MOLECULAR AND CELLULAR BIOLOGY, Feb. 1994, p. 944–950
0270-7306/94/$04.00+0

Vol. 14, No. 2

Two Distinct Transmembrane Serine/Threonine Kinases from *Drosophila melanogaster* Form an Activin Receptor Complex

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Received 6 October 1993/Accepted 2 November 1993

A transmembrane protein serine/threonine kinase, Atr-I, that is structurally related to receptors for members of the transforming growth factor-β (TGF-β) family has been cloned from *Drosophila melanogaster*. The spacing of extracellular cysteines and the cytoplasmic domain of Atr-I resemble most closely those of the recently described mammalian type I receptors for TGF-β and activin. When expressed alone in test cells, Atr-I is unable to bind TGF-β, activin, or bone morphogenetic protein 2. However, Atr-I binds activin efficiently when coexpressed with the distantly related *Drosophila* activin receptor Atr-II, with which it forms a heteromeric complex. Atr-I can also bind activin in concert with mammalian activin type II receptors. Two alternative forms of Atr-I have been identified that differ in an ectodomain region encompassing the cysteine box motif characteristic of receptors in this family. Comparison of Atr-I with other type I receptors reveals the presence of a characteristic 30-amino-acid domain immediately upstream of the kinase region in all these receptors. This domain, of unknown function, contains a repeated Gly-Ser sequence and is therefore referred to as the GS domain. Maternal *Atr-I* transcripts are abundant in the oocyte and widespread during embryo development and in the imaginal discs of the larva. The structural properties, binding specificity, and dependence on type II receptors define Atr-I as an activin type I receptor from *D. melanogaster*. These results indicate that the heteromeric kinase structure is a general feature of this receptor family.

The activins are members of the transforming growth factor β (TGF-β) family, an important group of cell growth and differentiation factors (17, 18, 25, 30). In mammalian cells, these factors interact with pairs of membrane proteins known as receptor types I and II, identifiable by their ligand-binding properties (4, 15, 19). The type II receptors for activin, TGF-β and bone morphogenetic protein (BMP), are transmembrane protein serine/threonine kinases that bind ligand with high affinity (2, 5, 8, 13, 16, 20, 21). However, the mammalian TGF-β type II receptor is unable to signal alone and requires the presence of the TGF-β type I receptor, with which it forms a complex (12, 14, 32). Receptor I, on the other hand, requires receptor II for TGF-β binding (32). These two receptors are therefore considered interdependent components of a heteromeric signaling receptor complex, a model that may apply to activin and BMP receptors as well.

Various human and mouse type I receptors for TGF-β and activin have been recently cloned, and, surprisingly, they also encode members of the transmembrane serine/threonine kinase family (1, 7, 9). Among these type I receptors, human TβR-I (9) and ActR-I (1) bind TGF-β and activin, respectively, when coexpressed with the corresponding type II receptors and signal as part of the resulting complex. ActR-I and its murine homolog, Tsk 7L (7), can bind TGF-β when co-overexpressed with the TGF-β type II receptor (1, 7), but this appears to be a low-efficiency interaction (1). TSR-I is a shared type I receptor that can bind either TGF-β or activin with high efficiency in concert with the respective type II receptors (1). Thus, the TGF-β family receptor structure emerging from these studies is that of a complex containing two distantly related transmembrane serine/threonine kinases that interact with the ligand in a cooperative manner.

Since a receptor structure containing two widely different protein kinase domains is unprecedented, these findings raised questions about the generality and conservation of this structure in species other than mammals. Structural homologs of the mammalian BMPs are present in *Drosophila melanogaster* (6, 22, 31), and the presence of an activin system in this organism is inferred from the existence of Atr-II (5). Atr-II is structurally related to activin type II receptors from vertebrates and binds human activin with high affinity. We now report the molecular cloning and biochemical characterization of Atr-I, a *Drosophila* receptor that binds activin only when coexpressed with type II receptors with which it forms a complex. The structural and ligand-binding properties of Atr-I are those of an activin type I receptor. These observations suggest that the heteromeric kinase receptor structure is broadly conserved in metazoa.

MATERIALS AND METHODS

cDNA cloning. *Atr-I* was identified in a two-step process involving PCR amplification and subsequent screening of PCR products. Total genomic DNA (100 ng) was incubated with primers A and B (2) and amplified by PCR (2). Amplified products in the 200- to 500-bp range were cloned into pBluescript KS between the *Bam*HI and *Xho*I sites. To enrich for sequences of interest, this library was screened under low-stringency conditions with a kinase-specific probe of the *Atr-II* gene (5). Positive clones were sequenced by the dideoxy-chain termination method with a Pharmacia Automated Laser Sequencer. Among these clones were several

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corresponding to the *Atr-I* gene. Using the *Atr-I* PCR product as a probe, we obtained full-length cDNAs of the 3.6- and 4.9-kb classes by screening 0- to 4-h and 0- to 8-h *Drosophila* embryo cDNA libraries kindly provided by Nick Brown (3). After subcloning the 3.6-kb insert of cDNA clone pNB40-1a into pBluescript, we generated a series of nested deletions for each strand by using an Erase-A-Base kit (Promega) and sequenced them. The sequence of the alternative form *Atr-I*$_2$, present in a 4.9-kb cDNA, was determined by using a series of oligonucleotides as sequencing primers for the entire extracellular and transmembrane domains.

Transfections and receptor assays. For COS-1 cell transient transfections, the *Sal*I-*Bam*HI fragment of *Atr-I* cDNA clone pNB40-1a was subcloned into pCMV5. The construction of Atr-II, ActR-II, and ActR-IIB in pCMV5 has been described previously (2, 5). For immunoprecipitations, the HA1 epitope of influenza virus hemagglutinin was introduced at the carboxy terminus of Atr-I as described previously for the TβR-II (32). For transient transfections, COS-1 cells were incubated with 2 μg of plasmid per ml diluted in Dulbecco's modified Eagle's medium containing 10% NuSerum (Collaborative Research), 400 μg of DEAE-dextran per ml, and 100 μM chloroquine and assayed 48 h posttransfection (2). Affinity labeling and iodination of activin A were carried out as previously described (2). For immunoprecipitations, affinity-labeled cells were solubilized in lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA) in the presence of protease inhibitors (the same as for affinity labeling) at 4°C for 20 to 30 min. Insoluble debris was removed by microcentrifugation at 16,000 × *g* for 5 min, and receptors were immunoprecipitated by incubating the lysate with polyclonal antibody raised against bacterially expressed Atr-II (5) or with anti-HA monoclonal antibody 12CA5 for 1 h at 4°C and then adsorbing it to protein A-Sepharose (Pharmacia). Beads were washed six times in lysis buffer with 0.1% Triton X-100, and bound protein was eluted by heating in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing dithiothreitol.

In situ and Northern hybridization assays. For in situ hybridization to mRNA, embryos from a *y,w* stock were collected, dechorionized with 50% bleach, and fixed for 20 min with 4% formaldehyde in phosphate-buffered saline–heptane (2:1). The embryos were devitellinized by being washed in methanol and hybridized with either sense (as negative controls) or antisense *Atr-I* probes labeled with digoxigenin-UTP (Boehringer Mannheim). The probe used contained the central region of the *Atr-I* gene and hybridized to all three mRNA species. Hybridization and detection were done as previously described (28), except that post-hybridization washes were extended to 2 days to reduce background. For Northern (RNA) blots, total RNA was prepared from staged embryos, larvae, or adults and poly(A)$^+$ RNA was isolated with a PolyATract kit (Promega). A 2.5-μg sample of poly(A)$^+$ RNA was loaded per lane. Hybridization with random-primed probes was carried out under standard conditions.

RESULTS

Searching for additional members of the serine/threonine kinase receptor family in *D. melanogaster*, we obtained a PCR product encoding a novel sequence (Fig. 1). Screening of embryonic fly cDNA libraries with this product yielded seven isolates of a 3.6-kb cDNA, which we now refer to as *Atr-I*. The *Atr-I* gene is located in the 45A1-2 interval on the right arm of the second chromosome (data not shown), as determined by hybridization of an *Atr-I* cDNA probe to polytene chromosomes (24). The predicted amino acid sequence of Atr-I shows the structural features of a transmembrane serine/threonine kinase (Fig. 1A and B).

The kinase domain of Atr-I resembles most closely (60 to 72% amino acid sequence identity [Fig. 1C]) that of mammalian type I receptors for TGF-β and activin (1, 7, 9), as well as related human orphan receptors (28a, 33), their rat homologs (11), and RPK-1 from chicken cells (27). The Atr-I kinase domain is more distantly related (37 to 40% amino acid sequence identity) to those of the type II receptors (2, 8, 13, 16, 20, 21), including the *Drosophila* activin receptor Atr-II (5). The extracellular region of Atr-I shows little sequence similarity to other receptors and is larger than those of serine/threonine kinase receptors from vertebrates. However, the spacing of the 10 extracellular cysteines in Atr-I resembles the spacing in the other type I receptors (Fig. 1A and B and 2), and includes the cysteine box motif near the transmembrane region that is characteristic of the serine/threonine kinase receptor family (5, 19). In addition, a comparison of Atr-I with the other type I receptors revealed the presence of a conserved 30-amino-acid region immediately preceding the kinase domain (Fig. 2). This region is rich in serines and threonines and contains a repeated Gly-Ser sequence in the middle.

A second class of cDNA, *Atr-I*$_2$, is represented by one clone of 4.9 kb obtained in the same library screening. This clone encodes a product in which a 70-amino-acid sequence replaces a 49-amino-acid sequence in the extracellular region of Atr-I near the transmembrane domain (Fig. 3). Aside from the cysteine box, which is included in this region, there is virtually no similarity between the two alternative sequences.

Atr-I transfected alone in monkey COS cells did not bind radiolabeled TGF-β1, activin A or BMP-2, as determined by binding assays and receptor cross-linking assays (Fig. 4a; data not shown). Given the possibility that Atr-I is a type I receptor, we determined its binding activity when coexpressed with *Drosophila* Atr-II. After cross-linking to receptor-bound ^{125}I-activin A, COS cells cotransfected with Atr-I and Atr-II yielded specifically labeled products of 70 to 90 kDa that correspond to the affinity-labeled Atr-II protein (5) and an additional product of 100 kDa that corresponds in size to Atr-I cross-linked with activin (Fig. 4a). The larger size of this product compared with labeled mammalian type I receptors (65 kDa) (1, 4, 32) correlates with the presence of a larger extracellular region and more N-linked glycosylation sites in Atr-I. Cotransfection of Atr-II with Atr-I$_2$ gave results similar to those obtained with Atr-I, except for the expected slightly larger size of the Atr-I$_2$ product (Fig. 4a).

Atr-I had little effect on the activin-binding affinity of Atr-II, which was high (K_d = 400 pM [Fig. 4b]). However, expression of Atr-I decreased markedly the level of Atr-II expression (Fig. 4a), which was reflected in a decrease in the number of activin-binding sites (Fig. 4b). This effect was also observed when other type I receptors were cotransfected with unrelated membrane receptors (1) and is therefore considered nonspecific. The mouse activin receptors ActR-II and ActR-IIB also supported activin binding to Atr-I (Fig. 4c), indicating that Atr-I can interact with type II receptors from widely divergent species.

A characteristic of mammalian TGF-β type I receptors is their ability to form a complex with the type II receptor (32). This property was also shown by Atr-I and Atr-II in the presence of activin, as demonstrated by the ability to copre-

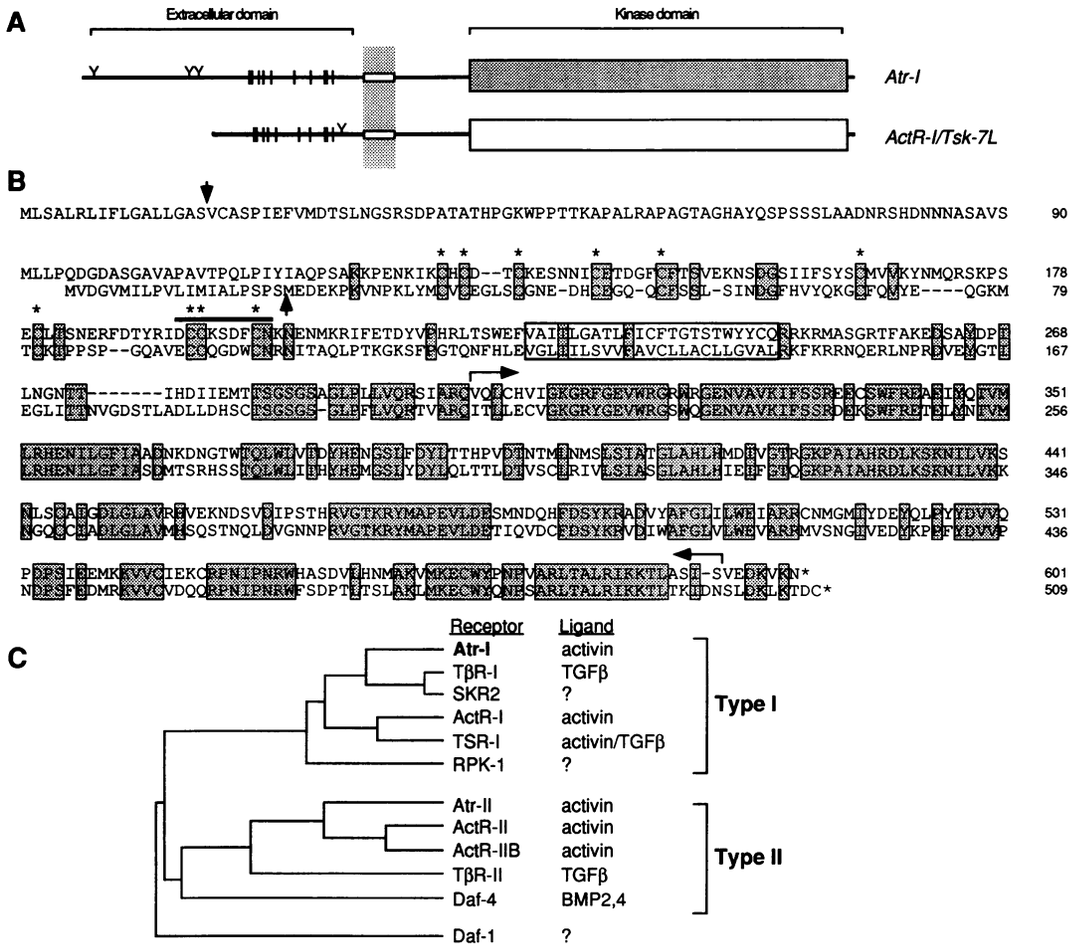

FIG. 1. Schematic representation and comparison of Atr-I and ActR-I. (A) Schematic representation of Atr-I and ActR-I (1) or Tsk-7L (7). Shown are the 10 extracellular cysteine residues (vertical bars), potential glycosylation sites (Y), transmembrane region (shaded box), and the serine/threonine kinase domain (large box). (B) Deduced amino acid sequence of Atr-I and alignment with the amino acid sequence of human ActR-I. Indicated are the potential signal peptidase cleavage sites (vertical arrows), the 10 conserved cysteines in the extracellular domain (asterisks) including the cysteine box (overline), the transmembrane domains (open box), and the limits of the kinase domain (arrow brackets). Amino acids conserved in both receptors are shown (shaded boxes). (C) Relationships between the kinase domains of this receptor family. Shown is a relationship dendrogram with the kinase domains of Atr-I, TβR-I (9), ActR-I (1, 21a), TSR-I (1), Atr-II (5), ActR-II (20), ActR-IIB (2), TβR-II (16), Daf-4 (8), and Daf-1 (10).

cipitate Atr-I with Atr-II by using anti-Atr-II antibody (Fig. 4d) or to coprecipitate Atr-II with antibody against an influenza virus hemagglutinin epitope engineered into the C terminus of Atr-I (Fig. 4d). Thus, these results demonstrated that Atr-I is an activin receptor with characteristics analogous to those of the mammalian type I receptors: it binds ligand with high affinity and specificity depending on the presence of a type II receptor with which it forms a complex.

The members of the TGF-β superfamily identified to date in *D. melanogaster* are the decapentaplegic (dpp) (22) and 60A (6, 31) products, both of which have close structural resemblance to mammalian BMPs and are implicated in fly development. Atr-I did not bind radiolabeled human BMP-2 (data not shown) when cotransfected with the *Caenorhabditis elegans daf-4* gene, which encodes a BMP-2 type II receptor (8). These results and the fact that BMP-2 and dpp appear to be functionally equivalent (23, 26) argue that Atr-I is not a receptor for dpp.

In situ hybridization assays with an *Atr-I* probe showed that this mRNA is maternally deposited into oocytes (Fig. 5A) and is widely distributed during development in the embryo (Fig. 5B) and in imaginal discs of the larva (Fig. 5C). *D. melanogaster* expresses three Atr-I transcripts of approximately 4.9, 4.0, and 3.6 kb at variable levels (Fig. 5D), the source of this diversity being unknown at present. The 4.0- and 3.6-kb mRNAs appear to be predominantly maternal as evidenced by their abundance in adult females but not males and their high level in 0- to 3-h embryos. However, the 4.9-kb mRNA appears to be predominantly zygotic and is present throughout development.

DISCUSSION

Biochemical and genetic data argue that mammalian TGF-β and activin receptors contain two components or receptors I and II. Receptor I requires the presence of receptor II to bind ligand, and both are required for signaling (1, 9, 14, 32). The heteromeric nature of these receptors and their predicted kinase specificity toward serine and threonine residues represent important departures from the well-

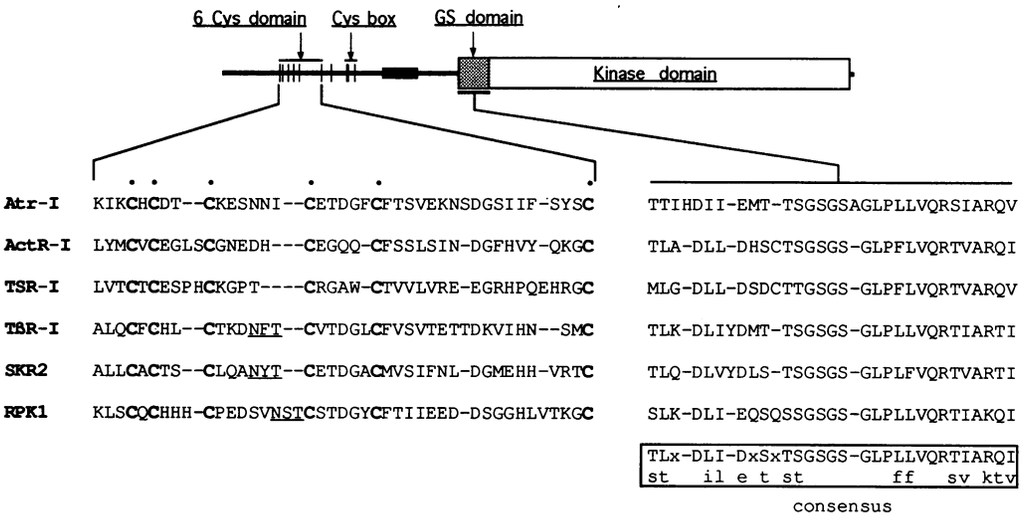

FIG. 2. Atr-I contains domains characteristic of type I receptors. The scheme emphasizes two domains that are characteristic of type I receptors as shown by their presence in Atr-I. The spacing of the six N-terminal cysteines is similar in all known type I receptors (Atr-I, ActR-I/Tsk7L [1, 7, 21a], TSR-I/R3 [1, 11], and TβR-I [9]) and in related orphan receptors (SKR2/R2 [11, 28a, 33] and RPK1 [27]). The three C-terminal cysteines in the extracellular domain constitute the cysteine box motif also present in all known type II receptors for the TGF-β family. The GS domain is adjacent to the kinase region, contains a central SGSGS motif, and is 25% serine and threonine.

established growth factor tyrosine kinase receptor model (29). The present identification of Atr-I as a type I receptor from *D. melanogaster* argues that the heteromeric kinase receptor structure is broadly conserved.

Atr-I was cloned as a new member of the serine/threonine kinase receptor family. Its closest relatives are the mammalian type I receptors TβR-I, ActR-I, Tsk 7L, and TSR-I (1, 7, 9, 21a) and various orphan receptors (11, 27, 33). Although most type I receptors can be distinguished biochemically from type II receptors by their smaller affinity-labeled products (2, 19, 32), Atr-I does not follow this pattern. The extracellular domain of Atr-II is larger than that of most members of the TGF-β receptor family except the BMP type II receptor from *C. elegans* Daf-4 (8), which is of a size similar to Atr-I. Both forms of Atr-I contain 10 extracellular cysteines, whose spacing is similar to that in the other type I receptors (Fig. 2). The three most C-terminal cysteines show the cysteine box arrangement that is present in type I as well as type II receptors (5, 19). Interestingly, the region containing the cysteine box in Atr-I exists in two widely divergent variants that may arise by alternative splicing, an event that generates different variants of the mouse activin receptor ActR-IIB (2). No gross differences in binding activity have been detected so far between the two Atr-I isoforms.

Atr-I binds activin with high affinity in concert with Atr-II, forming a complex that can be immunoprecipitated with antibodies directed against an epitope tag attached to Atr-I. Atr-I can also bind activin when coexpressed with mammalian activin type II receptors, indicating that the interaction is not strongly dependent on species-specific determinants. However, Atr-I did not bind BMP-2 when cotransfected with Daf-4, arguing that Atr-I is not a receptor for the BMP-2-related *Drosophila* factor dpp.

Ligand recognition appears to be mediated primarily by receptor II since this receptor can bind ligand in the absence of receptor I (32). Although the various type II activin receptors (ActR-II, ActR-IIB, and Atr-II) differ considerably in extracellular amino acid sequence, they show an identical spacing of cysteines in this region. Aside from the cysteine box, however, this cysteine pattern is very different from that of type II receptors for TGF-β or BMP (8, 16), suggesting that the cysteine pattern in type II receptors is a key determinant of ligand-binding specificity. In contrast, the type I receptors show extracellular domains with a similar pattern of cysteines irrespective of their ligand-binding specificity (Fig. 2). Aside from the cysteines, the ectodomain sequences of type I activin receptors Atr-I, ActR-I, and TSR-I are almost as divergent from each other as they are from other type I receptors. This region is nevertheless highly conserved between species, as shown, for example, by the human and mouse versions of ActR-I (1, 7), or the human and rat versions of TSR-I (1, 11). Therefore, the differences in sequence between Atr-I and the known mam-

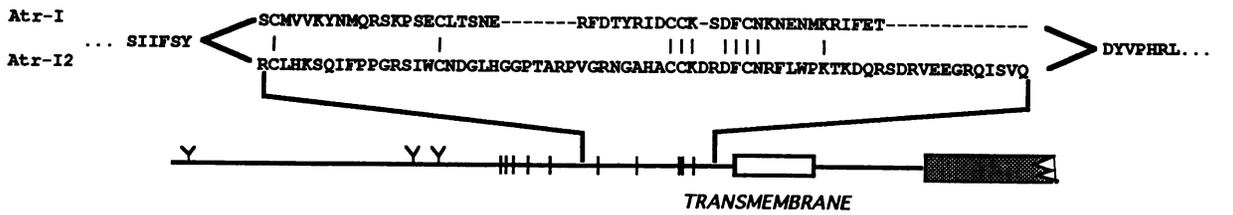

FIG. 3. Schematic representation and amino acid sequence of the Atr-I_2 variant. The approximate location of this region in the extracellular domain is indicated.

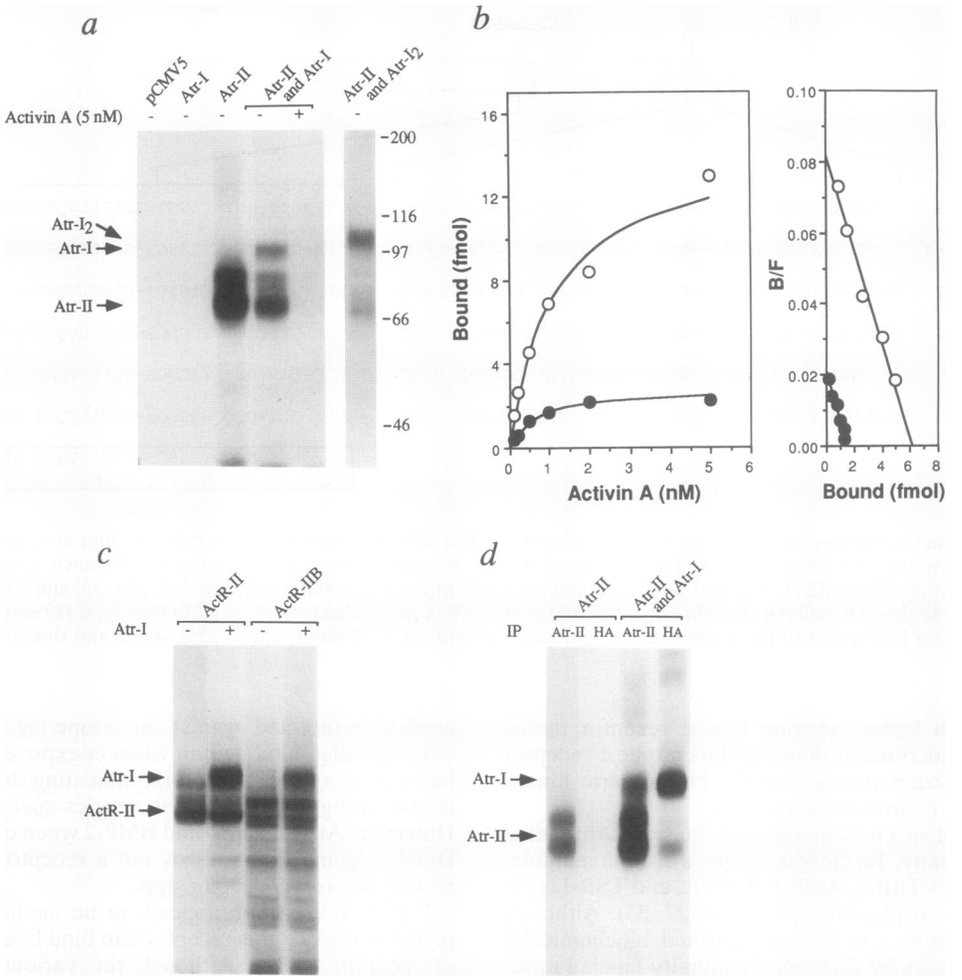

FIG. 4. Atr-I has the properties of an activin type I receptor. (a) COS-1 cells transfected with the indicated cDNAs in pCMV5 or pCMV5 vector alone were affinity labeled with 500 pM of ^{125}I-activin A in the presence (lane +) or absence (lanes −) of 5 nM unlabeled activin A. Detergent extracts from these cells were subjected to SDS-PAGE and autoradiography. Arrows indicate the affinity-labeled products. The positions of the molecular weight markers (in thousands) are indicated. (b) COS-1 cells transfected with Atr-II (open circles) or Atr-II and Atr-I (solid circles) were incubated with increasing concentrations of ^{125}I-activin A, and the bound radiolabeled ligand was quantitated. Total specific binding (left) and the Scatchard analysis of the equilibrium binding data (right) are shown. (c) COS-1 cells transfected with ActR-II or ActR-IIB either alone (lanes −) or together with Atr-I (lanes +) were affinity labeled with 500 pM of ^{125}I-activin A, and detergent extracts were analyzed by SDS-PAGE and autoradiography. (d) COS-1 cells were transfected with Atr-II alone or together with Atr-I containing an HA1 epitope. Cells were affinity labeled with 500 pM of ^{125}I-activin A, detergent lysates were subjected to immunoprecipitation with either a polyclonal antiserum against Atr-II or a monoclonal anti-HA1 antibody, and samples were analyzed by SDS-PAGE.

malian activin receptors are not necessarily a consequence of the divergence between these species and could be of functional significance.

The cytoplasmic region of Atr-I is occupied almost entirely by the kinase domain. This domain shows the closest similarity (60 to 72% amino acid sequence identity) to that of the other type I receptors and is very divergent from the kinase domains of type II receptors. Atr-I contains almost no C-terminal tail following the kinase domain, another property shared with the mammalian type I receptors (1, 7, 9). In addition, a sequence comparison between the cytoplasmic domains of Atr-I and other receptors in the type I subfamily reveals a region of similarity between them that is located immediately upstream of the kinase domain (Fig. 2). This region, of 30 amino acids (31 in Atr-I), contains a high proportion of serine and threonine residues. Its function is unknown. It contains a characteristic SGSGS sequence and is henceforth referred to as the GS domain.

Maternal *Atr-I* transcripts are abundant in the oocyte and widespread during embryo development and in the imaginal discs of the larva. *Atr-II* transcripts are also contributed maternally to the embryo, but the pattern of expression after cellularization is enhanced in the posterior end and the invaginating mesoderm. It later becomes prominent in the fore and midgut regions (5). The broader pattern of Atr-I expression raises the possibility that its product functions in concert with other members of the serine/threonine kinase receptor family to mediate diverse responses during development. In mammalian cells, a given type I receptor may interact with more than one type II receptor (1).

The occurrence of heteromeric kinase receptor complexes for activin in organisms from *D. melanogaster* through humans argues that this singular structure is a general feature of receptors for this family of cytokines in a wide variety of organisms. The presence of two widely different kinase domains in the same receptor complex raises the

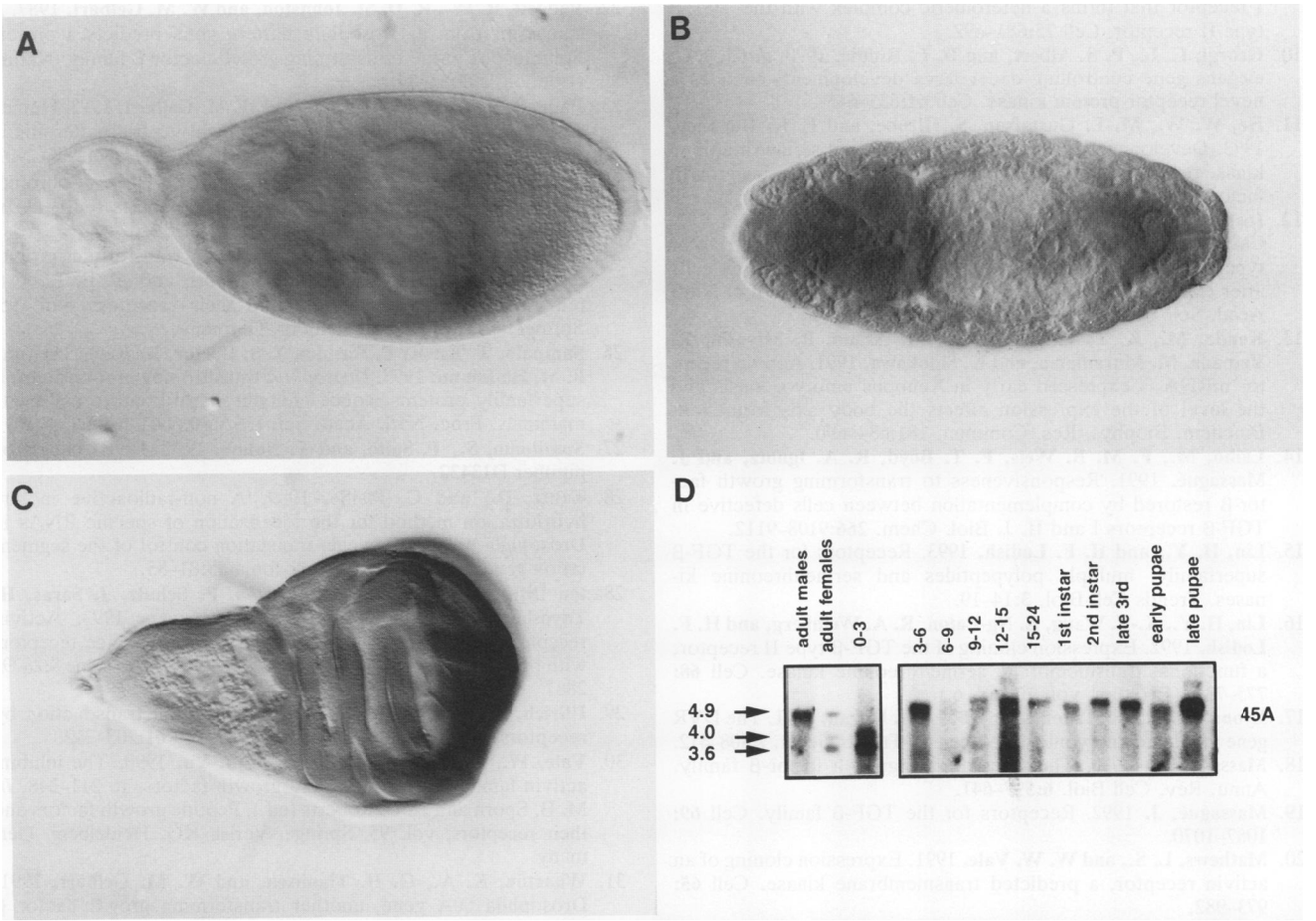

FIG. 5. Analysis of Atr-I expression. (A) Stage 10 to 11 egg chamber showing Atr-I transcripts in both nurse cells and the developing oocyte. Probing with sense Atr-I probe as a negative control showed that the signal was specific. (B) Dorsal view of a stage 14 embryo showing uniform expression of Atr-I. (C) Wing imaginal disc showing high-level expression of Atr-I during the third instar larval stage. All imaginal discs showed similar levels of staining, with no distinct pattern of staining within individual discs. (D) Developmental profile of Atr-I mRNA. The fractionated RNA species were blotted to a nylon membrane and hybridized with a full-length Atr-I probe. Rehybridization of these blots with ribosomal protein gene RP49 showed that similar amounts of RNA were loaded in each lane (results not shown).

possibility that signaling by these receptors has unique features. These might involve separate substrates for each kinase, common substrates undergoing phosphorylation on separate sites, or one receptor kinase acting on the other and thereby generating the first step of a signaling cascade within the receptor complex itself.

ACKNOWLEDGMENTS

This work was supported by awards from the Cancer Research Coordinating Committee of the University of California and National Institutes of Health grant GM47462 to M.B.O. and by National Institutes of Health grant CA34610 and Cancer Center Support Grant P30-CA08748 to J.M. J.L.W. and L.A. are the recipients of postdoctoral fellowships from the Medical Research Council of Canada. J.M. is a Howard Hughes Medical Institute Investigator.

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