Sustained *In Vivo* Inhibition of Protein Domains Using Single-Chain Fv Recombinant Antibodies and Its Application to Dissect RGMa Activity on Axonal Outgrowth

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Antibodies are powerful tools for delineating the specific function of protein domains, yet several limitations restrict their *in vivo* applicability. Here we present a new method to obtain sustained *in vivo* inhibition of specific protein domains using recombinant antibodies. We show that long term *in vivo* expression of single-chain Fv (scFv) fragments in the developing CNS can be achieved through retroviral transduction. Moreover, specific scFvs generated against the N- and C-terminal domains of the repulsive guidance molecule, RGMa, prevent proper axon targeting in the visual system. This work reveals a previously unappreciated role for the RGMa N-terminal domain in axon guidance, and provides a novel, broadly applicable and rapid procedure to functionally antagonize any protein domain *in vivo*.

Key words: repulsive guidance molecule; developing visual pathway; recombinant antibodies; scFvs; protein domains; axon guidance

Introduction

While considerable progress has been made in developing technologies that allow the elucidation of the activity of whole proteins (e.g., siRNA), it is more difficult to study the activity of individual protein domains *in vivo*. Such methodology is critical to fully understand protein function. One popular approach is to inject antibodies that bind selectively to specific protein motifs, thereby inhibiting their function. For instance, this has been used to study membrane and matrix protein functions in the developing and adult CNS (Hudson, 1999; Waldmann, 2003). However, after injection in animals, antibody concentration decreases over time, and the targeted protein domains reacquire function (Monnier et al., 2001). Injecting genetically engineered cells that express antibodies over weeks is one solution to this problem (Noel et al., 2000), but does not solve other limitations inherent to the use of antibodies. Indeed, these are large molecules that may not diffuse well in tissues, and the Fc fragment of full-length antibodies may generate an immune response (Hudson, 1999; Waldmann, 2003). To address the latter problem, antibodies may be enzymatically digested to produce Fab fragments (Fabs) that lack the Fc fragment (Avci et al., 2004). However, because Fabs are rapidly cleared, neutralization by the means of Fab fragments can only be achieved for relatively short periods of time *in vivo*

(Demignot et al., 1990). In recent years, small recombinant antibodies, such as single-chain Fv (scFv) fragments (Bird et al., 1988; Skerra and Plückthun, 1988), have been developed and expressed in mammalian cells (Meissner et al., 2001; Wurm, 2004) and very recently in living organisms (Zuber et al., 2008). Thus, the ideal approach to study protein domains over a longer time period would exploit small antibodies that both lack the Fc fragment and can be expressed in a defined spatial–temporal manner. scFvs are regarded as the minimal structural component of an antibody required for antigen-binding activity (Bird et al., 1988; Skerra and Plückthun, 1988). While they have been widely used for *in vitro* studies or as therapeutics (Carter, 2001; Lev et al., 2004), to date no studies have addressed the possibility of using scFvs for studying protein domains *in vivo* for extended periods of time. By expressing scFvs in a viral vector, we developed a method for sustained *in vivo* inhibition of protein domains. Here we show that our method can be used to neutralize protein domains in the developing visual system. Furthermore, we exploited this new technique to reveal new insight into the mechanism through which the repulsive guidance molecule, RGMa, regulates axonal pathfinding.

Materials and Methods

scFv construction and expression. Hybridomas producing antibodies (Abs) against N- and C-terminal RGMa peptides, spanning regions 70–149 and 195–349 aa, respectively, were obtained using immunized BALB/c mice (Immuno-Precise Antibodies). To clone scFvs, variable heavy (V_H) and light (V_L) chains were amplified by reverse transcription-PCR using primer sets (Larrick et al., 1989). V_H and V_L were then joined by a 15 aa linker (Gly₄Ser)₃, and inserted into pSecTag2B (Invitrogen) to add a signal peptide, His and myc tags for purification and detection. Control scFvs were constructed (1) from human anti-myc hybridoma

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Figure 1. RGMA expression. **A**, WB analysis of RGMA in reducing (+DTT) or nonreducing conditions (–DTT). Under reducing conditions blots performed with an anti-C-RGMA antibody display two bands corresponding to uncleaved RGMA (55 kDa) and C-RGMA (33 kDa). Under nonreducing conditions only one band, which corresponds to uncleaved RGMA, is apparent. This indicates that under nonreducing conditions N-RGMA is attached to C-RGMA via a disulfide bridge. **B**, Diagram of RGMA. After cleavage, N-RGMA remains associated to C-RGMA via a disulfide bridge. **C**, Schematic representation of RGMA constructs used in our experiments. Top drawing represents wild-type RGMA. Bottom drawing represents an RGMA construct in which N-RGMA has been removed (N-del). Here, a signal peptide was placed before C-RGMA. **D**, WB analysis of RGMA constructs after transfection in COS7 cells. Lane 1 (N-del), In cells transfected with the mutant where N-RGMA has been removed, C-RGMA is not present in the membrane fraction (Memb.). Lane 2 (RGMA), When full-length RGMA is expressed, analysis shows that C-RGMA is present in membrane preparations (memb.). Lane 3 (N-del), C-RGMA is present in whole cell lysates (cells), which indicates that N-del is normally expressed. **E**, Living cells, that have been transfected with RGMA and N-del constructs, were stained with an anti-C-RGMA and revealed with a FITC labeled secondary antibody. This cell surface staining indicates that N-del mutant is not present at the cell surface. Wild-type RGMA is targeted toward the cell surface.

line [Ctrl1; Developmental Studies Hybridoma Bank (DSHB)] and (2) from the aberrant κ chain produced by hybridoma cells (Ctrl2).

scFvs were cloned into an RCASBP(B) viral vector. To produce viruses, DF1 cells were transfected with RCAS-scFv constructs using Lipofectamine 2000 (Invitrogen), and supernatants were collected, pooled, and concentrated by centrifugation (21,000 rpm, 2 h). Titer was determined by infecting DF1 cells with serial viral dilutions and immunostaining for the viral protein gag (AMV-3C2 Ab; DSHB). Titers of $1\text{--}5 \times 10^8$ IU/ml were used for *in ovo* injection.

In ovo injection and DiI tracing. Eggs (White Leghorn) were purchased from Frey's Hatchery and incubated at 38°C in a high-humidity chamber. At embryonic day 2 (E2), a small window was cut in the egg shell and the extraembryonic membrane was removed to make the embryo accessible for injection. A small amount of viral solution mixed with 1/10 volume of 0.25% fast green solution (Molecular Probes) was injected in the tectum using a very fine glass drawn capillary. The opening is then resealed with tape and the egg is placed back in the incubator until E15, when a small DiI crystal (Molecular Probes) was placed in the dorsal part of the right eye for labeling axons targeting the anterior part of the optic tectum. At E17, the embryos were killed and the tecta were fixed in 4%

PFA. Each tectum was cut into two halves and DiI tracing of axons was viewed on a fluorescent microscope (Olympus BX61). Digital Images of the tecta were taken and processed using Photoshop (Adobe).

Western blot analysis. To check whether scFvs bind to RGMA, Western blotting (WB) was performed on (1) membrane fractions of COS7 cells transfected with RGMA or (2) E9 tecta. Membrane fractions were prepared by resuspending transfected cells/tecta in HB buffer (25 mM HEPES, 25 mM KCl, 5 mM MgCl_2 , pH 7.4) containing 1% protease inhibitors mixture (Sigma-Aldrich), and passing the cells through 25 gauge and 30 gauge needles, respectively. The suspension was then overlaid on 50% (350 μl) and 5% (150 μl) sucrose gradients and centrifuged at 28,000 rpm for 10 min using a Beckman SW 60 Ti rotor. The membrane fraction was then taken out and spun on a microcentrifuge at 4°C for 10 min at maximum speed (Tassew et al., 2008).

For Western blotting, viral supernatant from RCAS-scFv-transfected DF1 cells was used as primary antibody, followed by incubation with anti-myc antibody (9E10; DSHB). An IR-dye-conjugated secondary antibody (1:5000; LI-Cor Biosciences) was used and the nitrocellulose membrane was scanned on Odyssey Infrared Imaging System (LI-Cor Biosciences).

To check expression of scFvs, supernatant from RCAS-scFv-transfected DF1 cells was purified using Ni-NTA resin (Invitrogen) and samples were run in 12% SDS-PAGE gel. After transfer to a membrane, bands were revealed using anti-His primary antibody (1:1000; ABM) and IR-conjugated secondary antibody (1:5000; LI-Cor Biosciences).

To check expression in the brain, E9 tecta were taken from RCAS-scFv-injected E2 embryos and homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1% NP40, and 1% protease inhibitors). The lysate was passed through a 25 gauge needle until homogenized and incubated on ice for 30 min and centrifuged. Western blotting was performed as above, and bands were revealed using a His primary antibody and IR-dye-tagged anti-mouse secondary.

Retroviral transgene detection by immunostaining. Sections were prepared for immunostaining from E9 tecta that were injected with virus. After blocking and incubating with anti-His (1:200) or anti-gag antibody, sections were washed with PBS and then incubated with Alexa-555-conjugated secondary (1:500; Molecular Probes) diluted with DAPI solution (1 $\mu\text{g/ml}$).

To ascertain that the scFvs were still expressed at E17, sections were prepared from virus-injected tecta that showed abnormal projections after visualization under the microscope. Immunostaining was performed as above.

RGMA cell surface staining and signal intensity measurements. DF1 cells were cotransfected with RCAS-scFv, RGMA, and GFP-expressing constructs. Forty-eight hours after transfection, cells were incubated with anti-His antibody (1:1000) followed by fixation with 4% PFA for 10 min. Cells were washed with PBS, and incubated with Alexa-555-conjugated secondary antibody (1:2000; Molecular Probes) diluted in DAPI (1 $\mu\text{g/ml}$), after which they were treated with 0.1% Triton X-100/PBS for 2 min. Digital images were taken with a fluorescent microscope (Olympus BX61) and processed with Adobe Photoshop.

For quantitative analysis, all images were acquired with identical

Figure 2. scFv expression and biological activity. **A**, DF1 cells were transfected with empty plasmid (Mock) or scFv constructs (ctrl1, ctrl2, 7A2, 8B6, 2E11, 4A6) and the cell supernatant blotted with anti-His antibody. A 25 kDa band in scFv-transfected cells indicates that scFvs are normally secreted by DF1 cells. **B**, Membranes from COS7 cells transfected with RGMA or an empty plasmid (Mock) were blotted and probed with RGMA-scFvs. In blots probed with 8B6- and 2E11-scFvs, both against C-RGMA, two bands are apparent in RGMA-transfected cells. These two bands correspond to C-RGMA (33 kDa) and uncleaved RGMA (55 kDa). In blots probed with 4A6 and 2E11, which recognize N-RGMA, one band corresponding to uncleaved RGMA (55 kDa) is apparent. **C**, Membranes from E9 tectum were prepared and blotted using C-RGMA- and N-RGMA-scFvs. In these experiments, RGMA-scFvs recognize only RGMA in membrane preparation from tectum. **D**, Beads coated with Neogenin were incubated with RGMA-AP in the presence of scFvs. After incubation, beads were washed and submitted to WB analysis using an anti-RGMA antibody. In the presence of control scFvs (scFv-ctrl1 and scFv-ctrl2), a strong band corresponding to RGMA-AP is visible. Incubation with anti C-RGMA- and N-RGMA-scFvs strongly reduces the intensity of the band. **E**, Quantification of four independent experiments performed in which RGMA-AP was pulled down with Neogenin reveals a significant reduction (for *p* values, see supplemental Table 7, available at www.jneurosci.org as supplemental material) of the interaction between RGMA and Neogenin in the presence of C-RGMA (yellow) and N-RGMA (blue) scFvs when compared with control scFvs (red). **F**, SH-SY5Y cells were incubated with RGMA-AP in the presence of RGMA- and control-scFvs. RGMA-AP binding to the cells was revealed using a colorimetric reaction.

brightness and contrast settings and the entire area of the cell was outlined and its intensity measured using ImagePro 5.0 software. Only His-stained cells that were GFP positive were taken for quantitative analysis.

In situ hybridization. *In situ* hybridization was performed as described by Monnier et al. (2002). Chick embryos were infected at E2, and at E8 tecta were removed and frozen in Tissue Tek (Sakura Finetek) on dry ice. For *in situ* hybridization on sections, 14 μ m thick sections were cut on a cryostat (Leica) and processed according to Monnier et al. (2002). DIG-labeled antisense RNA probes were generated using the Dig DNA labeling and detection kit from Roche. Sections were developed with 330 μ g/ml 4-nitroblue tetrazolium chloride and 160 μ g/ml 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-HCl, and 5 mM MgCl₂, pH 9.6. Plasmids encoding ephrin A2 and ephrin A5 were obtained from Dr. E. Matsunaga (Hirose, Wako, Japan) (Matsunaga et al., 2006).

Active caspase-3 staining. For active caspase-3 staining, E2 embryos were infected and at E9 tecta were removed and fixed 30 min in 4% PFA in PBS. They were immersed in 30% sucrose for cryoprotection and then directly frozen in Tissue Tek (Sakura Finetek). Sections were cut, incubated with the anti-caspase-3 antibody (Santa Cruz) at room temperature (RT) for 2 h, and stained with Cy3-conjugated anti-mouse IgG (Molecular Probes) for 30 min in the dark. After rinsing with PBS, the sections were stained with DAPI and mounted in 10% glycerol in PBS.

Pull-down assay. Neogenin was coupled to activated-CNBr Sepharose beads (Pharmacia). Neogenin beads were added to tubes containing preincubated RGM-AP and scFv and allowed to bind for 3 h at RT. Beads were washed three times with PBS, and SDS loading buffer was added. Samples were boiled and run in SDS gel. WB was performed with an anti-C-RGMA Ab (8B6), and bands were revealed with an IR-dye-conjugated secondary Ab and scanned on Odyssey. Integrated intensities of the bands were measured using the Odyssey application software and the values expressed

← Presence of RGMA-scFvs reduces binding to SH-SY5Y cells when compared with controls. **G**, Quantification of three independent experiments reveals a significant reduction of RGMA-AP binding to SH-SY5Y cells when compared with controls (for *p* values, see supplemental Table 7, available at www.jneurosci.org as supplemental material). **H**, Chick tecta were infected with an empty RCAS virus (Mock) or RCAS virus that express scFvs, and 7 d after infection, immunohistochemistry was performed to reveal scFv expression. Representative pictures show that after infection with RCAS expressing ctrl1-, 8B6-, and 7A2-scFv a strong staining is apparent all over the optic tectum. Mock infection resulted in background fluorescence. The two panels in the right are magnifications of the pointed area indicated in the 8B6 and Mock experiments. **I**, Chick tecta were infected with an empty RCAS virus (Mock) or RCAS virus that express scFvs, and 7 d after infection, WB analyses were performed. A 25 kDa band indicates that scFvs are normally expressed in chick tecta.

Summary of retino-tectal phenotypes in scFv experiments						
Constructs	Total Number	(1) No TZ	(2) Anterior Termination	(3) Posterior Termination	(4) Aberrant Turns	No Phenotype
RCAS	6	0	0	1 (17%)	0	5 (83%)
scFv-ctrl1	8	0	0	1 (12%)	0	7 (88%)
scFv-ctrl2	8	0	0	0	0	8 (100%)
8B6-scFv	14	3 (21%)	2 (14%)	11 (78%)	5 (35%)	3 (21%)
7A2-scFv	11	4 (36%)	1 (9%)	8 (73%)	7 (63%)	3 (27%)
4A6-scFv	13	3 (23%)	2 (15%)	11 (84%)	11 (84%)	2 (15%)
2E11-scFv	10	3 (30%)	2 (20%)	7 (70%)	5 (50%)	2 (20%)

Figure 3. *In ovo* expression of C- and N-RGMA scFvs produce reproducible phenotypes in the embryonic chicken visual system. Thirteen days after infection with RCAS viruses, temporal retinal fibers were labeled with a Dil crystal. The target area in the optic tectum is presented in these panels. **A**, In experiments where embryos were infected with empty virus, fibers target a well defined terminal zone (TZ). **B**, Magnification of **A**. **C**, **D**, Infection with two different control scFvs does not perturb axonal pathfinding. **E–H**, After infection with C-RGMA-scFvs (8B6 and 7A2) or N-RGMA-scFvs (2E11 and 4A6) aberrant axonal trajectories are observed. Asterisks indicate fibers that establish arborizations outside the TZ. The hypothetical TZ is represented in each panel. **I**, Schematic representation of the phenotypes observed. Presentation of the types of errors observed in scFv experiments. The phenotypes included (1) absence of TZ, (2) anterior termination, (3) posterior termination, and (4) aberrant turns. **J**, Quantification of errors observed in control and scFv experiments.

relative to scFv-Ctrl1 set to 1 (supplemental Table 7, available at www.jneurosci.org as supplemental material).

RGMA-AP cell surface staining and quantification. Unfixed SH-SY5Y cells that express Neogenin (data not shown) were incubated for 2 h with RGMA-AP (5 nM) plus scFvs (5 nM), washed, fixed, and heated to inactivate endogenous AP activity. AP activity was revealed using

p-nitrophenyl phosphate as substrate. Quantitative assays were terminated when reaction product was visible, but not saturated. Images were acquired with identical brightness and contrast settings and the cell area measured using ImagePro 5.0 software. The intensity was determined for 20 individual cells from three independent experiments (supplemental Table 7, available at www.jneurosci.org as supplemental material).

Results

RGMA has two fragments that may be involved in retinal axon targeting

RGMA acts as a contact-dependent repulsive factor and inhibits outgrowth from temporal retinal axons through interaction with the transmembrane protein, Neogenin (Stahl et al., 1990; Monnier et al., 2002; Rajagopalan et al., 2004; Matsunaga et al., 2006). RGMA is expressed in a low anterior high posterior gradient in the optic tectum, and *in vivo* perturbation of this gradient leads to pathfinding errors for temporal retinal axons (Matsunaga et al., 2006). RGMA is a glycoprotein that is proteolytically cleaved into C-terminal (C-RGMA) and N-terminal (N-RGMA) fragments (Monnier et al., 2002). C-RGMA is bound to the membrane via a glycosylphosphatidylinositol (GPI) anchor (Monnier et al., 2002), and because membranes from cells that express RGMA inhibit outgrowth, it has been assumed that C-RGMA is the only active fragment (Monnier et al., 2002). However, our examination of processed RGMA reveals that N- and C-RGMA remain connected following proteolytic cleavage due to a stable disulfide bond (Fig. 1*A,B*). This finding raises the possibility that both RGMA fragments may contribute to axon guidance during development. To address this issue, we considered performing ectopic expression of the individual RGMA fragments in the chick visual system. C-RGMA was cloned with a signal peptide and its expression analyzed. Surprisingly, it was not targeted toward the cell surface (Fig. 1*C–E*). For this reason the *in vivo* activity of C-RGMA alone cannot be assessed by overexpression of this fragment. Therefore, to assess the function of RGMA domains in the developing visual system, we developed a protocol in which scFv fragments against both domains were generated and expressed *in ovo* (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

Generation and expression of scFvs

To generate hybridoma cell lines, mice were immunized with RGMA fragments spanning the amino acids 195–349 (C-RGMA) and 70–149 (N-RGMA), respectively. The RGMA fragments used

for immunization were selected on the basis of experiments showing that polyclonal antibodies against these fragments prevent binding of RGMa to Neogenin (Rajagopalan et al., 2004). After fusion and screening, hybridoma cell lines against both RGMa fragments were identified (supplemental Fig. 2, available at www.jneurosci.org as supplemental material), and total RNA was extracted. Heavy and light chain variable regions were amplified, joined with a 15 aa linker, and inserted into a pSecTag2B vector to add a signal peptide for secretion and His-Tag for detection, and then transferred into an RCASBP(B) viral vector. All constructs were sequenced (supplemental Tables 1–6, available at www.jneurosci.org as supplemental material). Western blot analyses performed on supernatants of DF1-transfected cells demonstrate that these scFvs are secreted *in vitro* (Fig. 2A). Viruses that produce scFvs were collected from supernatants of cells transfected with RCAS-scFv constructs. In experiments in which antibodies are used to study domain functions, controls as a rule consist of an irrelevant antibody. For these studies, we constructed two such controls, one scFv against human myc (scFv-ctrl1) and another against the aberrant κ chain produced by our hybridoma cell lines (scFv-ctrl2) (Fig. 2A). Neither of these control-scFvs recognizes RGMa or any chick protein (Evan et al., 1985) (supplemental Fig. 3, available at www.jneurosci.org as supplemental material).

RGMa-scFvs recognize and block RGMa activity

The parental monoclonal antibodies specifically recognize N-RGMa and C-RGMa in membrane preparation from cells transfected with RGMa (supplemental Fig. 2A, available at www.jneurosci.org as supplemental material). To demonstrate that RGMa scFvs also retain the properties of the parental antibodies we performed Western blotting analysis on membranes from RGMa-transfected cells as well as on membrane from the tectum (Fig. 2B,C). C-RGMa scFvs recognized the predicted two bands that correspond to uncleaved RGMa and C-RGMa, which remain bound to the membrane. Also as expected, N-RGMa scFvs recognized only one band corresponding to uncleaved RGMa, as N-RGMa is released from the membrane fraction under the reducing conditions used in this experiment (5 mM DTT). These scFvs are highly specific for RGMa as they did not recognize RGMb, its closest relative in the RGM family (supplemental Fig. 3B, available at www.jneurosci.org as supplemental material). Both N-RGMa and C-RGMa antibodies interfered with the RGMa/Neogenin interaction *in vitro* (supplemental Fig. 4, available at www.jneurosci.org as supplemental material), therefore, we tested whether scFvs also retained this important biological property. The efficiency of RGMa pull-down with Neogenin coated beads was strongly reduced in the presence of both N- and C-RGMa scFvs, when compared with control scFvs (Fig. 2D,E; supplemental Table 7, available at www.jneurosci.org as supplemental material). Also the binding of RGMa-AP to Neogenin-expressing SH-SY5Y cells was significantly altered by the presence of both N- and C-RGMa-scFvs (Fig. 2F,G; supplemental Table 7, available at www.jneurosci.org as supplemental material). These results demonstrate that both RGMa fragments can be targeted to influence binding to Neogenin, which alters our current view of the RGMa/Neogenin interaction.

scFv expression and function in the developing chick visual system

To study scFv expression in a developing CNS, we infected the E2 chick mesencephalon with RCAS-scFvs. Immunohistochemistry and Western blot analyses demonstrate that scFv expression in

the optic tectum was maintained throughout the establishment of retinal projections between E3 and E17 (Fig. 2H,I; supplemental Fig. 5, available at www.jneurosci.org as supplemental material). Thus, RGMa scFvs have the potential to neutralize RGMa fragments throughout the period of visual connection formation in the developing chick.

Encouraged by these results, we decided to study retinal axon pathfinding after scFv injection. siRNA silencing of RGMa in the chick optic tectum is known to perturb axonal pathfinding (Matsunaga et al., 2006). Moreover, polyclonal antibodies raised against the same C-RGMa domain that we used for hybridoma preparation interfere in the RGMa/Neogenin interaction, thereby abolishing the guidance effect of RGMa on temporal axons *in vitro* (Rajagopalan et al., 2004). Based on these results, we expected that C-RGMa scFvs (8B6- and 7A2-scFvs) would induce pathfinding errors in the tectum if the scFvs effectively inhibited C-RGMa function. Viruses expressing C-RGMa scFvs were injected at E2 and pathfinding studied using DiI staining of retinal axons. Infection with 7A2- and 8B6-scFvs perturbed pathfinding of temporal fibers (Fig. 3E,F). Several abnormal phenotypes were visualized including (1) anterior terminations, (2) absence of terminal zone, (3) aberrant trajectories, and (4) posterior terminations, all of which indicate that retinal axons misread RGMa topographic information within the optic tectum. In these experiments <24% (27% for 7A2-scFv and 21% for 8B6-scFv) of the embryos did not display any abnormal phenotype (Fig. 3J).

In addition, since N-RGMa scFvs (2E11- and 4A6-scFvs) were also found to interfere with the RGMa/Neogenin interaction *in vitro*, we tested whether inhibiting N-RGMa would have any effects on retinal axon pathfinding in the tectum. The results obtained with N-RGMa scFvs were highly similar to those obtained with C-RGMa scFvs (Fig. 3G,H). Here also, abnormal phenotypes indicate that in the presence of N-RGMa scFvs, temporal axons misread the RGMa topographic information. In <17% (15% for 4A6-scFv and 20% for 2E11-scFv) of the embryos no aberrant axonal pathfinding was observed (Fig. 3J). As expected, injection of virus alone had no abnormal phenotype (Fig. 3A,B), and importantly, injection of the two control scFvs also caused no noticeable abnormal phenotype (Fig. 3C,D) and >93% of the embryos (88% for scFv-ctrl1 and 100% for scFv-ctrl2) were normal (Fig. 3J).

Cell surface staining revealed that scFvs did not affect RGMa secretion, confirming that RGMa is properly transported toward the cell surface (supplemental Fig. 6, available at www.jneurosci.org as supplemental material). Tissue integrity and the number of caspase-3 positive cells appeared similar both in control and RGMa-scFv experiments (supplemental Fig. 7, available at www.jneurosci.org as supplemental material). Furthermore, *in situ* hybridization demonstrates that ephrin A2, ephrin A5, and RGMa expression patterns were not affected by RGMa-scFvs (supplemental Figs. 8–10, available at www.jneurosci.org as supplemental material). Thus the axon targeting phenotype arises from scFv inhibition of processed RGMa.

Discussion

Recent studies using loss of function as well as gain of function experiments in the chick embryo demonstrated that RGMa is involved in retinotectal pathfinding (Matsunaga et al., 2006; Tassew et al., 2008). We have also shown that RGMa is proteolytically processed into 33 kDa C-terminal and 11 kDa N-terminal fragments in cell culture as well as *in vivo* (Monnier et al., 2002). Because C-RGMa remains linked to the cell membrane via a GPI anchor and membranes that express RGMa inhibit

axonal outgrowth, it has been assumed that C-RGMA is the business end of this molecule and the N-RGMA fragment has received scant attention (Rajagopalan et al., 2004). Our data showing that N-RGMA remains linked to C-RGMA via a disulfide bridge challenges this assumption. Also, we demonstrated that C-RGMA is not secreted when expressed without N-RGMA, therefore, we could not study the function of C-RGMA alone using ectopic expression.

To study the function of the 2 RGMA domains, we considered using Fab fragments, however, the chick visual system development takes 15 d (Nakamura and O'Leary, 1989), a time frame too long for continued inhibition of targeted protein domains with Fabs. To study the function of RGMA protein domains in the developing CNS, we developed a novel method in which recombinant antibodies (scFvs) are used to neutralize protein domains *in vivo* and exploited this approach to reveal new insight into the mechanism through which RGMA regulates axon pathfinding. Here we engineered both monoclonal antibodies and scFvs against the two RGMA domains and studied their action in the developing visual pathway. We show that scFvs can be expressed over a 2 week time period in the developing chick CNS and thus can be used to study protein domain functions. It has been described that scFvs retain their binding specificity but may have different affinity from the parental antibody (Skerra and Plückthun, 1988). Here we demonstrated that indeed our RGMA-scFvs specifically recognize RGMA and thus may be used for *in vivo* studies. Collectively, our results provide the first evidence that both C- and N-terminal fragments of RGMA interact with Neogenin and contribute to retinal axon guidance. They reveal that C-RGMA is not the only RGMA fragment involved in axon guidance and that N-RGMA also plays a crucial role in this process. In a recent work, we demonstrated that RGMA may influence axonal outgrowth when it is expressed both in the direct environment of growing fibers or on fibers themselves (Tassew et al., 2008). Thus, although we clearly demonstrate that our scFvs act by blocking the interaction between RGMA and Neogenin, further experiments are needed to determine the effects of tissue-specific RGMA domains.

In addition, our data demonstrate that scFvs are an invaluable tool for developmental studies. The scFvs that we developed may be very important tools to decipher the biological functions of RGMA during CNS development. We show that *in ovo* scFv expression in chick embryos provides sustained neutralization of protein domains, and thereby allows functional studies to be undertaken that have not been possible to date. With the abundance of hybridoma cell lines currently available, this procedure provides an avenue for high-throughput assessment of biological functions to be rapidly conducted *in vivo*.

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