Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 8333–8337, November 1989
Biochemistry

Structure and activity of the sevenless protein: A protein tyrosine kinase receptor required for photoreceptor development in *Drosophila*

(induction/intercellular communication/phosphorylation)

MICHAEL A. SIMON, DAVID D. L. BOWTELL, AND GERALD M. RUBIN

Howard Hughes Medical Institute and Department of Biochemistry, University of California at Berkeley, Berkeley, CA 94720

Contributed by Gerald M. Rubin, August 4, 1989

ABSTRACT **The sevenless gene encodes a putative protein tyrosine kinase receptor that is required for the proper differentiation of the R7 photoreceptor cells of the *Drosophila* eye. We have expressed the sevenless protein in *Drosophila* tissue culture cells and studied its synthesis, processing, and activity. Our results show that the sevenless protein possesses protein tyrosine kinase activity. The protein is first synthesized as a 280-kDa glycoprotein precursor that is subsequently cleaved into 220-kDa amino-terminal and 58-kDa carboxyl-terminal subunits that remain associated by noncovalent interactions. The 220-kDa subunit is glycosylated and contains most of the extracellular portion of the protein, and the 58-kDa subunit is composed of a small portion of the extracellular sequences and the intracellular protein tyrosine kinase domain. This complex is subsequently cleaved into either 49- or 48-kDa carboxyl-terminal fragments with concomitant degradation of the rest of the protein.**

The *Drosophila* compound eye consists of several hundred identical subunits, called ommatidia. Each ommatidium is composed of 8 photoreceptor cells and 12 non-neuronal accessory cells. This precise array of cells develops from an initially unpatterned epithelium during the last larval instar and pupal periods (1, 2). Cells are instructed to appropriate differentiation pathways by signals produced by neighboring cells (3) in a process of progressive recruitment that is not dependent on cell lineage (1, 4). Studies of the nature of these signals and their receptors are crucial to understanding how the eye develops.

Mutations that inactivate the sevenless gene cause a single cell, the photoreceptor R7, to be absent from each ommatidium (5). A cell occupies the proper position to become R7 in the developing ommatidium but becomes a lens-secreting cell rather than a photoreceptor cell (6). Studies of ommatidia mosaic for sevenless function have demonstrated that the sevenless gene product is required only in the developing R7 cell (5–7). Thus these observations suggested that the sevenless gene product is required either for the reception of a signal that acts to help specify the fate of the R7 cell or for the proper execution of the pathway that leads to R7 development.

Conceptual translation of sevenless cDNAs (8–10) predicts a 288-kDa membrane protein with a large extracellular domain and a cytoplasmic domain possessing extensive homology to protein tyrosine kinases. Such a protein would be well suited for transducing a signal required for R7 differentiation by activation of its intracellular kinase domain in response to binding of a ligand to its extracellular domain. The observation that sevenless protein is expressed in the pre-R7 cell prior to any morphological signs of photoreceptor differentiation is consistent with a role for the sevenless protein in transducing a signal that specifies the R7 fate (11, 12).

An understanding of the structure and activity of the sevenless protein is a prerequisite for understanding the molecular mechanisms of sevenless-mediated signaling. Toward this end, we have studied sevenless protein that has been expressed in *Drosophila* tissue culture cells. Our results show that the sevenless protein has protein tyrosine kinase activity and that the mature sevenless protein is formed by proteolytic cleavage of a precursor.

MATERIALS AND METHODS

***Drosophila* Cell Culture.** *Drosophila* Schneider line 2 (SL2) cells were maintained at 25°C in M3 medium supplemented with 10% (vol/vol) fetal calf serum (13, 14). Transfections and selections of SL2 cells were performed using Ca_2PO_4 G418 (GIBCO) at 1 mg/ml, and 10 μg of plasmid DNA per 5×10^6 cells as described (15). Cells were metabolically labeled with [^{3}H]mannose by overnight incubation in M3 medium containing [^{3}H]mannose (1 mCi/ml; 30 Ci/mmol; 1 Ci = 37 GBq; Amersham). Cells were labeled with methionine for 14 hr in M3 medium containing [^{35}S]methionine (1 mCi/ml; 800 Ci/mmol; Amersham) and only 2% of the normal amount of methionine, Yeastolate (Difco), and Bacto Peptone (Difco). Cells were labeled with phosphate for 14 hr in M3 medium containing $^{32}P_i$ (2 mCi/ml; carrier-free; Amersham) and only 2% of the normal amount of phosphate.

Sevenless Expression Vector. The actin–sevenless gene was made by fusing a 2.7-kilobase DNA fragment containing the actin 5C promoter (ref. 16; provided by M. Krasnow, Stanford University) to a 13.1-kilobase *Nar* I–*Eco*RI fragment of genomic DNA that extends from 60 base pairs to the 5′ side of the probable initiation codon to 510 base pairs to the 3′ side of the polyadenylylation site (9, 10). This cassette and a 4.3-kilobase *Eco*RI–*Bam*HI fragment from the pcopneo vector (15) were cloned into a modified pUC19 vector to give the sevenless expression vector.

Membrane Preparation. Solubilized crude membranes were prepared at 4°C as follows. Frozen cells (2×10^7 cells per ml) were resuspended in 20 mM Tris·HCl, pH 7.5/5 mM KCl/1 mM $MgCl_2$ and disrupted with a Dounce homogenizer (pestle A). The cells were centrifuged at $1000 \times g$ for 5 min. The pellet was extracted again with 0.5 vol of the same buffer. The combined supernatants were centrifuged at $40,000 \times g$ for 45 min. The resulting pellet was resuspended (10^8 cells per ml) in membrane extraction buffer (MEB) [2% (vol/vol) Triton X-100/20 mM Tris·HCl, pH 8.0/150 mM NaCl/0.1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride)] and homogenized repeatedly. The sample was then centrifuged at $100,000 \times g$ for 45 min. This supernatant was the solubilized crude membrane protein preparation.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: IGF-1, insulin-like growth factor 1.

Analysis of Proteins. The antisera used in this study have been described (11, 17). Antisera CT and NCT correspond to sera 114 and 112, respectively, in ref. 11. Antibody staining of cells was performed using rhodamine-conjugated anti-mouse antibodies (Amersham) as described (18). Immunoblots were performed as described with slight modifications (19) using an iodinated second antibody $F(ab')_2$ fragment (16 μCi/μg; Amersham). The blocking solution and antibody diluent were 50 mM Tris·HCl, pH 7.5/250 mM NaCl/5% (wt/vol) dried milk. The wash solution was 50 mM Tris·HCl, pH 7.5/250 mM NaCl/0.25% Tween 20. Immunoprecipitations were performed at 4°C as follows. Cells were lysed in lysis buffer (20 mM Tris·HCl, pH 8.3/150 mM NaCl/1% Triton X-100/1% sodium deoxycholate/0.1% SDS/50 mM NaF/100 μM Na_3VO_4/2 mM EDTA/1 mM phenylmethylsulfonyl fluoride), repeatedly passed through a syringe to shear the DNA, and then centrifuged in a microcentrifuge for 5 min. The supernatant was incubated with antiserum (2.5 μl/ml) for 45 min. Pansorbin cells (Calbiochem) were used to collect IgG immune complexes and then washed four times with lysis buffer. Gel filtration chromatography was performed using Sephacryl S-400 (Pharmacia). Crude membrane proteins were prepared as described above except that the MEB was prepared with 2% (vol/vol) CHAPS {3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate} rather than Triton X-100. Most of the detergent was then removed by passage through a Sephadex G-75 column preequilibrated with 20 mM Tris·HCl, pH 7.5/150 mM NaCl/1 mM dithiothreitol/0.05% CHAPS. The sample was then concentrated 5-fold using a Centriprep-30 device (Amicon). Protein aggregates were removed by centrifugation at 100,000 $\times$ *g* for 45 min. The sample (5 ml) was separated on a 100 $\times$ 2.5 cm column with a flow rate of 25 ml/hr.

Kinase Assays. Solubilized membrane protein preparations were incubated with antiserum (2.5 μl/ml) for 20 min and then with 25 μl of protein A-Sepharose CL4-B beads (Pharmacia) for 20 min. The beads were washed twice with MEB and once with kinase assay buffer (KAB; 25 mM Tris·HCl, pH 8.0/0.1% Triton X-100/5 mM $MgCl_2$/5 mM $MnCl_2$/0.5 mM dithiothreitol). For assays of enolase phosphorylation, the beads were then suspended in 135 μl of KAB containing 10 μM ATP (with 1 μCi of [γ-^{32}P]ATP) and 15 μl of acid-denatured rabbit muscle enolase (ref. 20; Sigma). Assays of sevenless phosphorylation were performed in the same manner except that no enolase was added and carrier-free [γ-^{32}P]-ATP (10 μCi) was used. Phospho amino acid analysis was performed as described (21, 22).

RESULTS AND DISCUSSION

Expression and Structure of Sevenless Proteins. We have studied the synthesis and processing of sevenless protein expressed in *Drosophila* tissue culture cells rather than in developing eye imaginal discs because of the low level of sevenless expression in discs and the difficulties of isolating large quantities of eye discs. The protein was expressed in *Drosophila* SL2 cells that were stably transfected with a plasmid containing an actin 5C-driven sevenless gene. The actin promoter-sevenless cassette of this plasmid is able to rescue the sevenless mutation phenotypically in transgenic flies and, therefore, must encode an active sevenless protein (23). A single-cell clone (SL2-sev) that produces sevenless protein was derived from the mixed transfectants by limiting dilution.

The cellular location of the sevenless protein in the SL2-sev cells was examined by indirect immunofluorescence using antisera that recognize either the carboxyl-terminal 13 amino acids (antiserum CT) or epitopes near the amino terminus of the protein within the predicted extracellular domain (antiserum ECD1; ref. 17). The two antisera showed the same pattern of distribution of sevenless protein in the permeabilized cells (Fig. 1). The majority of immunoreactive material was localized in intracellular structures that may represent membrane vesicles. Similar large vesicles containing sevenless protein have been observed in eye imaginal discs (11). Antibody staining of live, unpermeabilized cells revealed that a portion of the sevenless protein is expressed on the surface of the SL2-sev cells. Antiserum ECD1 easily detected cell surface staining, whereas antiserum CT failed to detect any extracellular antigen. These results confirm the prediction from the deduced amino acid sequence that the bulk of the amino-terminal four-fifths of the predicted sequence would be either exposed on the extracellular surface of the cell or within the lumena of vesicles and the carboxyl-terminal tyrosine kinase domain would be within the cytoplasm.

We next examined the synthesis and processing of the sevenless protein. Immunoprecipitation of [^{35}S]methionine labeled proteins with either antiserum CT or antiserum ECD1 showed a complex pattern of sevenless proteins (Fig. 2*A*). Both antisera precipitated major bands corresponding to proteins of 280, 220, and 58 kDa. The antiserum CT also recognized proteins of 48 and 49 kDa. In addition, a minor band of 59 kDa was detected in some experiments. Immunoprecipitation of sevenless proteins from pulse–chase labeled SL2-sev cells demonstrated that the 280-kDa form is the direct precursor of the 220- and 58-kDa proteins and that the 49- and 48-kDa forms are subsequent products of cleavage of the 58-kDa protein (Fig. 3).

We next performed immunoblots using antisera CT and ECD1 to determine which portions of the 280-kDa precursor protein were contained in each of the smaller subunits (Fig. 2*B*). Antiserum CT recognized the 280-, 58-, 49-, and 48-kDa forms, whereas antiserum ECD1 recognized the 280- and 220-kDa proteins. Thus, the 58-, 49-, and 48-kDa proteins are derived from the carboxyl terminus of the 280-kDa precursor, and the 220-kDa protein comprises its amino-terminal portion. We can estimate the approximate positions of the

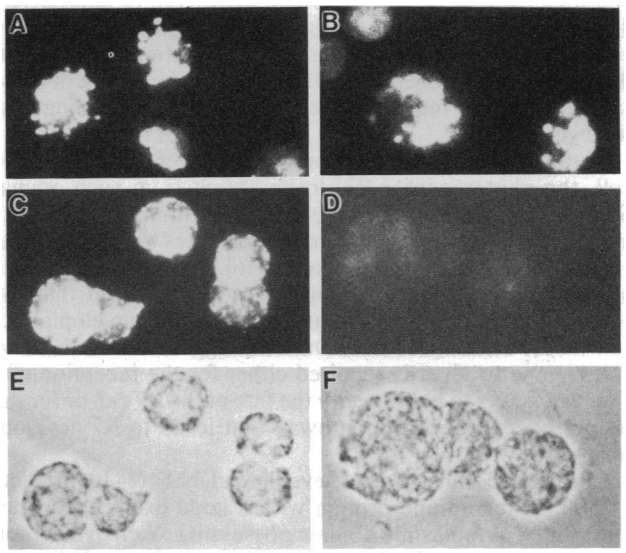

FIG. 1. Indirect immunofluorescence of SL2-sev cells. Fixed and permeabilized SL2-sev cells were stained with either antiserum ECD1 (*A*) or antiserum CT (*B*). Large intracellular structures that appear to be vesicles were stained with both antisera. Unfixed live cells were stained with either antiserum ECD1 (*C*) or antiserum CT (*D*). (*E* and *F*) Phase-contrast micrographs of the fields shown in *C* and *D*, respectively. Staining of unfixed cells is only observed with antiserum ECD1, which recognizes an epitope from the predicted extracellular domain of the protein. Staining of untransfected SL2 cells revealed no immunofluorescence (data not shown). (×1000.)

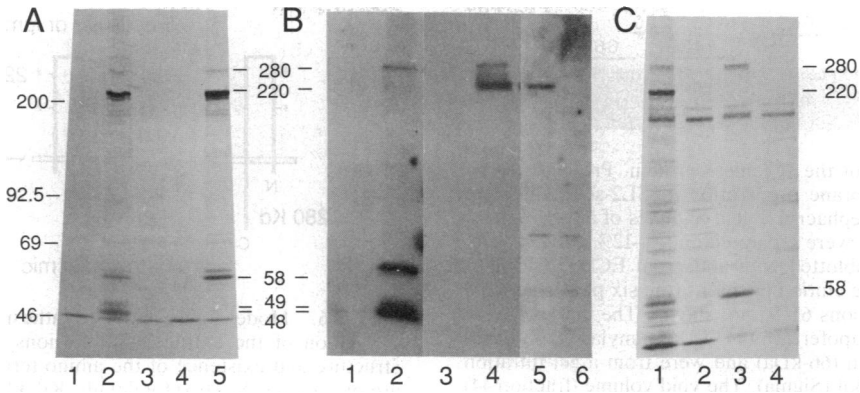

FIG. 2. Analysis of sevenless proteins. (*A*) Immunoprecipitation of sevenless proteins. [^{35}S]Methionine-labeled proteins were immunoprecipitated from either SL2 or SL2-sev cells and separated on a 5–12% polyacrylamide/SDS gel. Lanes: 1, SL2 cells with antiserum CT; 2, SL2-sev with antiserum CT; 3, SL2-sev cells with antiserum CT plus 50 μg of CT peptide; 4, SL2 cells with antiserum ECD1; 5, SL2-sev cells with antiserum ECD1. (*B*) Immunoblot of sevenless proteins. Membrane proteins from either SL2 cells (2×10^6 cells per lane), SL2-sev cells (2×10^6 cells per lane), wild-type (Canton S) dissected eye imaginal discs (250 per lane), or sev^{d2} (12) eye discs (250 per lane) were electrophoresed on a 5–14% polyacrylamide/SDS gel. The proteins were blotted and probed with either antiserum ECD1 or antiserum CT. Lanes: 1, SL2 cells with antiserum CT; 2, SL2-sev cells with antiserum CT; 3, SL2 cells with antiserum ECD1; 4, SL2-sev cells with antiserum ECD1; 5, wild-type eye discs with antiserum ECD1; 6, sev^{d2} eye discs with antiserum ECD1. (*C*) Effect of SDS on the association of the 220- and 58-kDa subunits. [^{35}S]Methionine-labeled proteins from either SL2 (lanes 2 and 4) or SL2-sev (lanes 1 and 3) cells were immunoprecipitated with antiserum CT. The extracts used for lanes 3 and 4 were treated by the addition of 2% SDS and incubation at 42°C for 10 min. SDS was then diluted 1:10 by the addition of lysis buffer. The extracts used for lanes 1 and 2 were treated by incubation at 42°C for 10 min, diluted 1:10 with lysis buffer, and then supplemented with SDS to the same final concentration as the extract used for lanes 3 and 4. The ability of antiserum CT to indirectly immunoprecipitate the 220-kDa subunit was eliminated by SDS treatment (lane 1 versus lane 3), indicating that the 220- and 58-kDa subunits do not associate by covalent bonds. The labeling was for only 8 hr and, therefore, the 49- and 48-kDa forms are not apparent.

cleavage sites from the sizes of the 58-, 49-, and 48-kDa proteins. The cleavage that generates the 220- and 58-kDa forms probably occurs in the extracellular domain ≈80 residues from the beginning of the putative transmembrane sequence that separates the extracellular domain from the kinase domain. At this position in the protein sequence there is a stretch of nine arginine residues that may be the cleavage site for the sevenless precursor. The cleavage of the insulin receptor into α and β subunits occurs after a similar run of four basic amino acids (24, 25). The cleavages that generate the smaller 49- and 48-kDa subunits must occur very near the transmembrane region since the transmembrane and cytoplasmic domains have a combined predicted mass of 48.8 kDa. That these forms are found in membrane preparations suggests that the cleavage may occur immediately adjacent to the extracellular face of the membrane.

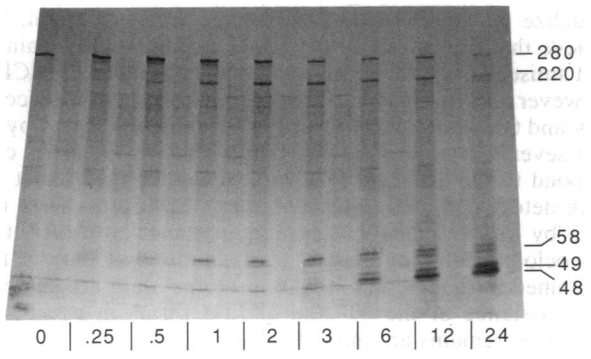

FIG. 3. Pulse–chase analysis of sevenless proteins. SL2-sev cells were placed in M3 medium lacking methionine for 6 hr. [^{35}S]-Methionine (5 mCi/ml) was then added to the medium and the cells were incubated for 15 min before the addition of an equal volume of complete medium. Samples were removed for immunoprecipitation at the times (in hr) after the start of the chase as indicated below each pair of lanes. The samples were divided into equal portions and immunoprecipitated with antiserum CT either in the absence (left lane of each pair) or presence (right lane of each pair) of 25 μg of the peptide used to raise antiserum CT. The proteins were separated on a 5–14% polyacrylamide gradient/SDS gel.

The observation that the 220- and 58-kDa polypeptides are both indirectly immunoprecipitated by antisera that are specific for either subunit indicates that these proteins are physically associated. The cleavage of an initial precursor into two subunits is known to occur for other protein tyrosine kinase receptors such as the insulin and insulin-like growth factor 1 (IGF-1) receptors (for review, see ref. 26). The native form of the insulin receptor is an $\alpha_2\beta_2$ tetramer that is bound together by $\alpha\alpha$ and $\alpha\beta$ disulfide bonds. We, therefore, sought to determine whether the sevenless protein has a similar structure. Two experiments indicate that the interaction between the sevenless α (220 kDa) and β (58 kDa) subunits does not involve disulfide bonds. First, the pattern of polypeptides detected by either antiserum ECD1 or antiserum CT is not affected when the samples are not reduced before electrophoresis (data not shown). Furthermore, pretreatment of the extracts with 2% SDS at 42°C for 10 min before immunoprecipitation is sufficient to destroy the association (Fig. 2*C*). The inability of the 49- and 48-kDa proteins to interact with the 220-kDa α subunit suggests that the site of the interactions between the α and β subunits is likely to be between the cleavage sites that generate the 58-kDa β form and the 49- and 48-kDa forms.

Gel filtration of membrane preparations from SL2-sev cells was used to examine whether multiple α and β subunits might be present in the native sevenless protein. The protein was visualized by immunoblotting the column fractions with antiserum ECD1 (Fig. 4). The 220/58-kDa sevenless protein complex has an apparent Stokes' radius of ≈8.5 Å. This radius corresponds to an approximate molecular mass of 650 kDa for a globular protein. This size is consistent with an $\alpha_2\beta_2$ structure for the 220/58-kDa sevenless protein; however, analysis of purified sevenless protein will be needed to confirm this suggestion.

The possible glycosylation and phosphorylation of the various forms of sevenless protein was examined by immunoprecipitation of labeled proteins from SL2-sev cells. The 280-kDa precursor and the 220-kDa subunit were detected after labeling cells with [^{3}H]mannose, indicating these subunits are glycosylated (Fig. 5*A*). This is consistent with the prediction that the 220-kDa subunit contains the majority of

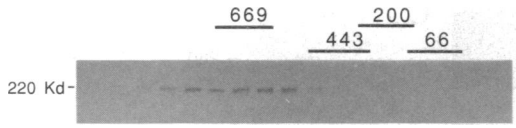

FIG. 4. Gel filtration of the sevenless protein. Proteins solubilized from a crude membrane preparation of SL2-sev cells were chromatographed using Sephacryl S-400. Samples of alternate fractions (10 min per fraction) were separated on a 5–12% polyacrylamide/SDS gel and immunoblotted with antiserum ECD1. The lines above the blot indicate the elution positions (the six peak fractions) of marker proteins. Fractions 61–95 are shown. The markers were thyroglobulin (669 kDa), apoferritin (443 kDa), amylase (200 kDa), and bovine serum albumin (66-kDa) and were from a gel filtration molecular weight marker kit (Sigma). The void volume (fraction 44) was determined by fractionation of dextran blue. The elution position of the sevenless protein corresponds to a Stokes' radius of 8.5 Å and a molecular mass of ≈650 kDa for a globular protein. Kd, kDa.

the extracellular sequences. Immunoprecipitation of [^{32}P]-labeled protein revealed that only the 49- and 48-kDa forms contain detectable amounts of incorporated phosphate (Fig. 5*B*). Phospho amino acid analysis of these proteins revealed that the 49-kDa protein contains both phosphoserine and phosphotyrosine and the 48-kDa subunit contains predominantly phosphoserine (Fig. 5*C*). Purification and assay of the various forms of sevenless protein will be required to address the effect of these phosphorylations on sevenless activity. However, hyperphosphorylation of receptor tyrosine kinases is often associated with ligand binding and receptor activation. Furthermore, removal of the extracellular portion of the insulin receptor by cleavage *in vitro* has been shown to activate the kinase activity (27).

Our view of sevenless synthesis and processing is illustrated in Fig. 6. The structure of the sevenless protein is similar to the insulin and IGF-1 receptors in that an initial precursor is cleaved into two polypeptides that remain associated. Moreover, the apparent size of the sevenless complex is consistent with an $\alpha_2\beta_2$ structure like that of these receptors. The sevenless protein is, however, distinct in at least two ways. The extracellular domain of the sevenless protein is much larger than those of the insulin and IGF-1

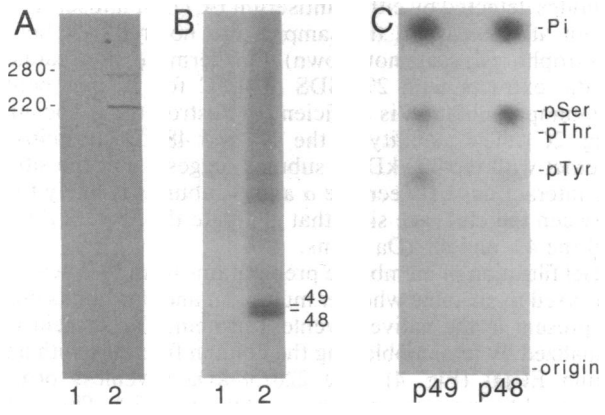

FIG. 5. Modification of the sevenless protein in SL2-sev cells. (*A*) Glycosylation of the sevenless protein. SL2 (lane 1) and SL2-sev (lane 2) cells were labeled with [^{3}H]mannose and immunoprecipitated with antiserum CT. (*B*) Phosphorylation of the sevenless protein. SL2 (lane 1) and SL2-sev (lane 2) cells were labeled with [^{32}P]phosphate and immunoprecipitated with antiserum CT. (*C*) Phospho amino acid analysis of 49- and 48-kDa forms of sevenless protein. The 49- and 48-kDa phosphorylated sevenless proteins (p49 and p48, respectively) were excised from the gel and analyzed for phospho amino acid content. The position of the various phospho amino acids was determined by ninhydrin staining of nonradioactive markers. pSer, phosphoserine; pThr, phosphothreonine; pTyr, phosphotyrosine.

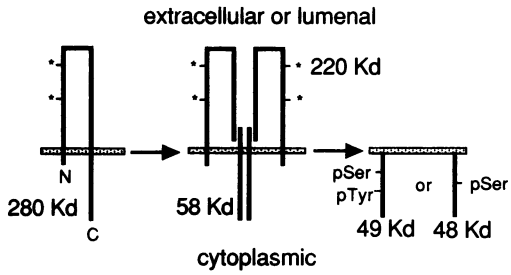

FIG. 6. Model for sevenless synthesis and processing. *, Glycosylation of the extracellular portions of the protein. The $\alpha_2\beta_2$ structure and existence of the amino-terminal cytoplasmic domain are speculative. See text for details. Kd, kDa. Stippled boxes, plasma membrane.

receptors. Furthermore, the α and β subunits of sevenless are not disulfide bonded as are those of the insulin and IGF-1 receptors.

Analysis of the predicted sevenless coding sequence has suggested that the sevenless protein may also be distinct from the insulin and IGF-1 receptors in another way. The initiating methionine is not immediately followed by a hydrophobic signal sequence. Instead, the initial hydrophobic region of the sevenless protein is separated from the putative initiating methionine by ≈60 amino acids. This has led to a suggestion that the hydrophobic sequences might serve as an amino-terminal anchor rather than a cleaved signal sequence and that the sevenless protein might have a small cytoplasmic domain at its amino terminus (9, 10). A similar structure has been proposed for the product of the c-*ret* gene (28). Our attempts to verify this prediction by producing antiserum that recognize this region and by directly sequencing the amino-terminus of the 220-kDa subunit have failed due to poor antigenicity of peptides derived from the amino-terminal region and blockage of the amino-terminal residue, respectively. The proposed structure is drawn with the amino-terminal hydrophobic region uncleaved, but this aspect of the model remains speculative (Fig. 6).

We have tried to determine whether the pattern of synthesis and processing that occurs in eye discs corresponds to the pattern observed for sevenless protein in SL2-sev cells. Antibody ECD1 does recognize the 220-kDa form of the sevenless protein in crude membrane preparations from dissected eye discs (Fig. 2*B*). However, our attempts to visualize the smaller subunits have been unsuccessful. We believe that this is due to the considerably weaker staining that antiserum CT always shows relative to antiserum ECD1. However, another group has reported (12) the existence of 250- and 60-kDa proteins that are specifically detected by an anti-sevenless monoclonal antibody. These most likely correspond to the 280- and 58-kDa sevenless proteins that we have detected. The smaller 49- and 48-kDa forms were not seen by this antibody in eye disc extracts. However, this monoclonal antibody reacts with an epitope that is not contained in the 49- and 48-kDa proteins (data not shown). The existence of the 49- and 48-kDa forms in eye discs, therefore, remains an unresolved issue.

Activity of Sevenless Protein. The predicted sequence of the sevenless protein displays strong similarity to members of the protein tyrosine kinase family. To test directly the protein kinase activity of the sevenless protein, we incubated immunoprecipitates of sevenless protein from crude membranes with [γ-^{32}P]ATP and an exogenous substrate. We chose acid-denatured enolase as a substrate because of its ability to be phosphorylated by a number of tyrosine-specific protein kinases (20). For these experiments the sevenless protein was precipitated with either antiserum CT or an antiserum (NCT) that recognizes an epitope near the car-

boxyl terminus of the protein. Immunoprecipitates containing sevenless protein showed elevated levels of enolase phosphorylation compared to immunoprecipitates that do not (Fig. 7*A*). Phospho amino acid analysis of the labeled enolase shows that the increase is due to tyrosine phosphorylation (Fig. 7*B*). These results strongly support the prediction from sequence homology that the sevenless protein is a tyrosine-specific protein kinase.

The ability of the sevenless protein to autophosphorylate was examined by performing phosphorylation assays in the absence of the enolase substrate. Immunoprecipitates from SL2-sev cells were capable of phosphorylating polypeptides that comigrate with the [^{35}S]methionine labeled 59-, 58-, 49-, and 48-kDa subunits (Fig. 7*A*). Comparison of the limited protease digestion products of these four bands with those of the [^{35}S]methionine-labeled 58-kDa sevenless protein subunit demonstrated that all four of these proteins are products of the actin–sevenless gene (data not shown). Analysis of tryptic phosphopeptides derived from each of the bands revealed a total of three major and five minor tyrosine-phosphorylated peptides. The relative intensity of labeling of each tryptic fragment was different for each of the four phosphorylated sevenless bands. This analysis indicates that the sevenless protein is capable of serving as a substrate for tyrosine phosphorylation at multiple sites by extracts that contain active sevenless protein. Since the immunoprecipitates of extracts from the SL2 cells were virtually devoid of protein tyrosine kinase activity (as measured in the enolase assay), we conclude that the phosphorylation of the sevenless protein in immunoprecipitates of SL2-sev cells is catalyzed by one or more of the forms of sevenless protein.

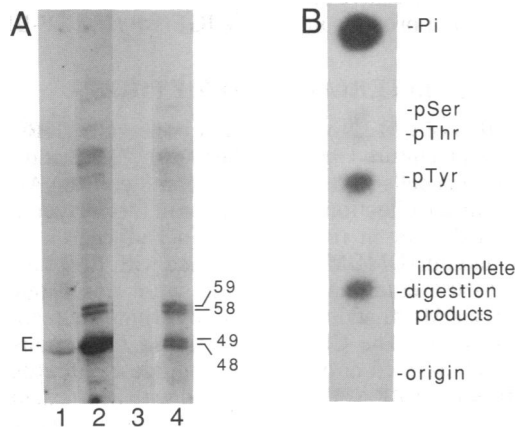

FIG. 7. Kinase activity of sevenless protein. (*A*) Phosphorylation of enolase by immunoprecipitates (with antiserum NCT) containing sevenless protein. Immunoprecipitates of either SL2 or SL2-sev cells were incubated with [γ-^{32}P]ATP in the presence or absence of acid-denatured enolase. The products were then electrophoresed on a 5–14% polyacrylamide gradient/SDS gel and autoradiographed. The position of enolase is marked by an "E". Lanes: 1, SL2 cells with enolase; 2, SL2-sev cells with enolase; 3, SL2 cells without enolase; 4, SL2-sev cells without enolase. The data are from nonadjacent lanes of a single gel. (*B*) Phospho amino acid analysis of the enolase phosphorylated by the SL2-sev activity. The enolase band from lane 2 in *A* was excised from the gel and analyzed for phospho amino acid content. The positions of the various phospho amino acids were determined by ninhydrin staining of nonradioactive markers. pSer, phosphoserine; pThr, phosphothreonine; pTyr, phosphotyrosine.

Concluding Remarks. In this report we have examined the structure and activity of the protein products of sevenless locus. The structure of the sevenless protein is most similar to that of insulin and IGF-1 receptors. The protein is synthesized as a large membrane-bound glycoprotein precursor that is subsequently cleaved into two subunits (α and β) that may form an $\alpha_2\beta_2$ complex. This complex is then degraded to a smaller subunit that contains the intracellular tyrosine kinase domain and is membrane associated. Two features of the sevenless structure are distinct from those of the insulin and IGF-1 receptors: the lack of intersubunit disulfide bonds and the much larger extracellular domain. The sevenless protein may, therefore, define a class of tyrosine kinase receptor molecules.

We thank Laurie Bost and Smadar Admon for help with antibody preparation and cell culture. M.A.S. is a Damon Runyon–Walter Winchell Fellow and D.D.L.B. is a C. J. Martin Fellow.

1. Ready, D. F., Hanson, T. E. & Benzer, S. (1976) *Dev. Biol.* **53,** 217–240.
2. Tomlinson, A. (1988) *Development* **104,** 183–193.
3. Tomlinson, A. & Ready, D. F. (1987) *Dev. Biol.* **120,** 366–376.
4. Lawrence, P. A. & Green, S. M. (1979) *Dev. Biol.* **71,** 142–152.
5. Harris, W. A., Stark, W. S. & Walker, J. A. (1976) *J. Physiol. (London)* **256,** 415–439.
6. Tomlinson, A. & Ready, D. F. (1987) *Dev. Biol.* **123,** 264–275.
7. Campos-Ortega, J. A., Jurgens, G. & Hofbauer, A. (1979) *Wilhelm Roux Arch. Entwicklungsmech. Org.* **186,** 27–50.
8. Hafen, E., Basler, K., Edstroem, J. E. & Rubin, G. M. (1987) *Science* **236,** 55–63.
9. Bowtell, D. D. L., Simon, M. A. & Rubin, G. M. (1988) *Genes Dev.* **2,** 620–634.
10. Basler, K. & Hafen, E. (1988) *Cell* **54,** 299–311.
11. Tomlinson, A., Bowtell, D. D. L., Hafen, E. & Rubin, G. M. (1987) *Cell* **51,** 143–150.
12. Bannerjee, U., Renfranz, P. J., Hinton, D. R., Rabin, B. A. & Benzer, S. (1987) *Cell* **51,** 151–158.
13. Shields, G. & Sang, J. H. (1977) *Drosoph. Inf. Serv.* **52,** 161.
14. Schneider, I. (1972) *J. Embryol. Exp. Morphol.* **27,** 353–365.
15. Rio, R. C. & Rubin, G. M. (1985) *Mol. Cell. Biol.* **5,** 1833–1838.
16. Bond, B. J. & Davidson, N. (1986) *Mol. Cell. Biol.* **6,** 2080–2088.
17. Bowtell, D. D. L., Simon, M. A. & Rubin, G. M. (1989) *Cell* **56,** 931–936.
18. Goding, J. W. (1986) *Monoclonal Antibodies: Principles and Practice* (Academic, New York).
19. Burnette, W. N. (1981) *Anal. Biochem.* **112,** 195–203.
20. Cooper, J. A., Esch, F., Taylor, S. S. & Hunter, T. (1984) *J. Biol. Chem.* **259,** 7835–7841.
21. Hunter, T. & Sefton, B. M. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 1311–1315.
22. Cooper, J. A. & Hunter, T. (1981) *Mol. Cell. Biol.* **1,** 165–178.
23. Bowtell, D. D. L., Kimmel, B. E., Simon, M. A. & Rubin, G. M. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6245–6249.
24. Ulrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M. & Ramachandran, J. (1985) *Nature (London)* **313,** 756–761.
25. Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J., Masiarz, F., Kan, Y. W., Goldfine, I. D., Roth, R. A. & Rutter, W. J. (1985) *Cell* **40,** 747–758.
26. White, M. F. & Kahn, C. R. (1986) in *The Enzymes*, eds. Boyer, P. D. & Krebs, E. G. (Academic, New York), Vol. 17, pp. 247–310.
27. Shoelson, S. E., White, M. F. & Kahn, C. R. (1988) *J. Biol. Chem.* **263,** 4852–4860.
28. Takahashi, M., Buma, Y., Iwamoto, T., Inaguma, Y., Ikeda, H. & Hiai, H. (1988) *Oncogene* **3,** 571–578.