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Oligomerization of the extracellular domain of Boss enhances its binding to the Sevenless receptor and its antagonistic effect on R7 induction

Evgueni A. Sevrioukov, Jason H. Walenta, Arisa Sunio, Meridee Phistry and Helmut Krämer*

Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX 75235-9111, USA
*Author for correspondence (e-mail: kramer@utsw.swmed.edu)

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SUMMARY

In the developing compound eye of *Drosophila,* neuronal differentiation of the R7 photoreceptor cell is induced by the interaction of the receptor tyrosine kinase Sevenless with its ligand Bride of sevenless (Boss), which is expressed on the neighboring R8 cell. Boss is an unusual ligand of a receptor tyrosine kinase: it is composed of a large extracellular domain, a transmembrane domain with seven membrane-spanning segments and a cytoplasmic tail. Expression of a monomeric, secreted form of the extracellular domain of Boss is not sufficient for Sevenless activation, and instead acts as a weak antagonist. Because oligomerization appears to be a critical step in the activation of receptor tyrosine kinases, we used oligomerized forms of the Boss extracellular domain to test their ability to bind to Sevenless in vivo and restore R7 induction in vivo. Oligomerization was achieved by fusion to the leucine zipper of the yeast transcription factor GCN4 or to the tetramerization helix of Lac repressor. Binding of these multivalent proteins to Sevenless could be detected in vitro by immunoprecipitation of cross-linked ligand/receptor complexes and in vivo by receptor-dependent ligand localization. However, neither R8-specific or ubiquitous expression of multivalent Exboss ligands rescued the *boss* phenotype. Instead, these ligands acted as competitive inhibitors for wild-type Boss protein and thereby suppressed R7 induction. Therefore the role of the transmembrane or cytoplasmic domains of Boss in the activation of the Sev receptor cannot be replaced by oligomerization.

Key words: *Drosophila*, Bride of sevenless, Receptor tyrosine kinase signaling, Ligand, Oligomerization

INTRODUCTION

Receptor tyrosine kinases (RTKs) control signaling pathways in developmental decisions as diverse as embryonic pattern formation (Perrimon, 1993), neuronal differentiation and cell death (Kaplan and Miller, 1997) and axonal guidance and brain segmentation (Friedman and O'Leary, 1996). During the development of the compound eye of *Drosophila*, every cell type requires signaling from the EGF receptor tyrosine kinase to adopt their proper cell fates (Freeman, 1996). One cell type, the R7 photoreceptor cell, requires the subsequent activation of a second RTK, the Sev receptor.

The only phenotype that has been observed for mutations in the Sev RTK is the transformation of the R7 photoreceptor neuron into a non-neuronal cone cell (Tomlinson and Ready, 1986). The Sev receptor is synthesized as a 280 kDa precursor protein, that is cleaved into an extracellular subunit of 220 kDa and a transmembrane and cytoplasmic subunit of 60 kDa which contains the tyrosine kinase domains (Basler and Hafen, 1988; Bowtell et al., 1988; Simon et al., 1989). In the eye disc, Sev is expressed in a dynamic pattern that is not restricted to the R7 cell precursor (Banerjee et al., 1987; Tomlinson et al., 1987).

Sev is activated by the Bride of Sevenless transmembrane ligand (Boss) which is composed of an extracellular domain of 498 amino acids (aa), a seven membrane-spanning domain (7TM), and a cytoplasmic tail of 115 aa (Hart et al., 1990; Krämer et al., 1991). Boss-containing membranes induce tyrosine phosphorylation of the Sev receptor (Hart et al., 1993b). The principal mechanism by which Sev activation is relayed to the R7 precursor nucleus appears to be the RAS/RAF/MAP kinase signaling pathway (Dickson and Hafen, 1994; Zipursky and Rubin, 1994).

Because of its 7TM domain, Boss is an unusual ligand for a receptor tyrosine kinase. One possible function of this domain could be to restrict the number of cells that have access to the inductive Boss ligand. The Sev receptor is expressed on the R7 cell precursor, which contacts the central R8 cell, as well as on four cone cell precursors that do not contact the R8 cell; all of these cells are competent to develop into R7 neurons upon activation of the Sev signaling pathway (Basler et al., 1991; Brunner et al., 1994; Dickson et al., 1992; Fortini et al., 1992; Van Vactor et al., 1991). By contrast to Sev expression, Boss expression is limited only to the R8 cell in each ommatidium (Krämer et al., 1991). Because Boss is anchored to the R8 cell membrane, only a cell that contacts R8 will have the Sev pathway activated. Therefore the R8-specific expression of the Boss ligand in combination with its membrane-anchor is instrumental in restricting R7 cell

development to a single cell in each ommatidium (Van Vactor et al., 1991).

However, spatial restriction of the inductive cue is not the sole function of the Boss transmembrane domain. An important role in Boss function was suggested for the transmembrane and cytoplasmic domains by their higher degree of conservation compared to the extracellular domain (Hart et al., 1993a). When the extracellular domain of Boss was expressed as a secreted protein (Exboss), it was not sufficient to induce R7 development. On the contrary, in a genetic background that was sensitized for R7 induction by a reduced level of Boss expression, the secreted Exboss protein acted as an antagonist (Hart et al., 1993b). These results indicated a direct requirement for the transmembrane or cytoplasmic domains in R7 induction.

One potentially attractive role for Boss's 7TM domain in R7-cell induction is that it could oligomerize the Boss ligand, allowing its activation domain to interact with Sev as a cluster. The importance of ligand clustering is consistent with a widely accepted model for how RTKs translate extracellular signals into intracellular responses (Schlessinger and Ullrich, 1992). In this model, ligand-induced oligomerization of the ectodomains of RTKs juxtaposes their kinase domains so that they can tyrosine-phosphorylate each other. Such ligand-induced dimerization has been demonstrated for the receptors of EGF (Gadella and Jovin, 1995; Lemmon et al., 1997), FGF (Spivak-Kroizman et al., 1994), and Steel (Philo et al., 1996).

In the Ephrin group of transmembrane ligands, the transmembrane domain appears to be instrumental in the ligand-induced oligomerization of RTKs. Whereas soluble, secreted forms of these ligands were inactive, antibody-induced oligomerization of the ligands restored their ability to activate their receptors (Davis et al., 1994). While these results suggested a role of the transmembrane or cytoplasmic domains in ligand oligomerization (Davis et al., 1994), an additional function of the cytoplasmic domains of the EphrinB transmembrane ligands was suggested by their phosphorylation upon binding to their receptors of the EphB subclass (Brückner et al., 1997; Holland et al., 1996).

In this paper, we tested whether a principal role of Boss's transmembrane or cytoplasmic domain is to oligomerize its activation domain, as was observed for the EphrinB ligands. To explore the role of Boss oligomerization in R7 induction, we generated oligomerized forms of Exboss and tested their ability to induce R7 development in vivo. We found that oligomerization enhanced binding of the extracellular domain of Boss to the Sev receptor, but it could not restore R7 induction. Instead, oligomerization of Exboss enhanced its antagonistic effects on R7 induction. Due to their strong binding to Sev, the oligomerized forms of Exboss acted as competitive inhibitors, thus preventing the binding of wild-type Boss to Sev and inhibiting R7 development. These results indicate a direct role of the 7TM or cytoplasmic domains of Boss in R7 induction.

MATERIALS AND METHODS

Generation of dimeric and tetrameric versions of Exboss

To generate Exboss-GCNa, amino acid 510 of Boss was fused to amino acid 249 of GCN4, which directly precedes the leucine zipper. Two additional variants of the Exboss-GCN dimer, b and c, contained a GCN4 leucine zipper shortened by one or two aa, respectively. For heat shock-controlled expression in S2 cells and transgenic flies, these constructs were inserted into the vector pHT4 (Schneuwly et al., 1987).

Similarly, Exboss-Lac was constructed by fusing aa 538 of Boss to the C-terminal 30 aa of Lac repressor that include its tetramerization domain (Alberti et al., 1993; Friedman et al., 1995; Lewis et al., 1996). For heat shock-controlled expression in S2 cells and transgenic flies, the Exboss-Lac construct was inserted into pCaSpeR-HS (Thummel and Pirrotta, 1992).

To achieve R8-specific expression, a Boss expression vector was constructed. A 7.5 kb *Xho*I/*Sal*I fragment from the genomic region of the *boss* gene is sufficient to rescue the *boss* phenotype (Hart et al., 1993a). At bp 4,423 (base numbering according to GenBank accession L08133), a *Sac*II restriction site was introduced that changed Ile539 into a glycine. Between this *Sac*II site and the *Stu*I site at bp 5,197, a linker containing a *Not*I and a *Kpn*I site was introduced. After insertion into the P-element vector pCaSpeR4 (Thummel and Pirrotta, 1992), the resulting vector contains all the 5′ and 3′ sequences that are sufficient for R8-specific expression of Boss and provides for the easy insertion of sequences replacing the transmembrane and cytoplasmic domains of Boss. Standard molecular biology methods were used for the generation of these constructs and their verification by DNA sequence analysis (Ausubel et al., 1994).

Genetics and generation of transgenic lines

Expression vectors based on pHT4 were injected into *h ry boss*^{1} flies, those based on pCaSpeR were injected into w^{1118} flies and crossed into the indicated genetic backgrounds using standard procedures (Ashburner, 1989; Rubin and Spradling, 1982). We obtained 1 line with pHT4-HS-Exboss-GCNa, 2 lines with pHT4-HS-Exboss-GCNb, 4 lines with pHT4-HS-Exboss-GCNc, 15 lines with pCaSpeR-HS-Exboss-Lac, 3 lines with pCaSpeR-gen-Exboss-GCNa, 6 lines with pCaSpeR-gen-Exboss-GCNb, 2 lines with pCaSpeR-gen-Exboss-GCNc and 4 lines with pCaSpeR-gen-Exboss-Lac. When more than one transgenic line was available, we chose two lines with P-element insertions on the 2nd or X chromosome for detailed analysis. For crosses into a *sev* background, *w sev*^{d2} was used.

Heat shock-induced expression of Exboss ligands in eye discs

Late third instar larvae were placed in perforated microfuge tubes containing 200 µl of fly food. Heat shocks were administered after placing the tubes in a PCR machine, either once for 30 minutes at 37°C for immuno-localization experiments or 4 times for 30 minutes at 37°C with 2 hour recovery periods at 25°C for testing antagonistic activities of different Exboss ligands. For examination of adult flies development proceeded at 25°C.

Expression and purification of Exboss proteins

Exboss proteins were expressed in S2 cells and purified by peanut-agglutinin affinity chromatography as previously described (Hart et al., 1993b). Cells were maintained in serum-free Sf900 medium (Gibco) for harvesting the proteins. The level of secretion of Exboss-Lac protein into cell supernatants was lower than that of the monomeric Exboss or the dimeric Exboss-GCNa proteins. In addition, we found the Exboss-Lac protein to be the least stable of these proteins, resulting in less enriched preparations for in vitro studies. Exboss is N-glycosylated (Krämer et al., 1991) and its binding to the peanut-agglutinin lectin is indicative of O-glycosylation (Hart et al., 1993b).

Determination of native molecular mass of Exboss proteins

Gel filtration on a 1 cm × 30 cm Superdex 200HR FPLC column was

used to determine the Stoke's radii of purified Exboss proteins. Cell supernatants or partially purified proteins (25 µl) were separated in running buffer (50 mM $NaPO_4$, pH 7.0, 150 mM NaCl) at a flow rate of 200 µl/minute (0.3 ml/fraction). Elution volumes for the different Exboss proteins were determined by western blot analysis of all relevant fractions. For the determination of Stoke's radii, the following standard proteins were used: myoglobin (1.9 nm), ovalbumin (2.8 nm), catalase (5.2 nm), ferritin (6.3 nm), and thyroglobulin (8.6 nm). To ensure that the large size of the Exboss-Lac protein was not due to unspecific protein aggregation during the purification, supernatants of expressing cells were used directly for molecular mass measurements.

Sedimentation coefficients were determined by centrifugation through 5-20% sucrose gradients in a SW41 Ti rotor at 5°C at 35,000 rpm for 20 hours for Exboss and Exboss-GCNa or for 14 hours at 28,000 rpm for Exboss-Lac. Sample size was 200 µl. Gradients were harvested into 500 µl fractions which were analyzed for the presence of the Exboss proteins by western blotting. Ovalbumin ($S_{20,w}$=3.6), catalase ($S_{20,w}$=11.4) and thyroglobulin ($S_{20,w}$=19.4) served as standard proteins. Native molecular mass was calculated from sedimentation coefficients and Stoke's radii using the Svedberg equation (Tanford, 1961).

Crosslinking experiments

2×10^6 Sev-expressing cells (Simon et al., 1989) were pelleted and resuspended in 250 µl Schneider's medium containing 200 µM chloroquine and incubated for 30 minutes at room temperature (RT). Partially purified Exboss ligands or control proteins were added at the following final protein concentrations: Exboss (6 µg/ml); Exboss-GCNa (6 µg/ml); Exboss-Lac (1 µg/ml) or rabbit IgG (6 µg/ml). Cells were then incubated in the presence or absence of the ligands for 30 minutes at RT. Unbound ligands were removed by two washes in PBS/chloroquine at RT. Dithiobis-sulfosuccinimidyl propionate (DSP) was added to a final concentration of 0.25 mM for 30 minutes at RT. Cells were pelleted and lysed in 1 ml of lysis buffer (100 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1% Triton X-100, 10 mM glycine, 0.5 mM PMSF, 2 µg/ml leupeptin, 1 µg/ml pepstatin) for 15 minutes at 4°C. The soluble fraction was cleared by centrifugation at 20,000 ***g*** for 15 minutes at 4°C. 20 µl of beads, coupled to anti-Boss NN1 antibodies, or anti-rabbit IgG antibodies for the control, were added to the soluble supernatant and incubated for 1 hour at 4°C. The beads were washed 4 times with lysis buffer containing 0.1% Triton X-100 and once in lysis buffer omitting Triton X-100. Proteins were eluted from the beads with 2% SDS and separated by 10 % SDS-PAGE after cleavage of the DSP crosslinker with 100 mM dithiothreitol (DTT). Immunoprecipitated Sev protein was detected by western analysis using mAb 150C3, which is directed against the C-terminal 59 kDa subunit (Banerjee et al., 1987).

Histology

Boss proteins in eye imaginal discs were visualized using affinity-purified anti-BossNN1 or anti-BossCT1/CT2 rabbit antibodies (Cagan et al., 1992; Krämer et al., 1991). Bound antibodies were detected using HRP-conjugated secondary antibodies and a modified protocol which considerably enhanced sensitivity. Dissected eye discs were fixed in PLP for 30 minutes, washed three times in PBS with 0.3% Saponin (PBSS) and blocked with 10% normal goat serum (NGS) in PBSS. Discs were incubated with primary antibody diluted in PBSS/10% NGS (1:3,000 for NN1 and 1:1,000 each for CT1/CT2) for 12 to 16 hours at RT, washed four times in PBSS and incubated for 3 to 5 hours with HRP-coupled secondary antibodies. Unbound secondary antibodies were removed by three washes in PBSS and the discs were refixed in 2% glutaraldehyde/PBS for 20 minutes. Discs were incubated two times for 10 minutes in Imidazole washbuffer (175 mM NaAcetate, 10 mM Imidazole, pH 7.2) before color development in chromagen solution (100 mM $NiSO_4$, 10 mM Imidazole, pH 9.2, 125 mM NaAcetate, pH 7.2, 0.3 mg/ml DAB, 2×10^{-3} % H_2O_2).

For double staining of eye discs, anti-bossNN1 rabbit antibodies and mAb150C3 (Banerjee et al., 1987) were used at dilutions of 1:3,000 and 1:2,000, respectively. FITC-conjugated goat anti-mouse and Cy3-conjugated goat anti-rabbit antibodies (Jackson Laboratories, West Grove, PA) were used at dilutions of 1:1000 and 1:2000, respectively. Images were aquired using a MRC1000 confocal microscope (Bio-Rad Labs, Hercules, CA) and processed as previously described (Krämer and Phistry, 1996).

Adult eyes were sectioned using standard procedures (Van Vactor et al., 1991). To analyze the antagonistic effects of oligomeric Exboss proteins we counted the number of R7 cells present and missing in each dorso-ventral column of ommatidia (Basler and Hafen, 1989a). Columns in which more than 50% of the ommatidia lacked R7 cells were scored as R7-minus.

RESULTS

The Exboss protein is secreted as a monomer

The secreted extracellular domain of Boss, Exboss, (Fig. 1) acted as an antagonist of the Sev receptor (Hart et al., 1993b). To test whether Exboss was secreted in a monomeric or oligomeric form, we measured its biophysical properties under non-denaturing conditions. We determined a sedimentation coefficient of 3.9 S by sucrose density centrifugation (Table 1). Gel filtration yielded a Stoke's radius of 4.7 nm. From these two values we calculated a molecular mass of 78 kDa for the native Exboss protein. The predicted molecular mass of the secreted Exboss protein is about 65 kDa, after potential N- and O-glycosylation are taken into account (Hart et al., 1993b; Krämer et al., 1991). This is in good agreement with the size of Exboss determined by SDS-PAGE (Fig. 2A). Therefore the calculated native molecular mass is close to the value expected for a monomer of Exboss.

The monomeric state of Exboss might explain its antagonistic effect on R7 induction. Exboss is sufficient to bind to Sev but might be unable to trigger receptor oligomerization which appears to be critical for RTK signaling (Schlessinger and Ullrich, 1992). Oligomerization of the Boss ligand by its transmembrane or cytoplasmic domains could therefore be a necessary requirement for Sev activation. To test this hypothesis we engineered oligomeric forms of Exboss and analyzed their effect on Sev binding and R7 induction.

Fusion to coiled-coil domains oligomerizes Exboss

A dimeric form of Exboss (Exboss-GCNa) was generated by fusion of Exboss to the leucine zipper derived from the GCN4 transcription factor (Fig. 1). A C-terminal 31-aa domain is sufficient to promote dimerization of secreted proteins (Table 1, and Blondel and Bedouelle, 1991). For in vitro studies, the Exboss-GCNa protein was expressed in S2 cells (Fig. 2). Successful dimerization of Exboss-GCNa was confirmed by determination of its molecular mass under non-denaturing conditions: a combination of sucrose density gradient centrifugation and gel filtration yielded a molecular mass of 165 kDa, consistent with Exboss-GCNa being a dimer of Exboss (Table 1).

To achieve higher order oligomerization, we developed a different approach. We expressed a fusion between Exboss and the Lac repressor tetramerization helix (Exboss-Lac) in S2 cells (Fig. 1B). A C-terminal 21-aa α-helix of Lac repressor is

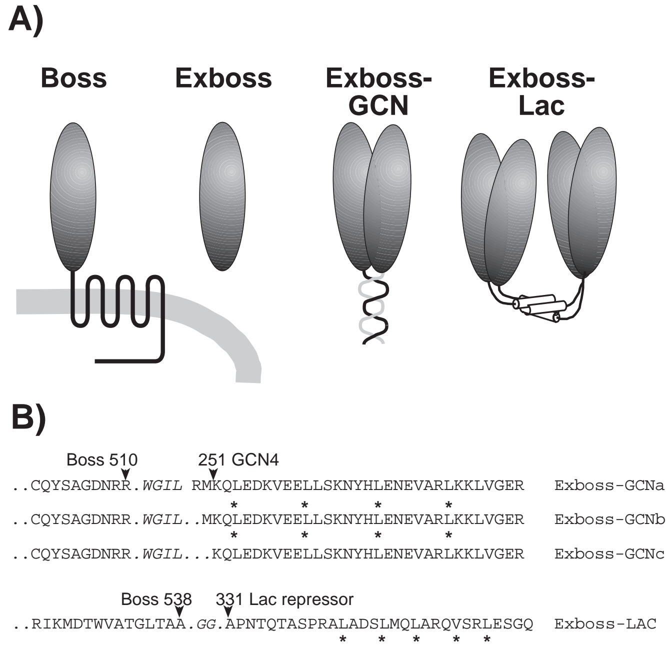

Fig. 1. Oligomers of Exboss were created by fusion to coiled-coil domains. (A) Schematic drawings outline the predicted structures of the wild-type Boss transmembrane protein (Hart et al., 1990) and the soluble Exboss protein consisting of the secreted extracellular domain of Boss (Hart et al., 1993b). The dimeric Exboss-GCN proteins were created by fusion to the leucine zipper of GCN4 (Blondel and Bedouelle, 1991), and the Boss-Lac protein utilizes the tetramerization helix of Lac repressor which forms a four-chain coiled-coil (Alberti et al., 1993; Friedman et al., 1995; Lewis et al., 1996). (B) Sequences at the points of fusion between the extracellular domain of Boss and the GCN4 or Lac repressor oligomerization domains are indicated. Note that Exboss-Lac contains 28 aa of the membrane-proximal domain of Boss not contained in Exboss. Italicized letters indicate spacer amino acids not derived from either of the proteins. Leucine residues critical for the oligomerization domains are indicated by asterisks.

sufficient to act as a tetramerization domain (Alberti et al., 1993; Friedman et al., 1995; Lewis et al., 1996). By gel filtration and sucrose gradient centrifugation we could separate the secreted Exboss-Lac protein into two peaks. Molecular mass determination suggested that one peak corresponded to a monomeric form. A second peak, that was more pronounced at higher concentrations of Exboss-Lac, exhibited a high molecular mass (Table 1) consistent with the formation of tetramers or higher order oligomers by the Exboss-Lac proteins.

Oligomeric forms of Exboss bind the Sev receptor in vitro

Binding of the Boss ligand to the Sev receptor has not been directly demonstrated by biochemical means. We reasoned that multivalent oligomeric forms of Exboss should facilitate detection of their interaction with Sev. To test this, we partially purified Exboss-GCNa and Exboss-Lac (Fig. 2A).

S2 cells expressing Sev (Simon et al., 1989) were incubated with the partially purified Exboss ligands. Upon binding, the ligand/receptor complexes were cross-linked using the reversible, membrane-permeable cross-linker DSP. The complexes were immunoprecipitated with anti-Boss antibodies, and co-immunoprecipitated Sev receptor was detected by western analysis. When the monomeric Exboss ligand was used, the level of Sev co-immunoprecipitation was low but significant (Fig. 2B, lane 2). Co-immunoprecipitation was strongly enhanced in the presence of oligomeric forms of the Exboss ligand (Fig. 2B, lanes 3 and 4).

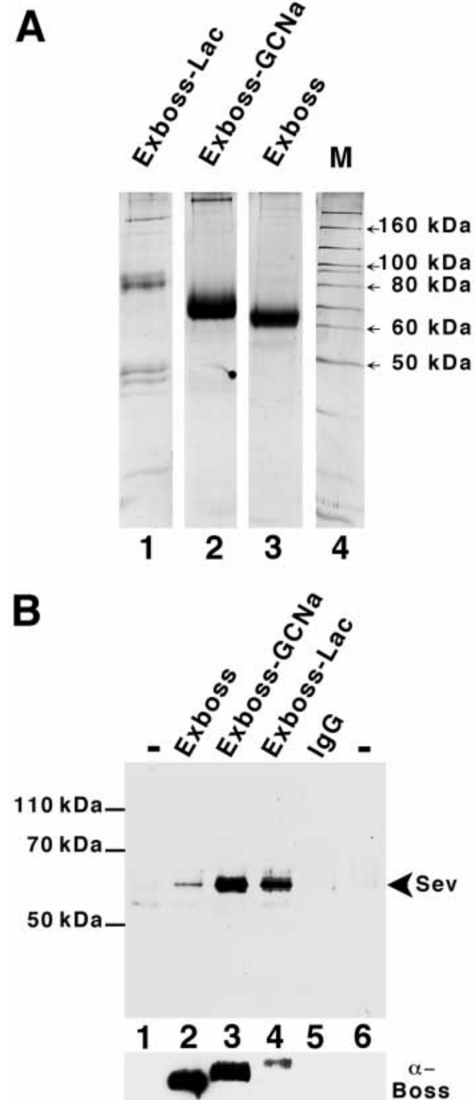

Fig. 2. Soluble oligomeric Boss proteins bind to Sev in vivo. (A) Partially purified preparations of Exboss-Lac (0.2 μg, lane 1), Exboss-GCNa (1 μg, lane 2) or Exboss (1 μg, lane 3) were separated by 10% SDS-PAGE and visualized by silver staining. Molecular mass markers were from Gibco (lane 4). (B) Cross-linked Sev receptor co-immunoprecipitates with oligomeric Exboss ligands. Sev-expressing S2 cells were incubated in the presence of no ligands (lanes 1 and 6), partially purified Exboss ligands (lanes 2-4), or nonspecific rabbit IgG as a control (lane 5). After cross-linking, the solubilized ligand-receptor complexes were immunoprecipitated using anti-Boss NN1 antibodies (lanes 1-4) or anti-rabbit IgG antibodies (lanes 5 and 6). Co-immunoprecipitated Sev receptor was detected by western analysis using mAb 150C3 (Banerjee et al., 1987). In the lower part of B, the relative amounts of Exboss ligands added to the cells were visualized by western blotting using anti-Boss NN1.

Table 1. Native molecular mass of Exboss proteins

Protein	Sedimentation coefficient* ($S_{20,w}$)	Stoke's radius† (nm)	Calculated native molecular mass‡ (kDa)	Molecular mass by SDS-PAGE§ (kDa)
Exboss	3.9	4.7	78	65
Exboss-GCNa	5.9	6.7	165	70
Exboss-Lac				
Peak1	4.2	5.1	87	80
Peak2¶	20	>10	>800	

*Sedimentation coefficients were determined by centrifugation on 5-20% sucrose density gradients.
†Stoke's radii were determined by gel filtration on a Superdex 200HR 10/30 column.
‡Native molecular mass was calculated from the Sedimentation coefficient and the Stoke's radii using the Svedberg equation (Tanford, 1961).
§Molecular mass was determined from partially purified fractions by SDS-PAGE and silver staining (Fig. 2). The identities of the Exboss protein bands were confirmed by western blotting.
¶The size of the second Exboss-Lac peak was outside the useful range of resolution for the Superdex 200HR column. Therefore the indicated Stoke's radius and the calculated molecular mass can only represent rough estimates. We propose that peak I is the monomeric Exboss-Lac protein, presumably dissociated because of the relatively low concentration of Exboss-Lac in the cell supernatants, while peak II represents multimeric Exboss-Lac.

Specificity of the co-immunoprecipitation was tested by several criteria. Co-immunoprecipitation was dependent on the presence of Exboss ligands (Fig. 2B, lane 1). Other soluble proteins, such as rabbit IgG (Fig. 2B, lane 5), did not co-immunoprecipitate Sev, and other transmembrane proteins expressed on the surface of S2 cells (e.g. Neuroligin; Ichtchenko et al., 1995) did not co-immunoprecipitate with Boss (data not shown). In summary, these experiments showed for the first time binding of purified Boss protein to the Sev receptor in vitro. Significantly, the enhanced binding of the oligomeric forms of Exboss demonstrated that they bind to the Sev receptor heterotetramer (Simon et al., 1989) with the increased avidity expected for functional multivalent ligands.

Dimeric Exboss-GCN ligands bind to Sev in the eye disc

To test whether the dimeric Exboss-GCN proteins can induce R7 development, we expressed them in eye imaginal discs using a vector containing all the boss promotor/enhancer regions necessary for R8-specific expression in the eye imaginal disc (Hart et al., 1993a). R8-specific expression of Exboss-GCNa was verified by its specific localization to the perinuclear region of R8 cells (Fig. 3) as had been described for wild-type Boss protein (Krämer et al., 1991).

In vivo binding of the dimeric Exboss-GCN protein to the Sev receptor was assayed by receptor-dependent ligand localization (Hart et al., 1993b). Similar to Exboss (Hart et al., 1993b), Exboss-GCN proteins were detected on the apical surface of eye imaginal discs in a distinctive pattern (Figs 3A,B and 4) resembling the dynamic Sev expression pattern (Tomlinson et al., 1987). This localization to Sev-expressing cells was confirmed by double-labeling of eye discs (Fig. 4) and its elimination in *sev*^{d2} mutant eye discs (Fig. 3C). We concluded that the dimeric Exboss-GCNa ligand could bind to the Sev receptor in the eye disc as it did in vitro.

Unlike ectopic expression of functional Boss protein (Van Vactor et al., 1991), expression of Exboss-GCNa protein in wild-type eye discs under control of the boss promotor did not induce extra R7 cells (Fig. 5B). As the threshold for induction of R7 precursor cells might be different than that of cone cell precursor cells (Rogge et al., 1992) we also tested the effect of Exboss-GCNa expression in a *boss*^{1} mutant background. No

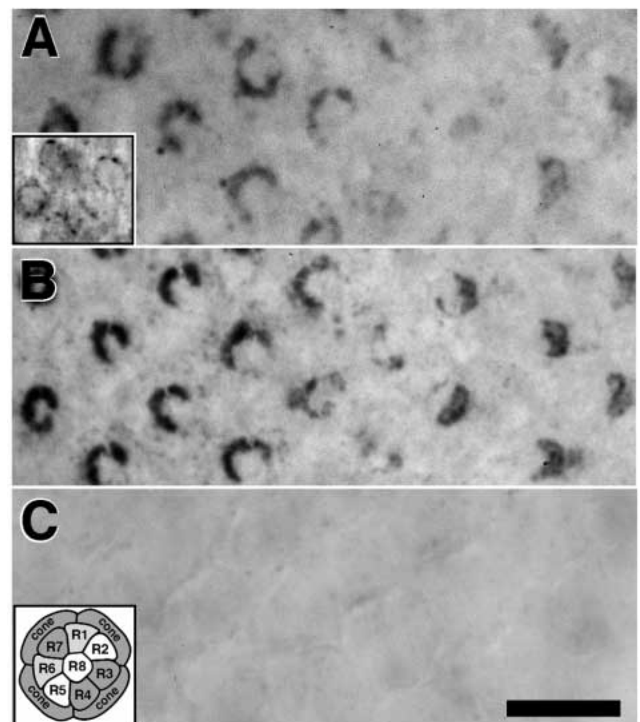

Fig. 3. Exboss-GCN proteins expressed in R8 cells bind to the apical surface of a subset of eye imaginal disc cells. (A-C) Third instar eye imaginal discs stained with anti-Boss antibodies. Exboss-GCNa (A) and Exboss-GCNb (B) expressed under control of the boss promoter localized to the apical surface of Sev-expressing cells. No such localization was detected for Exboss-GCNa in *sev*^{d2} mutant eye discs (C). The inset in A demonstrates R8-specific expression of Exboss-GCNa based on its specific localization to the perinuclear region of R8 cells. At this level, R8 cells can be identified by the unique position of their nuclei in the eye disc. Similar R8-specific perinuclear staining has previously been reported for wild-type Boss (Krämer et al., 1991). The inset in C summarizes the cell types which express Sev at different times of ommatidial development (compare Fig. 4). The right edge of A to C, corresponds to row 2 posterior to the furrow. Bar, 5 µm (for all panels). The relevant genotypes are: (A) P[w^+, gen-Exboss-GCNa]; *boss*^{1}, (B) P[w^+, gen-Exboss-GCNb]; *boss*^{1}, and (C) *sev*^{d2}; P[w^+, gen-Exboss-GCNa] *boss*^{1}.

induction of R7 cells was observed (Fig. 5D). Consistent with these findings, binding of the Exboss-GCNa protein to Sev in vitro did not induce tyrosine-phosphorylation of Sev (data not shown).

The GCN4 leucine zipper is a rigid dimerization domain that could fix the two Exboss subunits in a orientation relative to each other that is not favorable for Sev activation despite their ability to bind Sev. To address this possibility we expressed two additional forms of the Exboss dimer in which the α-Helix of the GCN4 coiled-coil domain was shortened by one or two aa, respectively (Exboss-GCNb and Exboss-GCNc; see Fig. 1B). As a consequence, the two Exboss subunits might be expected to be turned by about 100 or 200 degrees relative to the dimerization domain. Transgenic lines expressing these two proteins under control of the R8-specific boss promoter yielded the same results as observed with Exboss-GCNa. All three bound to the apical surface of Sev-expressing cells in the eye disc (Fig. 3 and 4), but none of the Exboss-GCN proteins restored R7 induction in a *boss* mutant background (Fig. 5, and data not shown). In summary, these results indicated that dimerization of Exboss had increased its avidity for the Sev receptor but it had not restored its ability to induce R7 development.

Dimeric Exboss-GCN ligands act as dominant-negative ligands

Given that the dimeric Exboss-GCN proteins bind to Sev but do not induce its activity, we wondered whether these ligands might act as antagonists inhibiting R7 induction. Previously, it has been demonstrated that monomeric Exboss could act as an antagonist of the Sev receptor (Hart et al., 1993b). However, this effect was weak: in *boss* heterozygous flies, the monomeric Exboss protein was not able to block R7 development; its antagonistic effect was only visible in a genetic background sensitized by a reduction in the level of Boss expression to a level at which only a fraction of R7 cells were induced (Hart et al., 1993b). Exboss-GCNa expressed under control of the *boss* promotor was not sufficient to compete with wild-type Boss and inhibit R7 induction (Fig. 5B). To test whether higher levels of expression might reveal an antagonistic function, Exboss-GCN proteins were expressed under control of the heat shock promotor. Exboss-GCN proteins, expressed under heat shock control, localized specifically to Sev-expressing cells in the eye disc (Fig. 6A and B).

Effects of dimeric Exboss-GCN proteins on R7 induction were tested by their expression in late third instar larvae carrying one wild-type copy of the *boss* gene. As Sev is activated in any given R7 precursor cell for at least 9 hours (see Discussion), larvae carrying the transgenes were heat-shocked 4 times for 30 minutes at 37°C with intervening 2 hour intervals at 25°C. In adult flies that developed from larvae expressing either of the three Exboss-GCN proteins, dorso-ventral stripes of ommatidia lacking R7 cells could be observed (Fig. 6C-E). The average width of the R7-minus stripes was 4 columns for Exboss-GCNa (n=2), 5 columns for Exboss-GCNb (n=6) and 3 columns for Exboss-GCNc (n=3). No such

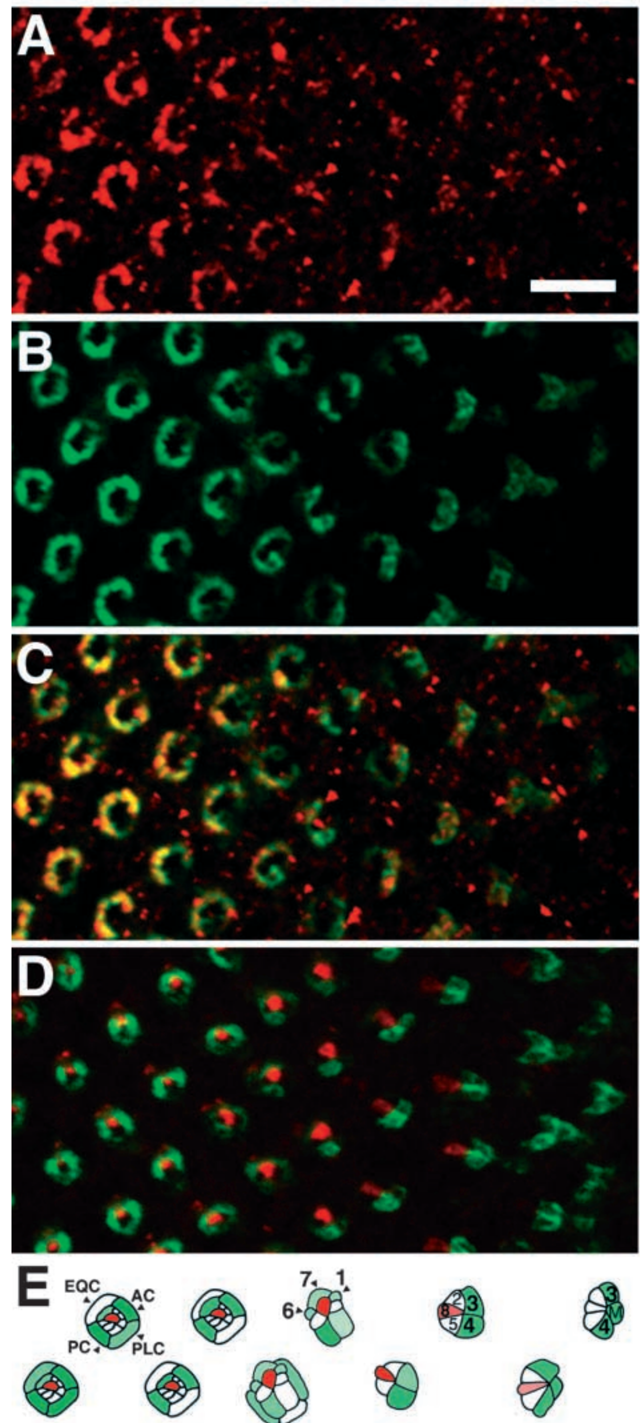

Fig. 4. Exboss-GCN proteins colocalize with the Sev receptor. Boss (red) and Sev (green) proteins were visualized by confocal microscopy of double-labeled eye discs expressing Exboss-GCNc under control of the boss promoter (A-C) or wild-type Boss protein (D). Exboss-GCNc localizes to a subset of ommatidial cells (A), in a pattern similar to the Sev receptor (B), as confirmed by the extensive colocalization in the merged image (C). The high level of background is presumably due to the diffusible nature of the Exboss-GCNc protein, no such background can be seen in a wild-type eye discs double-labeled for Boss and Sev (D). The schematic drawing in E represents the dynamic expression pattern of Sev (green) during early ommatidial development consistent with the original description (Tomlinson et al., 1987). Boss expression in R8 is shown in red. The right edge of A to E corresponds to row 2 posterior to the furrow. Bar, 5 μm (for all panels). The relevant genotypes are: (A to C) P[w^+, gen-Exboss-GCNc]; $boss^1$, (D) wild type.

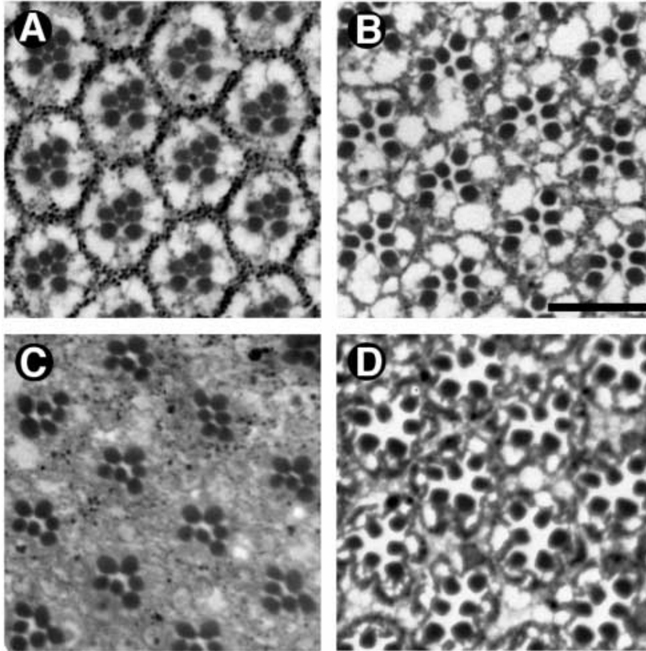

Fig. 5. Exboss-GCN proteins do not induce R7 development. Sections of eyes from adult flies with the following genotypes are shown: (A) wild type, (B) w^{1118}; P[w^+, gen-Exboss-GCNa], (C) w^{1118}; *boss*^{1}, (D) w^{1118}; P[w^+, gen-Exboss-GCNa] *boss*^{1}. Each ommatidium in panels A and B contains a single R7 cell, indicated by the small central rhabdomere. R7 cells are absent in D indicating that dimeric Exboss-GCN ligands cannot induce R7 development. Bar, 20 μm (for all panels).

stripes of 'R7-minus' ommatidia were observed in heat-shocked control flies, that were also heterozygous for *boss*^{1}, but lacked the Exboss-GCN transgenes (data not shown). The striped pattern is a consequence of the progression of eye development from posterior to anterior (Wolff and Ready, 1993). As the Sev requirement for each row of ommatidia is confined to a small window in development (Basler and Hafen, 1989a; Mullins and Rubin, 1991), so will be any effects of ubiquitously expressed antagonists of Sev activation.

Oligomeric Exboss-Lac ligands act as dominant-negative ligands

In the case of the Eph-receptors, secreted forms of their transmembrane ligands acted only as weak activators when dimerized. However, their activity could be potentiated by the formation of higher order oligomers (Davis et al., 1994). This is presumably due to their higher potential for more extended receptor crosslinking compared to the dimer. Based on this rationale, we wanted to test whether higher order oligomers of Exboss could function in R7 induction. Accordingly, we induced oligomerization of Exboss by fusion to the Lac tetramerization helix.

R8-specific expression of Exboss-Lac was not able to induce R7 development in a *boss*^{1} background (Fig. 7C). However, binding to the Sev receptor was evident by its localization to the apical surface of Sev-expressing cells in the developing eye disc (Fig. 7A and B). Like the dimeric Exboss proteins, Exboss-Lac inhibited R7 induction when expressed at high levels under heat shock control (Fig. 7D). Four 30 minute heat shock-induced pulses of Exboss-Lac expression resulted in an average of 2.5 columns that were R7-minus (n=4). In summary, oligomeric Exboss-Lac, just as the Exboss-GCN dimers, was able to bind to the Sev receptor in vivo, and acted as inhibitors of R7 induction.

Oligomeric forms of Exboss block R7 development by competing with wild-type Boss protein

The most straightforward interpretation of the antagonistic effects of the oligomeric forms of Exboss is that they to bind to the Sev receptor with high avidity and compete with wild-type Boss protein without activating Sev. While we cannot directly measure binding of the Boss ligand to Sev in vivo, Boss binding can be indirectly observed in the eye disc. Upon binding to the Sev receptor, the entire Boss transmembrane ligand is internalized into multivesicular bodies (MVBs) in R7 cells (Cagan et al., 1992; Krämer et al., 1991). Internalized wild-type Boss protein can be detected in MVBs in R7 cells using antibodies (anti-BossCT1 and anti-BossCT2) generated against the cytoplasmic domain of Boss (Fig. 8A, and Cagan et al., 1992). As this domain is not included in the oligomeric Exboss proteins, only the wild-type Boss protein is detected using the anti-BossCT1/2 antibodies. Upon heat shock-induced expression of Exboss-Lac, internalization of wild-type Boss into R7 cells was dramatically reduced (Fig. 8B). Inhibition of Boss internalization was also observed upon expression of the dimeric Exboss-GCN proteins (data not shown). The oligomerized forms of Exboss thus act as competitive inhibitors: they prevent binding of wild-type Boss to Sev, thereby blocking R7 induction.

DISCUSSION

The conserved 7TM and cytoplasmic domains of the Boss transmembrane ligand are required for its function in R7 induction, although the extracellular domain of Boss is sufficient for binding to Sev. One previously suggested explanation (Hart et al., 1993b) was based on the model that RTKs must dimerize or oligomerize to be activated (Schlessinger and Ullrich, 1992). In this paper, we tested the hypothesis that oligomerization of Boss's extracellular domain renders it able to activate Sev. We found that oligomerization enhanced Exboss binding to Sev, indicating that we had generated multivalent ligands. However, expression of these ligands inhibited, rather than activated, Sev signaling in the eye disc.

A ten-hour expression of these dominant-negative acting ligands resulted in a narrow dorso-ventral stripe of ommatidia lacking R7 cells. This stripe corresponds to the narrow time window in which Sev is required for R7 induction (Basler and Hafen, 1989a; Mullins and Rubin, 1991). Early photoreceptor cell development proceeds in a posterior to anterior wave across the eye imaginal disc at about 1.5 hours per row at 25°C in the late third instar larvae (Basler and Hafen, 1989a). At any given time, only a subset of R7 cells is in the process of being induced. Activation of Sev for every row of R7 precursor cells is restricted to a time window of about 9 hours based on the observation that Boss internalization can be observed from rows 6 to 11 posterior to the furrow (Krämer et al., 1991). Cells

more posterior are already committed to an R7 cell fate. During those 9 hours, Sev signaling is required for at least 6 hours to trigger R7 development (Mullins and Rubin, 1991); later expression of Sev appears to be inconsequential (Basler and Hafen, 1989a; Basler and Hafen, 1989b; Bowtell et al., 1989). In agreement with these previous results, we found that ubiquitous expression of Exboss oligomers for about 10 hours resulted in sufficient inhibition of Sev to block R7 development in a narrow stripe of about 3 to 5 rows (Figs 6 and 7).

Why do the oligomeric forms of Exboss not activate Sev? It does not appear likely that the tight association of the leucine zippers was occluding the interface of Exboss that is required for Sev binding, given that oligomerization actually enhanced receptor binding. A potential problem could be the specific spatial arrangement of the Exboss subunits in the Exboss-GCN dimers or Exboss-Lac oligomers. To address this issue we have tested the effect of the three versions of Exboss-GCN, which differ only in the orientation of their subunits relative to each other. Given the similarity of the effects of the three rotated Exboss-GCN forms and of Exboss-Lac, which used a different oligomerization unit, we do not believe that a specific spatial arrangement of the Exboss subunits is the cause of their dominant-negative effects. While we cannot rule out that oligomerization is necessary for Sevenless activation, the most straightforward explanation for our data is that the 7TM and cytoplasmic domains of Boss have an additional, different function in R7 induction.

The cytoplasmic or 7TM domains of Boss could be required for the correct conformation of the extracellular domain of Boss so that it can not only bind but also activate Sev. Integrins are an example for which control of ligand specificity by the intracellular domain has been demonstrated. A membrane-proximal cytoplasmic domain of integrins controls the affinity of the extracellular domains for their ligands (Hughes et al., 1995; O'Toole et al., 1994). Several attempts to distinguish between the roles for the cytoplasmic and 7TM domains of Boss using chimeras between Exboss and different transmembrane domains were inconclusive as none of these chimeric proteins were transported to the apical surface in eye discs (H. Krämer, unpublished observations and Hart et al., 1993b).

A requirement for the cytoplasmic domains has also been observed for ligands of the Notch receptor in *Drosophila*. For example, a mutation that removes the cytoplasmic and transmembrane domains of Serrate, *Beaded of Goldschmidt*, (Hukriede and Fleming, 1997) as well as secreted forms of Delta (Hukriede et al., 1997; Sun and Artavanis-Tsakonas, 1997) inhibit Notch signaling. This is likely due to the loss of

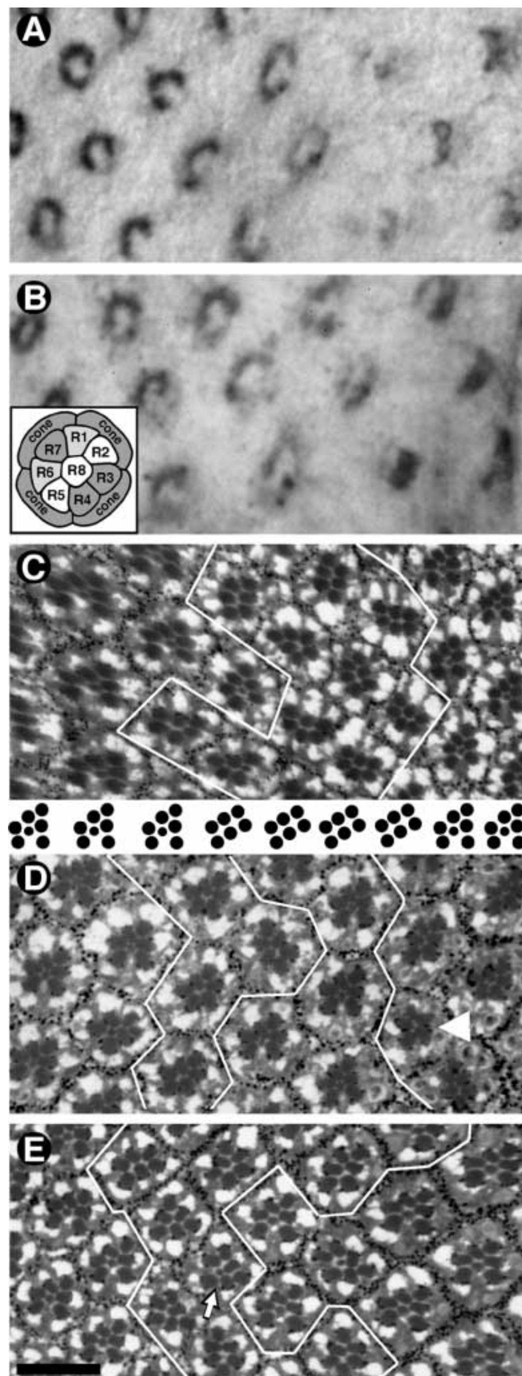

Fig. 6. Dimeric forms of Exboss bind to Sev in the eye disc and inhibit R7 induction. Heat shock-induced expression of Exboss-GCNa (A) or Exboss-GCNc (B) in $boss^1$ mutant eye discs was visualized by staining with anti-BossNN1 antibodies. The pattern of Exboss-GCN localization on the apical surface is indistinguishable from that of the Sev receptor (compare inset and Fig. 4). The right edge of A and B corresponds to row 2 posterior to the furrow. (C to E) Sections of eyes from adult flies that developed from larvae that were heat-shocked 4 times for 30 minutes at 37°C and allowed to recover for 2 hours at 25°C. Relevant genotypes of heat-shocked flies were (C) P[ry^+, HS-Exboss-GCNa] $boss^1$/+, (D) P[ry^+, HS-Exboss-GCNb]^{10}; $boss^1$/+, and (E) P[ry^+, HS-Exboss-GCNc]^{35}; $boss^1$/+. White lines indicate the borders between the posterior and anterior areas of wild-type ommatidia and the stripes of R7-minus ommatidia in between. An example for the scoring of R7-minus ommatidia is provided in the schematic drawing for panel C. In some cases we observed the appearance of two separate sevenless stripes, an example is shown in D. At a very low rate (about 1 in 200), ommatidia exhibited additional defects such as the lack of an outer photoreceptor cell (e.g. arrowhead in D). A few distally-displaced R8 cells (see legend to Fig. 7) could be noted in the stripe of ommatidia lacking R7 cells (arrow in E). Control eyes, lacking P-elements expressing oligomeric Exboss proteins, did not exhibit any loss of R7 cells under these conditions (data not shown). Bars: 4 µm (A,B); 20 µm (C-E).

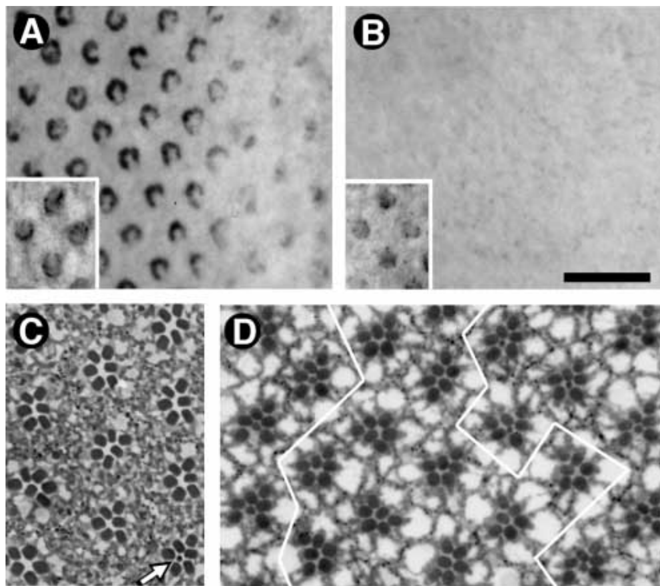

Fig. 7. Oligomeric Exboss-Lac binds to Sev and inhibits R7 induction. (A) Exboss-Lac was expressed under control of the boss promotor in a *boss*[1] background. Specific localization of Exboss-Lac to Sev-expressing cells (compare to Fig. 4) was visualized using anti-bossNN1 antibodies. No such localization was observed in *sev*[d2] mutant eye discs. (B) R8-specific expression of Exboss-Lac was revealed by its specific localization to the perinuclear region of R8 cells (inset in A), independent of the presence of Sev (inset in B). Expression of Exboss-Lac under control of the boss promotor had no phenotypic consequences in a wild-type background (data not shown) and did not induce R7 cells in a *boss*[1] background (C). In these lines, with the relevant genotypes P[w^+, gen-Exboss-Lac]; *boss*[1], we noticed a relatively high proportion of cells with central small rhabdomeres of up to 12% in some eyes (e.g. ommatidium marked with arrow). However, we concluded that these cells were distally displaced R8 cells, rather than R7 cells, based on the observation that, unlike R7 cells, they did not express fusions of *lac*Z to the R7-specific Rh3 and Rh4 promoters (Fortini and Rubin, 1990) and were not visible in the most distal sections in serial reconstructions (data not shown). (D) The antagonistic effect of Exboss-Lac on R7 induction was revealed by sections of eyes of adult flies of the genotype P[w^+, HS-Exboss-Lac]; *boss*[1]/+) that were heat-shocked as third instar larvae 4 times for 30 minutes at 37°C and allowed to recover for 2 hours at 25°C in between. R7-minus stripes, as described for the dimeric Exboss-GCN proteins, could be detected. Bar: 8 μm (A,B); 20 μm (C); 17.5 μm (D).

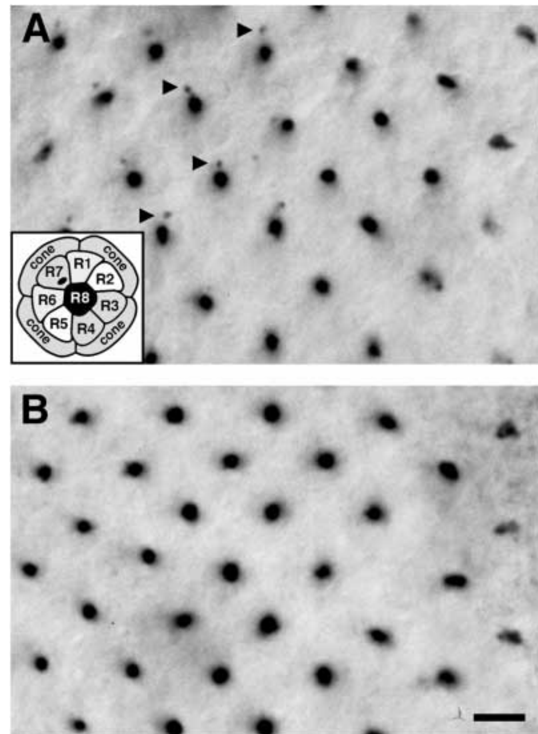

Fig. 8. Oligomeric Exboss acts as a competitive inhibitor for wild-type Boss. Third instar larvae were heat-shocked for 30 minutes at 37°C. After 1.5 hours at 25°C eye imaginal discs from wild-type (A) or P[HS-Exboss-Lac, w^+] larvae (B) were stained with anti-Boss CT antibodies, which recognize the cytoplasmic C-terminus of the wild-type Boss protein (Cagan et al., 1992) but not the secreted Exboss-Lac protein. Arrows indicate staining in multivesicular bodies in R7 cells in the wild-type eye disc (A). This staining is drastically reduced in eye discs expressing the Exboss-Lac protein (B). The right edge of both panels corresponds to row 3 posterior to the morphogenetic furrow. The inset in A indicates Boss localization relative to the position of photoreceptor cells in the ommatidia. Bar, 3 μm.

the cytoplasmic domain, as membrane-anchored forms of the Serrate and Delta proteins also act as dominant-negative ligands of the Notch receptor once their cytoplasmic domains are deleted (Sun and Artavanis-Tsakonas, 1996).

A complex role for the function of cytoplasmic domains of transmembrane ligands has been revealed in EphrinB ligands. Antibody-induced clustering obviated the requirement for their transmembrane and cytoplasmic domains for the activation of the EphB receptor tyrosine kinases (Davis et al., 1994). However, oligomerization appears to be only part of their function. In mice containing a protein-null mutant for the EphB2 receptor, some commissural axons were misguided (Henkemeyer et al., 1996). Interestingly, the ligands, rather than the EphB2 receptor, were expressed in these axons, raising the possibility that the ligand functioned in receiving and transducing a signal. Consistent with this idea is the finding that a mutant form of the EphB2 receptor, with β-Galactosidase replacing its tyrosine kinase domain, can rescue the axonal misguidance phenotype (Henkemeyer et al., 1996). This rescue is presumably due to the binding of the intact EphB2 ectodomain to the ligands. Additional support for bi-directional signaling between the EphB2 receptor and its ligands came from experiments demonstrating that EphrinB ligands respond to binding of a EphB2 receptor ectodomain by phosphorylation of conserved tyrosine residues in the ligands cytoplasmic tail (Brückner et al., 1997; Holland et al., 1996).

The structure of the Boss protein is reminiscent of seven transmembrane receptors, although no sequence homology has yet been identified. This similarity raised the possibility that Boss, like EphrinB ligands, may function not only as a ligand but also a receptor (Hart et al., 1990). One possible function for Boss as a receptor would be to act indirectly in R7 induction. However, constitutively active Sevenless receptor results in R7 induction even in the absence of Boss (Basler et al., 1991) arguing against a role of Boss as a receptor in the induction of R7 cell fate. A second possible

function for the Boss protein as a receptor is to affect R8 development. An R8-specific Rhodopsin, Rh5, is expressed in a subset of R8 cells that is paired with R7 cells expressing Rh3. Interestingly, in eye discs lacking R7 cells, R8 cells no longer express the Rh5 opsin (Chou et al., 1996; Papatsenko et al., 1997). These recent findings constitute the first indication that a signal from R7 influences gene expression in R8, and provide an assay to test the possibility that Boss acts as a receptor as well as a ligand.

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