The *scabrous* Gene Encodes a Secreted Glycoprotein Dimer and Regulates Proneural Development in *Drosophila* Eyes

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R8 photoreceptor cells play a primary role in the differentiation of *Drosophila* eyes. In *scabrous* (*sca*) mutants, the pattern of R8 photoreceptor differentiation is altered. The *sca* gene is predicted to encode a secreted protein related in part to fibrinogen and tenascins. Using expression in *Drosophila* Schneider cells, we showed that *sca* encoded a dimeric glycoprotein which was secreted and found in soluble form in the tissue culture medium. The *sca* protein contained both N- and O-linked carbohydrates and interacted with heparin. This Schneider cell protein was similar to protein detected in embryos. We showed that *sca* mutations, along with conditional alleles of *Notch* (*N*) and *Delta* (*Dl*), each affected the pattern of cells expressing *atonal* (*ato*), the proneural gene required for R8 differentiation. In normal development, about 1 cell in 20 differentiates into an R8 cell; in the others, *ato* is repressed. *N* and *Dl* were required to repress *ato* in the vicinity of R8 cells, whereas *sca* had effects over several cell diameters. Certain antibodies detected uptake of *sca* protein several cells away from its source. The overall growth factor-like structure of *sca* protein, its solubility, and its range of effects *in vivo* are consistent with a diffusible role that complements mechanisms involving direct cell contact. We propose that as the morphogenic furrow advances, cells secreting *sca* protein control the pattern of the next ommatidial column.

Cell-cell signals mediated both by diffusible factors and by direct cell contact are thought to be important during development. Studies of the *Drosophila* eye have contributed examples of interactions between neighboring cells (49). Definitive proofs of diffusible signaling have been harder to obtain. Here we have characterized the *scabrous* protein, a candidate secreted signal acting in eye development.

The *Drosophila* retina is made up of hundreds of 20-cell ommatidia, precisely arranged in a hexagonal array. The origin of the array during development is not fully understood. As the wave of differentiation crosses the eye imaginal disc from posterior to anterior, the ommatidia in each new column form out of phase with those in the previous column. Within each ommatidium, R8 photoreceptor cells are the first cell type to differentiate, and each R8 cell is required for differentiation of the other ommatidial cells. R8 cell determination is thus important for the entire retinal pattern (24, 33, 43).

Several lines of evidence suggest that determination of the R8 cells shares features with determination of neural cells from undifferentiated ectoderm in other parts of the nervous system. R8 differentiation requires the basic helix-loop-helix protein *atonal* (*ato*), similar to the proneural genes required for other neural cells (24). Loss of *Notch* (*N*) or *Delta* (*Dl*) gene function results in supernumerary R8 cells, similar to the neural hypertrophy that these neurogenic mutations cause elsewhere (3, 5, 32). Retinal differentiation is delayed by the helix-loop-helix genes *hairy* and *extramacrochaetae*, and these are also negative regulators of neural development elsewhere in the nervous system (8).

Neural cells are often determined through competition mediated by direct contact between cells expressing both proneural and neurogenic genes. One of the competing cells becomes

a neural precursor; the others are inhibited and lose proneural gene expression. *Notch* is the receptor and *Delta* is the cell surface ligand for the inhibitory cell signaling (1, 11, 21, 22, 39). However, in the developing retina, the R8 photoreceptor cells arise too far apart to be placed only by competition between neighboring cells. Within column 0, R8 cells are about 10 cells apart. R8 cells in column 0 are about six cells distant from the nearest R8 cells in column 1 (5, 47). In this arrangement, there are many cells that do not become R8 but that, not being neighbors of R8 cells, cannot have been inhibited after competition with an adjacent R8 precursor. Consistent with the limited range of cell contact interactions, reduction of *Notch* or *Delta* gene function leads to groups of adjacent R8 cells instead of single ones but does not convert all intervening cells to the R8 cell fate (5). Therefore, additional mechanisms and genes must be involved to prevent all cells from becoming R8 cells. One possibility is that a sequence of cell-cell interactions, of which that mediated by *N* and *Dl* is one, directs the fate of cells successively more distant from the R8 cells. A second possibility is that diffusible signals supplement direct cell contact to inhibit more distant cells.

The *scabrous* (*sca*) gene may encode one such diffusible signal in the eye. The *sca* gene sequence predicts a secreted protein (Fig. 1). Presence of a fibrinogen-related domain (FReD) makes *sca* the only *Drosophila* member of an expanding group of FReD proteins, including vertebrate fibrinogen and tenascin proteins (13). In *sca* mutants, the placement of R8 cells becomes irregular, and some ommatidia with multiple R8 cells develop (3, 29). The *sca* phenotype is cell nonautonomous in genetic mosaics, indicating that cell-cell signals are affected (3). In *N; sca* double mutants, R8 hypertrophy is greater than in *N* single mutants, indicating greater range of *sca* gene function (5).

Experimentally, it is difficult to distinguish between true diffusible action at a distance and effects mediated by relays of shorter-range signals. Many secreted factors may in fact be immobile outside the cell (for example, see reference 45).

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FIG. 1. Structures of *sca* sequences expressed in this study. (A) Main features of the wild-type sequence. Signal peptide cleavage is most likely after Ala-22 (arrow). Since this has not been determined experimentally, we continue to number residues from Met-1. The box (Asp-516–Glu-715) corresponds to the FReD and is 30 to 40% identical to other FReDs (2, 12). Cys-517 and Cys-543 are conserved in other FReDs and form an intrachain disulfide bond in fibrinogen chains. The same is true for Cys-662 and Cys-675. However, Cys-594 is not found in other FReDs. Cys-276, Cys-298, and Cys-716 lie in sequences which are not related to other known proteins. Asterisks indicate consensus glycosylation sites (Asn-347, Asn-562, Asn-593, Asn-635, Asn-721, Asn-762). Also indicated are regions recognized by antibodies. Studies with deleted proteins map the mAbsca1 epitope within the Leu-41–Ser-275 region. Polyclonal scaR antisera were obtained after immunization with a fusion protein of TrpE with Glu-578–Ala-774, but in vivo immunohistochemistry with truncation mutant alleles (22) shows that the scaR antiserum recognizes the Trp-698–Ala-774 region. Antipeptide antisera recognize Lys-257 to Ala-269 (scaA) and Ser-649 to His-661 (scaB). Below are shown primary structures of deleted proteins used in this paper.

Immunoelectron microscopy has not detected extracellular *sca* protein (5), and *sca* is partially homologous to the large hexameric fibrinogen and tenascin proteins. Fibrinogen polymerizes to an insoluble form after proteolytic cleavage to form fibrin. Polymerized fibrin is the major constituent of blood clots (14). Tenascins are components of extracellular matrix (17). It was thus possible that only adjacent cells would be exposed to secreted *sca*, in which case action at a distance would have to be relayed through other signals.

In this study, we sought to determine whether the biochemical properties of *sca* protein were consistent with the range of the *sca*-dependent signals in vivo, or whether a relay of other signals had to be invoked. We found that *sca* encoded a secreted, homodimeric glycoprotein that was soluble in culture cell medium. We examined the effects of *sca*, *N*, and *Dl* mutations on expression of the proneural gene *atonal* (*ato*) and report that all of these genes acted to repress *ato* expression. The inferred range of *sca* signaling was several cell diameters, and *sca* protein was found to have been taken up by cells across similar distances. Therefore, properties of the *sca* protein are consistent with the model that *sca* itself encodes a diffusible regulator of R8 determination.

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MATERIALS AND METHODS

Bacterial protein expression. PCR was used to replace 5' sequences from *pcsb* (29) with an *NdeI* restriction site, and this modified cDNA was inserted into the *NdeI* site of PET5a (42). Transcription by T7 polymerase led to the expression of *sca* protein with the N-terminal 22 residues (the putative signal peptide) replaced by Met. Protein was purified by electroelution from sodium dodecyl sulfate (SDS)-polyacrylamide gels and solubilized as described previously (19). The gel mobility was slower than expected (apparent molecular mass of ~110 instead of 87 kDa), but protein sequencing confirmed that the N terminus was as predicted. Because of the anomalous migration of *sca* proteins, migration of hybridoma protein was also determined on most of the gels in this study.

Antibody preparation. Polyclonal scaT antibodies were raised to all of the putative *sca* protein; scaR antibodies were raised to amino acids 578 to 774 (3, 29). To obtain antisera without use of denatured antigen, the *sca* open reading frame (the *EcoRV*–*SmaI* fragment from *pcsb*) was cloned into *SmaI*-digested phosphatase-treated pGEX-2T (41). Under optimal conditions ~2% of the *sca*-glutathione *S*-transferase fusion protein was soluble and could be recovered by glutathione *S*-transferase affinity. BALB/c mice were immunized with 20 μ g of fusion subcutaneous protein in Freund's complete adjuvant and boosted several times in Freund's incomplete adjuvant, and fusion was performed with spleen cells and myeloma P3X63-Ag8.653 cells as described previously (20). Supernatants were screened by affinity for T7 *sca* protein in an enzyme-linked immunosorbent assay, and the mAbsca1 subclone was obtained. Experiments with anti-immunoglobulin antibodies indicate that mAbsca1 is an immunoglobulin G3 class antibody. It may be worth mentioning that this is the only stable hybridoma line recovered from many promising subclones in six fusions involving experienced laboratories at the Albert Einstein College of Medicine and the University of California, Berkeley. After pristane priming, hybridoma cells were injected into BALB/c mice, and immunoglobulins were purified by protein G affinity (Pierce). The mAbsca1 antibody is available from the Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, Iowa City.

Two rabbit antipeptide antibodies, corresponding to residues 257 to 270 (C KLQQRTRKDGSSA; scaA) and residues 649 to 661 (SAIDDDRISQTH; scaB) of *sca* protein, were synthesized and purified by high-pressure liquid chromatography, and each was coupled to keyhole limpet hemocyanin. The coupled peptides were injected into rabbits (Hazleton Research Products, Inc.). Sera were affinity purified with peptides coupled to Actigel ALD (Sterogene).

Schneider cell expression. pHsca was obtained by cloning 0.4 kb from the *hsp70* gene promoter and 4.3 kb from the *pcpneo* vector (35) into the *HindIII* site in the *pcsb* polylinker. Transfection and selection were performed as described previously (35). Cell cloning was by limiting dilution in conditioned medium. The cell line obtained expressed *sca* proteins constitutively regardless of heat shock. pAct5CscA was obtained by transferring an actin 5C promoter-copia neomycin cassette (37) into the *pcsb* *HindIII* site as a Klenow enzyme-treated *NotI* fragment. pMTsca8 was obtained by inserting the *XhoI*–*BamHI* fragment from *pcsb*, made flush with Klenow enzyme, into *BamHI*-digested, Klenow enzyme-treated pRmHa3 (9). Deletion of the *sca* leader to the *XhoI* site at nucleotide 204 was required for good expression in Schneider cells.

Deletion constructs were made by using PCR to replace either the 5' or the 3' portion of *pcsb* with *KpnI* sites. 5' *EcoRI*–*KpnI* fragments, 3' *KpnI*–*BamHI* fragments, and *EcoRI*–*BamHI*-digested Bluescript (Stratagene) were ligated in three-way reactions. *XhoI*–*BamHI* fragments from the products were treated with Klenow enzyme and ligated with *BamHI*-digested, Klenow enzyme-treated pRmHa3 to give internally deleted expression constructs. pMTsca Δ 41-463, pMTsca Δ 41-514, pMTsca Δ 276-463, pMTsca Δ 276-514, pMTsca Δ 317-463, and pMTsca Δ 317-514 all have the indicated amino acids replaced by Gly and Ser encoded by the *KpnI* site.

The Schneider 2 (S2) cell line was maintained at 25°C in Schneider medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and penicillin (50 U/ml)-streptomycin (50 μ g/ml) (Gibco). For transfection, 20 μ g of DNA in 25 μ l of distilled water–25 μ l of Lipofectin reagent (Gibco) was added to 1.5 ml of log-phase cells (10⁶ cells per ml of unsupplemented M3 medium) overnight. For selection of stable lines, 4 μ g of pPC4 (26) was cotransfected; after 24 h, cells were grown in the presence of 5 μ g of α -amanitin per ml for 1 month.

Immunohistochemistry. S2 cells were washed in phosphate-buffered saline, settled onto gelatin-coated slides, and fixed in acetone (–20°C, 10 min). Blocking (10 min), antibody incubations, and washes were in 1% bovine serum albumin–0.1% saponin–0.15 M NaCl–10 mM phosphate (pH 7.4). The primary antiserum was scaT, the secondary antiserum was horseradish peroxidase-conjugated goat anti-mouse (Bio-Rad), and diaminobenzidine was the peroxidase substrate in the presence of 1.5 mM NiCl₂–1.5 mM CoCl₂.

Imaginal discs were fixed in 4% formaldehyde–0.1 M piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.9)–2 mM EGTA–1 mM MgSO₄ for 20 min on ice. Blocking for 1 h in 0.1 M NaPO₄ (pH 7.2)–5% normal goat serum–0.5% Nonidet P-40 (NP-40) was followed by incubation overnight (4°C) in anti-ato

FIG. 2. Expression of *sca* by cultured cells. (A) *Drosophila* S2 cells do not express scabrous protein. (B) scabrous protein is detected in transfected S2 cells. Many cells show punctate intracellular staining like that detected in vivo.

serum (23) diluted 1:50,000 and preadsorbed extensively with fixed homogenized *Drosophila* larvae. The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit (Bio-Rad). For *sca*/atonal double labeling, peripodial membranes were removed, and mAbsca1 ascites fluid diluted 1:200 was used in the primary antibody solution; fluorescein isothiocyanate-conjugated goat anti-rabbit and Texas red-conjugated goat anti-mouse (Jackson ImmunoResearch) were used as secondary antisera. Confocal analysis was performed with a Bio-Rad MRC600 instrument. For the heat shock experiment, wild-type (Oregon R) larvae in 1.5-ml microcentrifuge tubes were heated in water baths (38°C, 30 min). Recovery was at 25°C. Tissue was dissected in fixative and stained as described previously (5), using the scaR antiserum.

Electrophoresis of proteins was in 7% polyacrylamide gels in the presence of SDS. Two percent 2-mercaptoethanol was added to sample buffer for reducing gels. After transfer to nitrocellulose (Probind 45 membrane; Pharmacia), filters were blocked (0.15 M NaCl, 10 mM Tris-HCl [pH 7.0], 0.05% NP-40, 5% nonfat milk; 1 h). Antisera were used at the following dilutions: mAbsca1 ascites fluid, 1:20,000; scaA, 1:300; scaB, 1:100; and scaR, 1:1,000. Horseradish peroxidase-conjugated secondary antibodies (1:10,000; Bio-Rad) were visualized with ECL reagents (Amersham).

Pulse-chase labeling and immunoprecipitation. A cloned pMTsca8 transfectant (6×10^6 cells per ml) was induced with 0.7 mM CuSO₄ for 12 h. After 6 h in M3 medium lacking cysteine or methionine, 500 μ Ci of [³⁵S]methionine and [³⁵S]cysteine (Trans³⁵S-label; ICN) was added per ml of cells. After labeling, an equal volume of complete, unlabeled M3 medium was added. For analysis, cells and medium were separated by centrifugation. Cell pellets were resuspended in lysis buffer (0.3 M NaCl, 50 mM Tris-HCl [pH 8.0], 1.0% NP-40, 1.0% deoxycholate, 1 mM phenylmethylsulfonyl fluoride [PMSF]) and passed through a 22-gauge needle several times. Medium was recentrifuged at high speed; the supernatant was made 1× in lysis buffer and centrifuged again. Samples were incubated with the polyclonal scaT antiserum (25 μ l of 40×-diluted serum per ml of culture); after 1 h at 4°C, 10 μ l of Pansorbin cells (Calbiochem) was added; after 1 h at 4°C, the cells were centrifuged, and the pellets were washed with lysis buffer and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Controls consisted of untransfected S2 cells or addition of 50 μ g of unlabeled T7 sca prior to immunoprecipitation.

Glycosidase studies. ³⁵S-labeled immunoprecipitates were prepared as follows. For endoglycosidase H (endo H), pellets from 10⁷ cells were resuspended in 22 μ l of 0.1 M sodium citrate (pH 5.5)–0.1% SDS and boiled (3 min); 2 μ l of endo H (1 U/ml; Boehringer) and 1 μ l of 25 mM PMSF were added, and after 14 h at 37°C, another 1 μ l of endo H was added for 2 h more. In mock reactions, H₂O was added instead of enzyme. For *N*-glycanase, pellets were resuspended in 10 μ l of 0.5% SDS–0.1 M β -mercaptoethanol and boiled (3 min). Addition of 20 μ l of buffer (0.3 M NaPO₄ [pH 8.6], 10 mM EDTA, 2% NP-40, 1.25 mM PMSF) was followed by addition of 2 μ l of *N*-glycanase (250 U/ml; Genzyme). Incubation for 14 h (37°C) was followed by further addition of 1 μ l of enzyme for 2 h. H₂O was used for mock reactions.

TFMS. After 20 h of induction, 1 ml of medium was exchanged into 0.1 M ammonium carbonate over Sephadex G-50, lyophilized, and reacted with trifluoromethanesulfonic acid (TFMS) as described previously (15).

Embryonic proteins. Batches of 25,000 embryos were lysed (300 mM NaCl, 50 mM Tris-HCl [pH 8.0], 0.5% NP-40, 0.5% deoxycholate, 1 mM CaCl₂, 1 mM MgCl₂, 20 μ g of aprotinin per ml, 1 μ g of leupeptin per ml, 20 μ g of [4-amidinophenyl]-methanesulfonyl fluoride per ml). The solution was mixed, passed through 18-, 21-, and 25-gauge needles, and microcentrifuged (10 min, 4°C), and supernatants were immunoprecipitated with mAbsca1. Immunoprecipitates were electrophoresed and immunoblotted with the scaA rabbit antiserum.

***Drosophila* stocks.** Most strains have been described previously, as have the

temperature shift procedures for the *N^{ts}* allele and the *D^{ts}* genotype (*D^{ts}*/*D^{ts}*) (5). HSsca and 4Xrosca transformatant flies were provided by M. Mlodzik (16).

RESULTS

Cultured *Drosophila* cells glycosylate and secrete scabrous protein. Sca protein was not detected in Western blots (immunoblots) of *Drosophila* embryos, embryonic membrane fractions, perivitelline fluid, or dissected eye imaginal discs, although immunohistochemistry indicated that the protein should be present in these samples. To initiate biochemical characterization, S2 cells were transfected with plasmids designed to transcribe a *sca* cDNA, and similarity to the proteins in vivo was investigated later. Most of the experiments used a cloned cell line (MTsca8) derived from cells transfected with a metallothionein promoter-*sca* cDNA plasmid, but similar proteins were also detected after expression under control of the *Drosophila* heat shock protein 70 or actin 5C promoter. Main features of the *sca* sequence are shown in Fig. 1. Figure 2 shows immunolocalization of *sca* proteins expressed in S2 cells.

Metabolic labeling and pulse-chase analyses were performed to investigate the *sca* gene products. Labeled proteins were immunoprecipitated from the cell lysates and tissue culture media by using polyclonal antisera raised against the entire protein (scaT) (Fig. 3A and B). After 2 h, most of the labeled sca protein was outside the cells. [³⁵S]methionine first incorporated into 115-kDa intracellular proteins (form I) (Fig. 3A). About half this material was converted to 130 kDa (form II) within 15 min. Intracellular levels of both then decreased in the cell lysate as form II accumulated in the medium (Fig. 3B). Thus, form I is the precursor of form II, which is subsequently secreted.

Most of the sca protein was recovered in soluble form in the medium, although levels began to decay after 6 h. Negligible protein was recovered from material associated with the tissue culture dish (Fig. 3). This is in contrast to what has been found for certain other secreted proteins, which either remain associated with the cell surface or become bound to the culture vessel along with other material (6, 17, 25, 28, 29, 42). However, secreted sca protein bound quantitatively to a heparin-Sepharose column and required high salt concentrations to be eluted (Fig. 3C). Such heparin binding is consistent with in vivo interactions with heparan sulfate proteoglycans (6).

All of the sca proteins migrate more slowly than predicted by the *sca* cDNA sequence (87 kDa) and more slowly than bac-

FIG. 3. [35 S]methionine labeling of MTsca8 cells induced to express sca by addition of copper to the medium, determined by immunoprecipitation and autoradiography of sca proteins. (A) Cell lysates prepared after pulse-labeling. Times of labeling pulse and subsequent chase with cold methionine are indicated in minutes. Lane 1, untransfected S2 cells; other lanes, MTsca8 cells. For lane 2, sca protein synthesized in bacteria was added to the immunoprecipitation step as a competitor. Lanes 3 to 10, label is incorporated into two species, forms I and II. Form I is the precursor of form II. Lane 11, protein recovered from the medium (see panel B) comigrates with form II. Lane 12, negligible protein is recovered from material on the culture plate. (B) Analysis of culture media from the same experiment. Form II appears in the medium 30 min after labeling begins. (C) Interaction of scabrous with heparin. Shown are slot immunoblots after heparin-Sepharose column chromatography. Scabrous protein (2 μ g/ml in culture medium) was completely bound by heparin-Sepharose (Flowthrough) and was eluted from the column by high salt. Most sca protein was eluted between 0.4 and 0.7 M sodium chloride. Binding of this affinity is likely to be physiologically significant. Sizes are indicated in kilodaltons.

terially expressed sca (110 kDa). This finding suggests post-translational modification. The contribution of glycosylation was investigated by using three procedures. Endo H removes the immature, N-linked carbohydrates added in endoplasmic reticulum. N-Glycanase removes both immature and complex N-linked sugars. TMSF removes both N-linked and O-linked sugars. We found that form I contains about 10 kDa as a result of immature N-linked sugars (Fig. 4A). Since [35 S]methionine was incorporated directly into form I without appearance of lower-molecular-weight forms, addition of N-linked carbohydrate must be cotranslational. The 10-kDa probably corre-

FIG. 4. Glycosylation of sca proteins. (A) Lanes: 1 and 2, endo H treatment (lane 2) and mock reaction (lane 1) (endo H has little effect on form II, but form I is reduced to $\sim$ 110 kDa by removal of immature high-mannose carbohydrates); 3 and 4, N-glycanase (N-Glyc; lane 4) and mock reaction (lane 3). N-Glycanase should remove all N-linked sugars and also reduces form I to $\sim$ 110 kDa. In addition, form II is reduced to $\sim$ 120 kDa, indicating the presence of matured N-linked sugars in this protein. Two bands after N-glycanase treatment indicate additional modification of form II. (B) TFMS removes all carbohydrates from glycoproteins. Scabrous form II from culture medium (lane 1) was reduced to $\sim$ 110 kDa by TFMS treatment (lane 2). The mobility of this deglycosylated protein was similar to that of sca protein synthesized in bacteria (lane 3). We do not know if any remaining difference reflects another modification or a different position of signal peptide cleavage from the prediction used as a basis for the *Escherichia coli* protein. Sizes are indicated in kilodaltons.

sponds to fewer than the six potential glycosylation sites in the sequence. Form II was little affected by endo H, but N-glycanase removed about 10 kDa of mature N-linked carbohydrate (Fig. 4A). Since form II contained about 20 kDa of total carbohydrate (Fig. 4B), about 10 kDa must be O linked. The mature form II protein is secreted. The electrophoretic mobility of completely deglycosylated sca was similar to that of bacterial protein, and so glycosylation may be the major if not only posttranslational modification (Fig. 4B).

To test whether any of the sca proteins made by S2 cells were cleaved to a smaller form, Western blotting was performed with region-specific antibodies. All of the antibodies shown in Fig. 1 detected the same sca protein species, providing no evidence for proteolytic processing (Fig. 5).

On the basis of DNA sequence, the sca protein is predicted to contain eight cysteines (Fig. 1). When migration was compared on reducing and nonreducing SDS-gels, unreduced samples had apparent molecular masses close to 300 kDa, indicating disulfide-linked multimers (Fig. 6). The 300-kDa forms were excised, reanalyzed under reducing conditions, and found to contain only the form I and form II proteins already de-

FIG. 5. Region-specific antibodies. (A) Similar sca proteins (form II) detected with antibodies raised against fusion proteins. Lanes: 1, 3, and 5, S2 cells; 2, 4, and 6, HSSca cells; 1 and 2, mAbsca1; 3 and 4, scaT; 5 and 6, scaR (longer exposures still show only one band). (B) Antipeptide antibodies. Lanes: 1 and 3, medium from MTsca1 cells; 2 and 4, medium from S2 cells; 1 and 2, scaB; 3 and 4, scaA. Sizes are indicated in kilodaltons.

FIG. 6. Metabolically labeled cell lysates from the experiment shown in Fig. 3 were immunoprecipitated and analyzed without reduction. Most label appears first in an ~280-kDa protein which matures into an ~300-kDa protein. Some protein appears at around 110-kDa and similarly increases in apparent molecular mass with time. These monomer forms migrate more slowly under reducing conditions (see Fig. 3). Pulse and chase times are indicated in minutes; sizes are indicated in kilodaltons.

scribed, with no additional proteins (data not shown). In addition, monomer species run more rapidly without reduction, consistent with the existence of intrachain bonds as well (Fig. 6). Pulse-chase experiments indicate that the 300-kDa species formed immediately, consistent with multimerization and in-

terchain disulfide bond formation within the endoplasmic reticulum (Fig. 6).

Coexpression of a shorter sca protein was used to distinguish whether disulfide-bonded species were sca homodimers, trimers, or other structures. Plasmid pMTsca Δ 320-463 directs synthesis of sca protein in which amino acids 320 to 463 are replaced by the amino acids Gly and Thr encoded by a *KpnI* restriction site. All eight cysteines are still present. Monomeric sca Δ 320-463 has an apparent molecular mass of 90 kDa, which is increased to 220 kDa without reduction, compared with 130 and 300 kDa for wild-type sca (Fig. 7A and B). S2 cells cotransfected with pMTsca and pMTsca Δ 320-463 secreted three molecular weight forms, one corresponding to intact sca, one corresponding to the deletion, and one representing a single intermediate species which therefore must be heterodimeric (Fig. 7A). The heterodimer was not seen when cells were separately transfected and conditioned media were combined, eliminating the possibility of reassortment during isolation and gel loading (data not shown). This experiment showed that the secreted proteins were disulfide-linked sca dimers and not a complex with other proteins.

A still smaller protein, sca Δ 276-463, ran as a monomer on both reducing and nonreducing SDS-gels, suggesting that residues Cys-276 and Cys-298, which are deleted from this protein, might be involved in interchain bonds (Fig. 7B and C). Proteins encoded by pMTsca Δ 41-463 and pMTsca Δ 41-514, lacking most sequences amino terminal to the FR Δ , were also monomeric (Fig. 7B and C and data not shown). These results suggest that the amino-terminal portion of sca may be important for dimerization, although deletion experiments must be interpreted with caution since structures different from the wild-type protein may sometimes result (reference 23 and our unpublished results).

Similarity of sca products in cultured cells and flies. Using two approaches, we were able to detect sca proteins in vivo and found them similar to the proteins expressed by S2 cells.

Flies transformed with HSca constructs express immunohistochemically detectable sca following heat shock. This transient expression does not appear to have phenotypic effects

FIG. 7. Dimerization. (A) Cells were transfected or cotransfected with a full-length clone (pMTsca1) or an internal deletion mutant (Δ 320-463); amounts shown are micrograms per 10^6 cells. A mAbsca1 Western blot is shown; secreted proteins were electrophoresed without reduction. The Δ 320-463 protein (220 kDa) can form a single intermediate species (260 kDa) when coexpressed with wild-type protein (300 kDa), indicating heterodimer formation. (B) Wild-type and deleted proteins under reducing conditions, Western blotted with the scaR antiserum (the mAbsca1 epitope is deleted from sca Δ 41-463). Lanes: 1, wild-type sca; 2, sca Δ 320-463; 3, sca Δ 276-463; 4, sca Δ 41-463. (C) Same samples without reduction. A minor band at 300 kDa in all lanes is due to a scaB-cross-reactive protein and is seen in untransfected controls. Wild-type (lane 1) and sca Δ 320-463 (lane 2) proteins are largely dimers (300 and 220 kDa); sca Δ 276-463 (lane 3) and sca Δ 41-463 (lane 4) proteins are monomers. Sizes are indicated in kilodaltons.

FIG. 8. Scabrous expression in vivo. (A) Western blots of fly embryo and S2 cell extracts. Lanes: 1, HS*sca* transgenic embryos without heat shock; 2, HS*sca* embryos, 40-min heat shock (37°C), 5-min recovery; 3, HS*sca* embryos, 40-min heat shock, 30-min recovery; 4, *sca* proteins in cultured cells. Note similar gel mobility with in vivo protein in lane 2. (B) Western blots of immunoprecipitated embryo lysates (lanes 1 and 2) and of scabrous proteins from transfected cells (lanes 3 and 4), 25,000 3- to 6-h embryos per lane. Lanes: 1, *sca*^{BP2} null mutant; 2, Canton S wild type; 3, pMT*sca*1 cell lysate; 4, pMT*sca*1 culture medium. Most embryo protein (lane 2) seems comparable to the form I protein from transfected cells (lane 3). (C) Same as panel B except that samples were analyzed without reduction. Embryo protein is also dimerized. Sizes are indicated in kilodaltons.

(16). After Western blotting of embryo lysates, the expressed proteins appear similar to those made by the MT*sca*8 cells (Fig. 8A). However, since most of this protein must be expressed in cells that do not normally synthesize *sca*, it remained possible that *sca* was differently modified in its normal locations.

Expression of the endogenous gene was not detected by simple Western blotting, perhaps because of the small subset of cells expressing *sca*. We overcame this difficulty with a combined immunoprecipitation-immunoblotting procedure. We did not attempt mass isolation of imaginal discs because of the apparent short protein half-life in vivo. For example, *sca* protein becomes undetectable by immunocytochemistry within minutes of dissection of tissues (data not shown). Instead we examined *sca* protein expressed by the neurogenic ectoderm of embryos (29). Eggs from wild-type and *sca* strains were collected in population cages and aged to 4.5 ± 1.5 h, the stage at which the largest proportion of cells express *sca* (50). Lysates from 25,000 4.5 ± 1.5 -h embryos were first concentrated by immunoprecipitation and then electrophoresed. Western blotting identified *sca* proteins similar to the MT*sca*1 proteins (Fig. 8B and C). Thus, the major product of at least one tissue that normally expressed *sca* is similar in structure to the protein we have characterized in cultured cells.

R8 patterning by repression of *atonal*. To investigate the mechanism of *sca* function in vivo, we determined the effects of *sca* and other mutations on expression of the proneural gene *atonal*. R8 cell differentiation depends on *ato* autonomously, so spatial regulation of *ato* function may be required for the pattern of R8 cell differentiation (24).

In wild-type flies, *ato* expression is initiated by all cells just anterior to the morphogenetic furrow (24) (Fig. 9A). Expression is lost from all cells except the emerging R8 cell precursors. Loss of *ato* expression occurs in two phases. First, *atonal* disappears from about half of the cells but remains in groups of cells, called intermediate groups, evenly spaced across the furrow. The spacing of these cell groups gives *ato* expression the appearance of a sine wave across the posterior of the furrow. Subsequently, *atonal* further disappears from all but one cell per group, which is the R8 precursor (24, 25) (Fig. 9A).

The *sca* protein is expressed in R8 cell precursors and initially in groups of cells from each of which one R8 cell derives

(5, 29). Double labeling shows that *sca*-expressing cells are a subset of the *ato*-expressing cells, corresponding to the intermediate groups (Fig. 9B). However, *sca* is not detected earlier when all cells are *atonal* positive (Fig. 9B). *Notch* and *Delta* are expressed by all of the morphogenetic furrow cells, although levels are modulated later (5).

When *ato* expression was examined in flies with *N^{ts}* or *Dl^{ts}* genotypes, we found *ato* expressed in clusters of cells instead of single R8 precursors (Fig. 9C and D). This persistent *ato* expression implies failure to repress *ato* in cells near the R8 precursor.

More extensive *ato* expression was also seen in *N^{ts}*; *sca* double mutants than in *N^{ts}* single mutants (Fig. 9E). The appearance of spaced cell groups was lost, implying that *ato* expression was retained in many cells that would lie in the troughs of the wave in the wild type and so be among the first cells to repress *ato* (Fig. 9E). In the most severely affected individuals, all cells retained *ato* expression (Fig. 9E). Thus, in *N^{ts}*; *sca* double mutants, multiple modes of *ato* repression were lost, affecting both cells that might be neighbors of R8 cells in the wild type and many more distant cells. This finding means that *sca* mutations affect cells in addition to the cells that express the *sca* gene.

In *sca* single mutants, the pattern of *ato* was altered. Expression was largely restricted to single R8 precursor cells, but their placement was disordered and variable. The regular pattern of intermediate groups was not apparent (Fig. 9F). This finding also indicates that *sca* mutations affect the initial phase of *atonal* repression. It is difficult to explain exactly how the *ato* pattern in *sca* mutants arises, but repression must depend on N and Dl to a greater extent than in the wild type.

In addition to loss-of-function mutations, the pattern of ommatidia is made irregular by a uniform pattern of *sca* expression directed by the *ro* promoter (16). We found an altered pattern of *atonal* expression in this genotype. Intermediate groups were not seen, indicating more synchronous loss of *ato* from all but an irregular pattern of R8 precursor cells (Fig. 9G).

Range of *sca* diffusion in vivo. Both *sca* mutations and misexpression of *sca* protein change the pattern of *ato* repression, even though *ato* is first repressed in cells that never express *sca*. Thus, the first repression of *ato* must be regulated

FIG. 9. Proneural development in eye imaginal discs. Anterior is to the left in all panels. Except for panel B, these pictures of *ato* expression show a single focal plane in the morphogenetic furrow, and further positive nuclei are not in focus. Nevertheless, the pictures are representative of the total pattern. (A) Atonal protein in the wild type. Expression in nuclei of all cells on the left reduces to an array of single R8 cells on the right. As the reduction occurs, the pattern of *ato* expression appears wavy, reflecting loss of *ato* earlier in the troughs than in the peaks of the wave pattern. (B) Confocal projection of *ato* expression (green) with *sca* expression (red). The *sca* protein is found in the R8 cells. Sometimes the cytoplasmic *sca* vesicles lie above or below the R8 nucleus and so appear yellow when projected on top of the *ato* staining. *sca* expression begins in the intermediate groups of cells, which are the cells of prolonged *ato* expression in the peaks of the wave. Previous studies showed that *sca* is restricted to single R8 cells by column 0 (arrow) (5). (C) N^{ts1} restrictive temperature, 4 h. The arrow shows the position corresponding to column 0, in part determined for the mutant genotypes from the position of the unaffected, normally patterned cells to the posterior. Reduction of *N* function leads to excess R8 cells in and posterior to column 0. Groups of R8 cells derived from peaks of the *ato* wave are seen. (D) Dl^{ts} restrictive temperature, 4 h. Groups of R8 cells form, as in the N^{ts1} strain. (E) $N^{ts1}; sca^{UM2}$ restrictive temperature (4 h). Reduction of both *N* and *sca* function gives a continuous swath of R8 cells, as almost all nuclei retain *ato* protein in and posterior to column 0. (F) $N^{ts1}; sca^{UM2}$ permissive temperature. Reduction of *sca* function leads to a disordered arrangement of R8 cells. Anterior to column 0, the loss of *ato* expression occurs in a ragged pattern, indicating less temporal order to the repression of *ato* than in the wild type. (G) Ectopic expression of *sca* in the 4Xrosca genotype. An abnormal R8 cell array is formed by column 0. More anteriorly, levels of *ato* expression were reduced and intermediate groups were not seen, indicating more synchronous repression of *ato* in non-R8 cells.

by a signal from the intermediate groups which are located to the posterior. The spacing of intermediate groups both in the wild type and in N^{ts} mutants indicates that this signaling has to extend about three cell diameters (see Fig. 11). Certain antibodies detect *sca* protein spreading over this range.

When eye imaginal discs were dissected in the fixative solution and *scaR* antibodies were used for immunohistochemistry, the distribution of *sca* protein was broader and more diffuse than that detected by other antibodies (Fig. 10A). Protein was detected between and anterior to the intermediate groups and

FIG. 10. Scabrous at a distance from the intermediate groups. All tissues were dissected in fixative solution and labeled with the *scaR* antiserum. (A) Confocal projection of *sca* protein in the wild type. Protein is detected in a diffuse pattern, with halos of weaker staining surrounding the intermediate groups and R8 cells (in columns 2 and 3 especially). (B) Same preparation as panel A, showing both *sca* (red) and *ato* (green). Overlapping expression appears yellow. Scabrous is more widely detected than with other antibodies and extends anterior to the intermediate groups, into regions where *ato* expression is being repressed (compare with Fig. 9B). (C) Scabrous expression in the wild type, apical focal plane. Notice widespread *sca* immunoreactivity beyond the strongly expressing cells. (D) Scabrous expression in the wild type, 30-min heat shock followed by 30-min recovery. Scabrous proteins are detected only in intermediate groups and R8 cells, as seen with other antibodies (compare with Fig. 9B). (E) At 60 min after heat shock, *sca* proteins begin to reappear in other cells. (F) At 120 min after heat shock, *sca* proteins are as widespread as before heat shock.

surrounding the R8 cells in columns 2 and 3 (Fig. 10A). Double labeling confirmed that *sca* protein was detected within cells anterior to the intermediate groups (Fig. 10B). This could not be explained as low-level transcription in outlying cells because it was seen only with antibodies specific for the C-terminal half of the protein and because protein was also seen outside R8 cells in *Ellipse* mutants, in which *sca* expression is almost entirely R8 specific (reference 4 and data not shown).

Dispersion of *sca* protein from its site of synthesis was also apparent in a pulse-chase experiment using heat shock (Fig. 10C to F). After 30 min of heat shock, which is expected to affect transcription, mRNA processing, and translation of most genes (2, 28, 48), *sca* protein was detected only in intermediate groups and R8 cells. After 60 min, *scaR* antibodies began to detect more widespread *sca* protein, and after 120 min, the distribution was as broad as before heat shock (Fig. 10C to F).

DISCUSSION

Characterization of *sca* protein. Characterization of *sca* protein was important for two reasons. First, although developmental studies suggested a role in cell-cell signaling, secretion had not been demonstrated and partial homology with fibrinogen and tenascins was consistent with less diffusible roles. Second, the *sca* receptor is unknown, and *Drosophila* proteins interacting with recombinant *sca* protein expressed in bacteria have not been found (27a). As posttranslational modifications may be crucial in receptor-ligand interactions (see, for example, reference 36), it was essential to identify the form of the protein present in vivo in order to investigate interacting proteins.

The *sca* gene directs synthesis of a secreted glycoprotein dimer. Secretion is efficient and may be unregulated, since most of the *sca* protein synthesized in pulse-chase experiments was recovered from the medium. The time course of *sca* biosynthesis was similar to that observed for many secreted proteins in mammalian cells (e.g., colony-stimulating factor 1 [34]). N-linked carbohydrates appeared to be added cotranslationally, and dimerization and disulfide bond formation also occurred rapidly. In mammalian cells, these processes occur in the endoplasmic reticulum. N-linked sugars subsequently became complex, and O-linked sugars were added about 15 min later. In mammalian cells, these processes occur after transfer to the Golgi apparatus. The mature species was the only form detected outside the cell.

After secretion, all of the detectable *sca* protein was found in the medium. This is in contrast to what has been found for other *Drosophila* secreted proteins. For example, *dpp* secreted from Schneider cells is found mostly in material associated with the culture plates (30). We found that *sca* interacts with heparin, suggesting interaction with heparan sulfate proteoglycans in vivo. In vertebrates, proteoglycans are found both in the extracellular matrix and on cell surfaces and can interact with both matrix and soluble proteins.

As a soluble glycoprotein dimer, the *sca* protein resembles many growth factors and is less like the hexameric structures of fibrinogen and tenascins. We conclude that many properties of these and perhaps other FReD-containing proteins depend significantly on their distinct N-terminal sequences, not on the FReD sequence. In vivo histochemical detection of the *sca* C terminus alone is intriguing, but we have no biochemical evidence of proteolytic cleavage in tissue culture or in embryos and cannot distinguish whether such a cleavage might occur before or after endocytosis.

Immunohistochemistry has not yet detected the *sca* protein in extracellular compartments in vivo. Our studies with S2 cells

suggest that the posttranslational modifications should not have interfered with detection of secreted *sca* in situ by any of the antibodies. Tissue fixation may be one explanation, if the more accessible extracellular protein is overfixed by even the mildest treatments. Stability is another factor. The pattern of *sca* expression changes dynamically in vivo, indicating that the protein is short-lived. In addition, immediate tissue fixation is necessary for detection even of intracellular *sca* protein. The short lifetime, combined with dispersion outside the cell, may be a further reason for failure to detect extracellular protein in situ. Antibodies to the C-terminal part of *sca* detect intracellular *sca* protein several cell diameters from the apparent synthetic source, implying that the protein does travel to other cells.

Function of the *sca* protein in vivo. Previously we showed that *sca* mutations affect R8 differentiation. We inferred that R8 was the default fate for morphogenetic furrow cells and that *sca* and *N* were parts of pathways needed for the selection of other cell types (5). Recently the *ato* gene was identified as the basic helix-loop-helix proneural gene responsible for R8 determination, suggesting that all pathways regulating R8 cells should affect *ato* function. We show here that mutations in the *sca*, *N*, and *Dl* genes each affect *ato* expression, suggesting that all these genes work through pathways that repress *ato* transcription.

Repression in cells adjacent to the R8 precursor depended on *N* and *Dl* function. Selection of the R8 precursor from these cells may be analogous to the competition for the neural fate which occurs in proneural cell groups elsewhere in the nervous system (1, 22, 38). Repression in more distant cells depended to a greater extent on *sca* function, since *ato* expression is maintained by many more cells in the *N^{ts}*; *sca* double mutant than in the *N^{ts}* single mutant. As *sca* is expressed only by cells close to each future R8 cell, more distant effects of *sca* mutations imply cell signaling. The *ato* expression pattern indicates that *sca* signaling must occur across several cell diameters (Fig. 9). Characterization of *sca* protein suggested that it could diffuse over this range. With certain antibodies, we detected *sca* protein several cells away from the intermediate groups (Fig. 10). These results are consistent with the idea that *sca* protein itself spreads several cell diameters from the intermediate groups and R8 cells and either represses *ato* expression or coordinates repression of *ato* expression at these locations.

Posttranslational regulation of *ato* activity may also occur. It is striking that *sca* expression is not seen when *ato* is present in all of the morphogenic furrow cells, since *sca* is thought to be a direct transcriptional target of proneural genes (29, 40) and *ato* is required for *sca* expression in the eye (24). In our view, temporal regulation of *ato* activity may be crucial to R8 patterning. The advance of the morphogenic furrow would ensure that as *ato* becomes active, the nascent column is exposed to a pattern of *sca* secretion from the posterior. In this way, the nascent column would be imprinted on its older neighbor (Fig. 11). It is possible that premature *sca* expression contributes to the ommatidial spacing defects seen in *hairy*, *emc* mutant cells, in which proneural gene function is derepressed (8).

Formal distinction of a diffusible role for *sca* from the alternative as a step in a relay of signals probably requires identification of the *sca* receptor. We previously suggested that *N* might be a *sca* receptor (3, 29), and both genes act similarly to repress *ato* expression. However, genetic studies are equally consistent with a distinct *sca* receptor acting in parallel to *N* (see reference 23 for discussion). Many attempts to demonstrate association between *sca* and *N* proteins by coimmunoprecipitation, a variety of cell adhesion assays, or direct binding experiments with labeled *sca* protein have been negative (our

FIG. 11. Features of *ato* and *sca* expression in the morphogenetic furrow. Circles represent *ato*-expressing cells; those also expressing *sca* are shaded. As the morphogenetic furrow advances, a single R8 cell from each intermediate group will continue to express both *ato* and *sca*. Singling out the R8 cell precursor requires *N* and *Dl* function. In addition, *ato* will be repressed in other cells posterior (right) of the line, leading to a similar but opposite-phase *ato* pattern for the next ommatidial column. We suggest that secreted *sca* protein contributes to *ato* repression because the predicted new boundary seems to map a few cell diameters from cells expressing *sca* and is altered in both *sca* loss-of-function and misexpression genotypes. Certain antibodies detect *sca* protein travel over this range. In our model, the expression of *sca* as one of the first *ato*-activated genes is crucial in determining the eye pattern as each new column develops next to its predecessor. In *sca*⁻ and misexpression genotypes, the action of *N*, *Dl*, and perhaps other genes can give only aberrant patterning of the eye.

unpublished results). Biochemical characterization of *sca* protein is an important step toward searching for a possibly novel receptor.

Implications for neural development in other tissues. Previously, direct cell contact has been thought to mediate signaling between proneural cells (39). We report that secreted proteins can also regulate proneural development. We find secreted *sca* to be important for interactions between successive ommatidial columns (Fig. 11). It is possible that this is most comparable to signaling between proneural groups. Communication between proneural regions is also implied by other experiments. In grasshoppers, ablation of neuroblasts after delamination reveals interactions with proneural regions that later arise in the overlying ectoderm (12). In the bug *Rhodnius prolixus*, in which epidermal sensory organs are intercalated during growth, pattern of the added sense organs is influenced by preexisting organs whose own proneural regions must have decayed long before (46). These interactions might also be explained by proteins secreted from differentiating neural cells

affecting nearby proneural groups. It seems possible that secreted signals also act within proneural regions and that during the evolution of compound eyes from individual ommatidia (10), proteins already secreted by the proneural cells assumed responsibility for interactions between ommatidia.

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