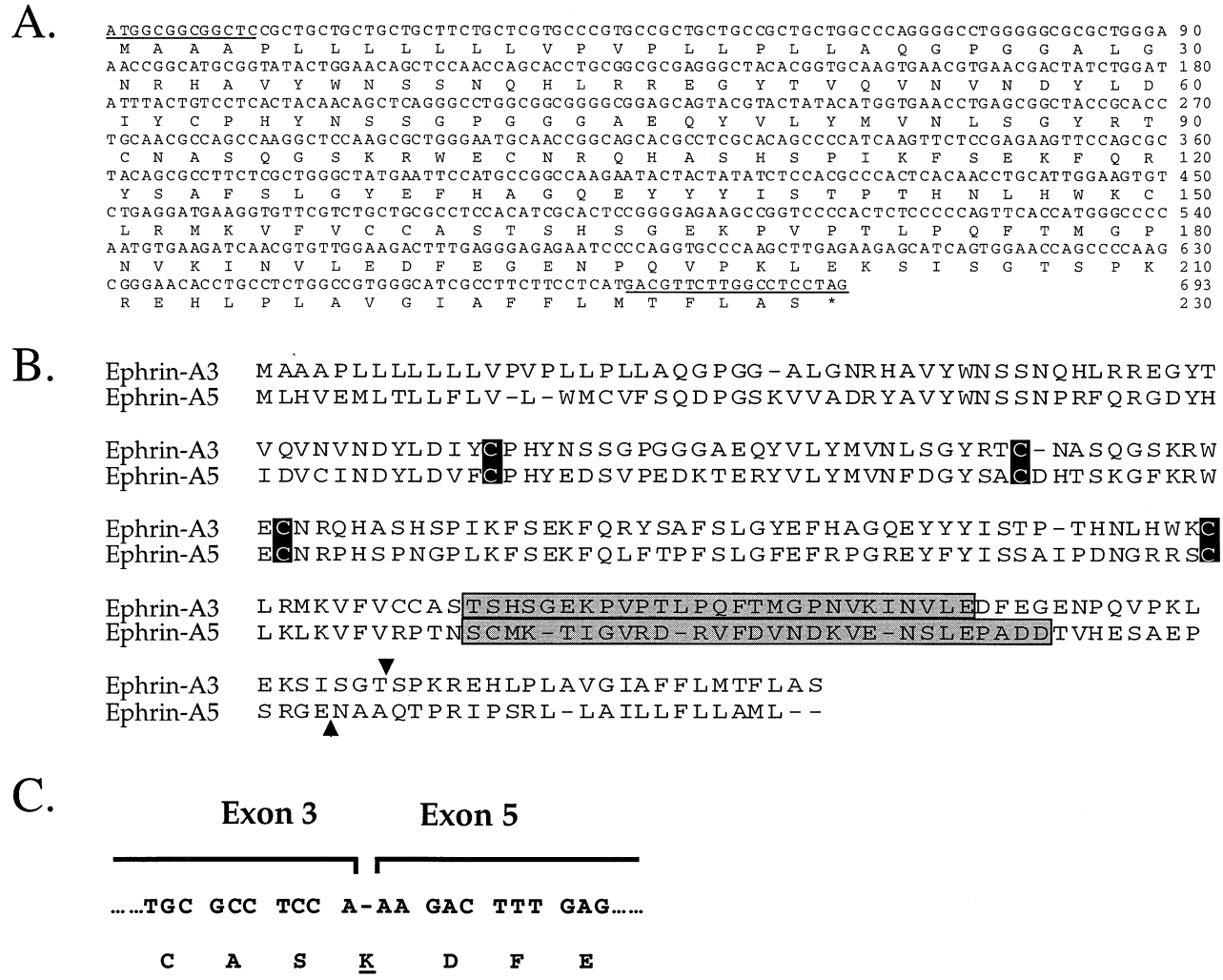

Fig. 1. Sequence of rat ephrin-A3 and its alignment with ephrin-A5. A: Nucleotide and the deduced amino acid sequences of rat ephrin-A3. The full-length sequence was amplified by PCR using primers derived from human ephrin-A3 (underlined). B: Alignment of ephrin-A3 with rat ephrin-A5. The four conserved cysteine residues (dark) and their corresponding regions deleted in the shorter isoforms (shaded) were indicated. Arrowheads represented the positions where the Fc region of human IgG was fused to the ligands for binding assay (see below). C: Alternative splicing of exon 4 resulted in the substitution of ^{188}Glu by Lys.

2.4. Co-culture of DRG explants with stable 293 cells that expressed different ephrin-A3 and ephrin-A5 isoforms

Stable lines of 293 fibroblasts were made by transfecting the cells with each isoform of ephrin-A3 and ephrin-A5 encoded in pCNA3neo (or the vector alone for the mock-transfected stable clones). The positive clones were selected by staining with Fc-tagged EphA5 at a concentration of 1 mg/ml. The expression of specific ephrin isoform by each individual clone was confirmed by RT-PCR using the primers that flanked the variable regions mentioned above (data not shown). For co-culture with chicken DRG explants, stable 293 (1×10^6) were plated 1 day prior to co-culture. DRG from embryonic day 8 chickens were placed onto the fibroblasts and neurite outgrowth was induced by nerve growth factor at 1 ng/ml. After 2 days incubation, the co-culture was fixed and immunostained with anti-neurofilament 160-kDa antibody (clone NN18, Sigma, MO, USA) diluted at 1:200. Two different clones of stable 293 for each ephrin isoform and the mock-transfected control were analyzed in the co-culture experiments.

3. Results and discussion

3.1. Cloning of rat ephrin-A3 and its isoform

The cDNA and protein sequences of rat ephrin-A3 had not been reported. In this study, the full-length ephrin-A3 was isolated from adult rat brain cDNA (Fig. 1A). While it shared 97% amino acid identity to human ephrin-A3 [3,15], it was 100% identical to the partial sequence of the mouse homolog [14]. A novel isoform of ephrin-A3 was identified from adult brain by RT-PCR. It lacked the highly variable 26 amino acid residues within the spacer region and the ^{188}Glu was substituted by a Lys residue (Fig. 1B, C). The deleted region corresponded precisely to the sequence encoded by exon 4 [14], indicating that the isoform was likely to be derived from alternative splicing. Interestingly, the deleted sequence corresponded to the region that was spliced to generate the ephrin-A5 isoform reported previously [17]. However, using the same approach, we were not able to isolate isoforms for other ephrins that contained insertion or deletion at the corresponding position. The longer isoforms of both ephrins that contained the variable regions were designated the α-isoforms, while their shorter isoforms were named as the β-isoforms.

3.2. Expression of the α- and β-isoforms

The expression profiles of individual isoforms of both ephrins were examined by RT-PCR. Both isoforms of ephrin-A3 and ephrin-A5 were expressed in the brain and their expression was developmentally regulated (Fig. 2A). Interestingly, while the β-isoforms remained less prominent throughout development, their relative expression increased considerably at postnatal stages of the brain. Moreover, both isoforms were found in different brain regions in adults, though the relative expression of the α- and β-isoforms appeared to be similar in distinct brain areas (Fig. 2B). Thus, the β-isoforms of ephrin-A3 and ephrin-A5, despite their lower abundance compared with the α-isoforms, are expressed early in development when precise axonal connections are being made, suggesting their potential involvement in axon guidance. In addition, the increased expression of the β-isoforms at late embryonic and early postnatal stages raised the possibility that the β-isoforms might perform specific functions in the brain.

The relative expression of ephrin isoforms outside the nervous system was also examined. Both isoforms were expressed in muscle and liver and the β-isoforms were less abundant (Fig. 2C). It was noteworthy that the expression of both ephrin-A3 and ephrin-A5 was drastically down-regulated in muscle during development. Ephrins had been implicated to

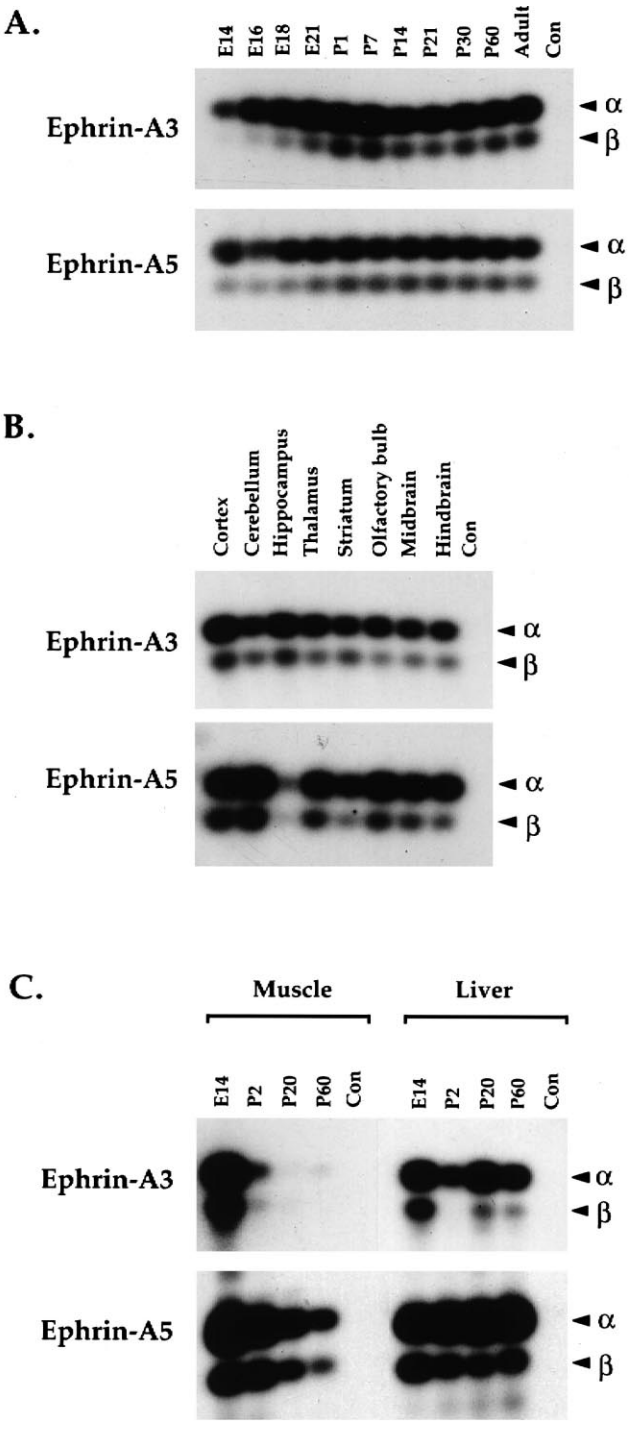

Fig. 2. Expression of ephrin isoforms as revealed by RT-PCR. A: Expression of isoforms at different developmental stages of the brain. The expression of the shorter β-isoforms of both ephrins increased at later developmental stages, though they remained less prominent compared with their respective long isoforms (α-isoforms). B: Both isoforms were expressed in different brain areas in adult rat. C: Both isoforms were expressed in muscle and liver, but the β-isoforms were less prominent. Down-regulation of both ligands was observed during muscle development. E14 to E21, from embryonic day 14 to 21. P1 to P60, from postnatal day 1 to 60. Con represented the negative control in which no RNA was added in the RT reaction (same result was obtained for the negative control without reverse transcriptase; data not shown).

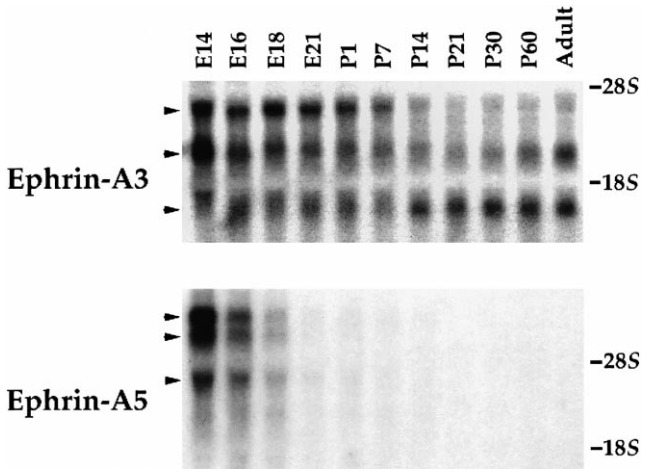

Fig. 3. Northern blot analysis of ephrin-A3 and ephrin-A5 in muscle during development. Multiple transcripts were observed for both ligands (arrowheads). Both ephrin-A3 and ephrin-A5 were relatively abundant at embryonic stages but the expression decreased along muscle development. The positions of the ribosomal RNA were indicated (18S and 28S).

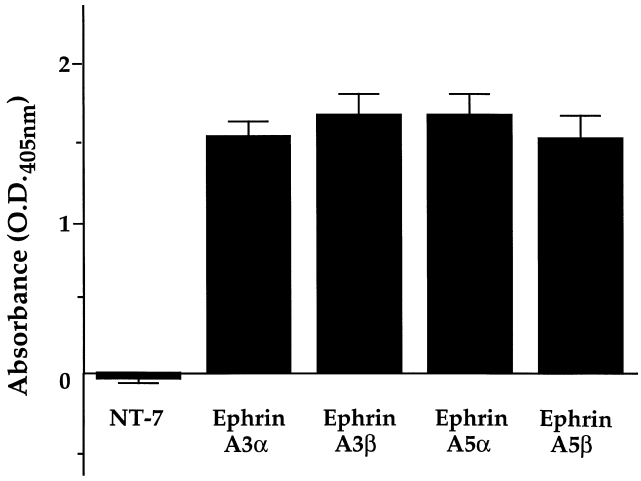

Fig. 4. Binding assay of ephrin isoforms to EphA3. Fc-tagged ephrin was added to stable 293 fibroblasts that over-expressed EphA3 and the absorbance represented the amount of ephrin-Fc fusion proteins bound to the EphA3-expressing cells. The negative value for NT-7, an unrelated Fc-fusion protein which acted as the negative control was obtained as a result of subtraction from the absorbance value of mock-transfected 293 cells. The differences in absorbance between the two ephrins and their isoforms were not significant ($P > 0.005$). The error bars represent S.D., $n = 5$.

confer rostral/caudal identity of skeletal muscles [13], yet the developmental expression profile of any ephrin in muscle remained unknown. We therefore attempted to examine the developmental regulation of ephrin-A3 and ephrin-A5 expression in muscle by Northern blot analysis (Fig. 3). Multiple transcripts for both ephrins were detected, in contrast to the single transcript observed for the other ligands [19–22]. Consistent with the RT-PCR analysis, down-regulation of ephrin-A3 and ephrin-A5 expression was observed. Indeed, both ligands were abundant in embryonic skeletal muscle, which was not revealed by previous reports that examined the spatial expression of ephrins in adult tissues. The high expression of ephrins in embryonic muscle supports their proposed roles in guiding sensory and motor neurons [13,23].

3.3. Binding of ephrin isoforms to EphA3

Binding assays were performed in order to test whether splicing of the corresponding variable regions from ephrin-A3 and ephrin-A5 would disrupt binding to their cognate Eph receptors. Each ephrin isoform was tagged with the Fc region of human IgG, and its binding to fibroblasts that over-expressed EphA3 was examined. The binding of EphA3 to the

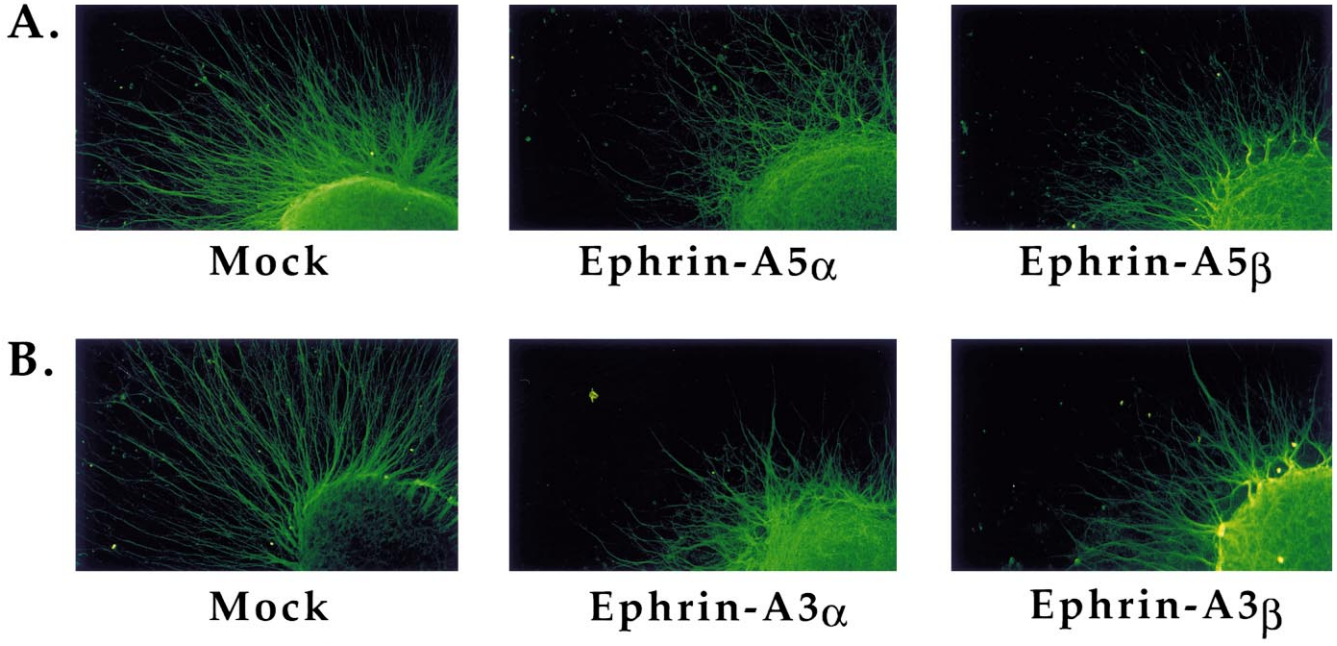

Fig. 5. Inhibition of neurite extension by ephrin-A3 and ephrin-A5. A: Stable 293 fibroblasts that expressed either the vector alone (mock) or individual isoform of ephrin-A5 were co-cultured with DRG explants. Both the α- and β-isoforms of ephrin-A5 could inhibit neurite extension from DRG. B: Similar approach demonstrated that both isoforms of ephrin-A3 inhibited neurite extension by DRG.

α- and β-isoform of both ephrins was similar, suggesting that their respective variable regions deleted in the β-isoforms were not essential in binding to the receptor (Fig. 4). In addition, EphA3 could be phosphorylated upon stimulation with the β-isoform of both ephrins (data not shown). These observations agree with the proposed structure-function model in which the more conserved N-terminal half of the molecule that contained the four invariant cysteine residues is involved in receptor binding, while the variable spacer region is not [15].

3.4. Repulsive guidance activities of ephrin isoforms

Although the variable regions were not involved in receptor binding, they might be important in mediating the repulsive guidance activities of ephrins. Ephrin-A5, when expressed in fibroblasts, had been demonstrated to inhibit neurite outgrowth of DRG explants [13], which was reminiscent of the repulsive guidance activities. In our study, DRG explants were placed onto a confluent layer of stable 293 fibroblasts that expressed the individual isoform of either ephrin and the resulting neurite outgrowth was observed. Both the α- and β-isoforms of ephrin-A5 inhibited neurite outgrowth of DRG, and the extent of inhibition was similar (Fig. 5A).

Despite its prominent expression in the nervous system, there was no evidence that ephrin-A3 can guide axon by repulsion. When DRG were co-cultured with stable 293 fibroblasts that expressed ephrin-A3, the neurite extension was inhibited (Fig. 5B). Therefore, our finding raised the possibility that ephrin-A3 might also act as a repulsive guidance cue for neurons. In addition, both the α- and β-isoforms could inhibit neurite extension (Fig. 5B).

The presence of both the α- and β-isoforms in developing brain and muscle, together with the findings that both isoforms are able to inhibit neurite outgrowth in vitro, suggested that they may co-operate in the development of topographic mapping. It is likely that both isoforms guide axons by repulsion. Although the two isoforms were indistinguishable in the binding and neurite inhibition assays, they might exhibit distinct receptor specificities. Alternatively, differential signal transduction pathways might be induced by the two isoforms. It was reported that the extent of ephrin oligomerization could influence the formation of signaling complexes and the subsequent cellular responses [24]. It remained to be seen whether the two isoforms might differentially activate the Eph receptors in a similar manner. The possibility of distinct receptor specificities as well as signaling pathways would represent a mechanism to increase the precision of topographic mapping.

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