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A cDNA clone for cytotactin contains sequences similar to epidermal growth factor-like repeats and segments of fibronectin and fibrinogen

(cell adhesion/differential RNA splicing/Arg-Gly-Asp sequence/extracellular glycoprotein/type III repeats)

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ABSTRACT **Cytotactin is an extracellular glycoprotein that influences neuron–glia interactions. It has been shown to appear in multiple forms that are differentially expressed in neural and non-neural tissues during vertebrate development. We report here the isolation and characterization of a cytotactin cDNA clone (λC801) that encodes 933 amino acids, equivalent to about half of a cytotactin polypeptide. Clone λC801 is an authentic cytotactin cDNA: it encodes a polypeptide that reacts with a monoclonal anti-cytotactin antibody and its deduced amino acid sequence is identical for 15 amino acids to the directly determined sequence of a CNBr fragment that reacted with the same antibody. Southern blot analyses with fragments of λC801 suggested that there may be only one cytotactin gene, but RNA transfer blots detected multiple mRNAs ranging in size from 6.5 to 8.0 kilobases. An 8.0-kilobase message and a M_r 240,000 cytotactin polypeptide were present in embryonic gizzard but not brain, while a 7.2-kilobase message and a M_r 220,000 polypeptide were present in brain but not gizzard. These results indicate that differential splicing of primary transcripts of the cytotactin gene yields various site-specific polypeptides. Sequence analyses of λC801 indicated that it specifies a region with extensive similarities to other proteins: the sequence begins with four consecutive epidermal growth factor-like repeats that are followed by eight segments that closely resemble each other and the type III repeats in fibronectin, and it ends with a 66 amino acid sequence similar to part of the β and γ chains of fibrinogen. One fibronectin-like repeat contains a single Arg-Gly-Asp sequence. The similarities with all three of these apparently unrelated proteins are extensive, suggesting that cytotactin has an evolutionary and possibly a functional relationship to each.**

Orchestration of the formation of epithelia and mesenchyme during animal morphogenesis requires coordination of the processes of cell growth, movement, and cell–cell and cell–substratum interactions by an interactive network of cell surface, cytoskeletal, and extracellular matrix proteins (1, 2). A variety of substrate-adhesion molecules (SAMs) are expressed at crucial times during embryogenesis and histogenesis, and these SAMs help to guide those primary developmental processes, particularly cell movement, that lead to pattern formation. Fibronectin (2), thrombospondin (3), and laminin (4) are among the well-characterized SAMs that mediate these morphoregulatory events.

Cytotactin is a recently discovered SAM that is expressed by a limited number of neural and non-neural tissues during embryogenesis (5). As isolated from embryonic brain, it is a large, disulfide-linked glycoprotein composed of three related but nonidentical polypeptide chains (M_r 220,000, 200,000, and 190,000; refs. 6 and 7). Tenascin (8) and brachionectin (9) appear to be similar or identical to cytotactin. In the brain, cytotactin is synthesized by glia and is involved in neuron–glia adhesion *in vitro* (6). One of its natural ligands is a chondroitin sulfate proteoglycan with a core protein of apparent M_r 280,000 (10); cytotactin also binds to fibronectin.

A restricted pattern of cytotactin expression is seen during development, most prominently in areas of cell migration (5, 11, 12). In contrast to fibronectin, which promotes the spreading and migration of cultured cells (2), cytotactin appears to inhibit cell migration. For example, migratory neural crest cells actively avoided painted strips of cytotactin that lay in their path (12). The inhibitory effects of cytotactin on crest cell migration were counteracted (12) when cytotactin was mixed with fibronectin, suggesting that mutual expression or interaction of these molecules in different ratios could differentially modulate cell movement.

To provide a structural basis for understanding the expression and activity of cytotactin and to compare cytotactin with other extracellular matrix proteins, we have isolated cDNA clones specific for the molecule. We describe here the characterization of a clone that encodes about half of a cytotactin polypeptide. Hybridization studies with this cDNA suggest that cytotactin is encoded by a single gene that is differentially spliced to give multiple mRNAs and polypeptides, two of which are preferentially expressed in brain and gizzard. Analysis of the cDNA sequences* reveals that the predicted protein sequence of cytotactin bears significant similarity to specific domains of human fibronectin and fibrinogen and contains a series of 31 amino acid cysteine-rich repeats that resemble the epidermal growth factor (EGF)-like repeats seen in a variety of other proteins including the Notch gene product of *Drosophila melanogaster* (13). These findings support the idea that cytotactin evolved by selective incorporation of genetic elements from a variety of evolutionary precursors.

MATERIALS AND METHODS

A 10-day embryonic chicken cDNA library (Clontech Laboratories, Palo Alto, CA) in bacteriophage λgt11 was screened (5×10^4 plaque-forming units per plate) on *Escherichia coli* Y1090 cells (14), using three independent antibodies specific for cytotactin polypeptides; antibody binding was detected with ^{125}I-labeled protein A.

For RNA transfer blots, 20 μg each of brain, liver, and gizzard total RNA was resolved by electrophoresis in formaldehyde-containing gels (15), transferred to nitrocellulose, and hybridized to ^{32}P-labeled DNA. Southern blot hybrid-

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Abbreviations: E*n*, embryonic day *n*; EGF, epidermal growth factor; SAM, substrate-adhesion molecule.

*These sequences are being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03641).

ization analysis of embryonic chicken liver DNA (10 μg) was performed as described (15). All filters were washed twice for 20 min at 65°C with 0.3 M NaCl/0.03 M sodium citrate/0.1% $NaDodSO_4$.

The DNA insert was liberated from CsCl gradient-purified phage λC801 by digestion with restriction endonuclease *Eco*RI. Two fragments, approximately 2.0 kilobases (kb) and 0.8 kb long, were purified by electrophoresis in agarose gels and inserted into the *Eco*RI site of Bluescript KS vectors (Stratagene, San Diego, CA), yielding subclones pEC802 and pEC803, respectively. For M13 sequencing, insert DNA from λC801 and pEC802 was digested with restriction enzymes, subcloned into bacteriophage vector M13mp18 or M13mp19 (16), and selected by plaque hybridization with purified ^{32}P-labeled cDNA from inserts pEC802 and pEC803 or subfragments thereof. DNA was sequenced by the dideoxynucleotide chain-termination method using the modified T7 DNA polymerase, Sequenase (17). Sequence data were compiled using the Staden ANALYSEQ programs (18) and used in data base†‡ searches with the Wilbur–Lipman fast search algorithm (19).

CNBr fragments of purified cytotactin (7) were separated by electrophoresis in $NaDodSO_4$/12% polyacrylamide gels. A single M_r 22,000 fragment reactive with the 1D8 monoclonal antibody was electroeluted and subjected to automated Edman degradation in the Rockefeller University Sequencing Facility.

RESULTS

Cytotactin cDNA clones were selected by screening a λgt11 library successively with two polyclonal anti-cytotactin antibodies and the monoclonal anti-cytotactin antibody 1D8. One clone, λC801, encoding epitopes recognized by all three antibodies, was selected for further characterization. Digestion of λC801 DNA with *Eco*RI yielded two fragments of 2.0 kb and 0.8 kb, which were isolated and subcloned into Bluescript KS.P; these clones were designated pEC802 and pEC803, respectively. Subclone pEC802 was placed at the 5′ end of λC801 on the basis of direct sequence of this portion of the λC801 DNA; pEC803 was placed at the 3′ end on the basis of restriction maps.

The insert from pEC802 hybridized to multiple RNA species in brain and gizzard that are sufficiently large to code for cytotactin polypeptides. Expression of a 7.2-kb RNA in brain increased between embryonic days 9 and 15 (E9 and E15; Fig. 1*A*, compare lanes 3 and 1). Over the same period, expression of an 8.0-kb RNA species in gizzard also increased (compare lanes 4 and 2), while no RNA from E15 liver reacted with pEC802 (lane 5). Consistent with these findings, the monoclonal anti-cytotactin antibody 1D8 recognized M_r 220,000 and 200,000 polypeptides in the E15 brain (Fig. 1*B*, lane 1) and M_r 240,000 and 200,000 species in the E15 gizzard (Fig. 1*B*, lane 2) but did not recognize any liver polypeptides.

Southern blot analyses (Fig. 1*C*) yielded single bands in *Xba* I, *Hin*dIII, and *Sst* I digests (4.3 kb, 5.5 kb, and 8.0 kb, respectively; lanes 2, 4, and 5) and two bands in an *Eco*RI digest (2.6 kb and 0.8 kb; lane 1) and a *Pst* I digest (2.3 kb and 0.7 kb; lane 3) when probed with the ^{32}P-labeled insert of pEC803; none of these enzymes cleaved the probe. These results are consistent with the notion that *Eco*RI and *Pst* I cleave within introns and that cytotactin is encoded by one or at most two genes in the chicken genome.

Sequence Analysis of pEC802 and pEC803. The complete DNA sequences of pEC802 and pEC803 are presented in Fig. 2 along with the deduced amino acid sequences. The pEC802 cDNA sequence contained only one continuous open reading frame (672 amino acids) that includes many cysteine

†EMBL/GenBank Genetic Sequence Database (1987) GenBank (Bolt, Beranek, and Newman Laboratories, Cambridge, MA), Tape Release 52.0.

‡Protein Identification Resource (1987) Protein Sequence Database (Natl. Biomed. Res. Found., Washington, DC), Release 12.0.

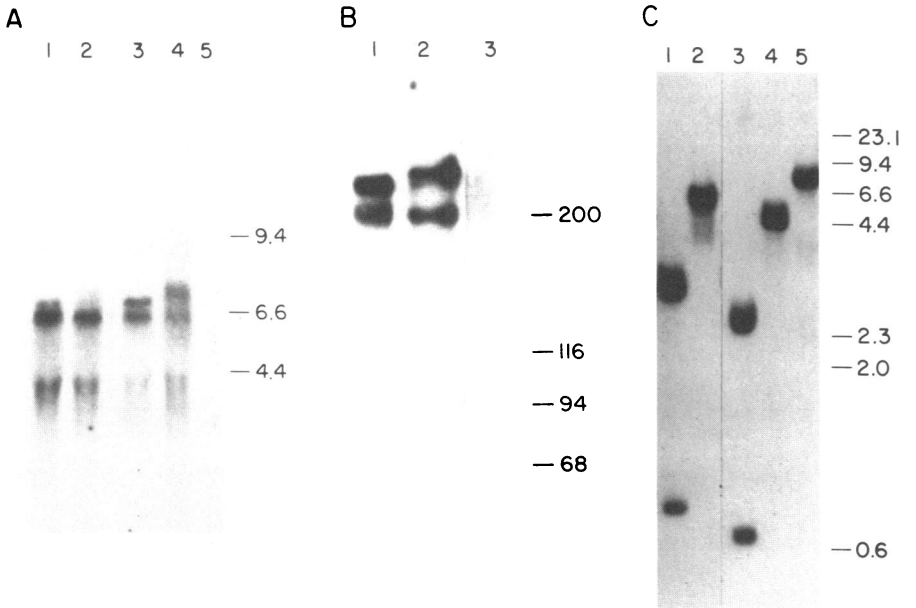

FIG. 1. Blot analysis of cytotactin-specific mRNAs (*A*), polypeptides (*B*), and genomic DNA (*C*). (*A*) RNA from the indicated organs was resolved in a 1.2% agarose/formaldehyde gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled pEC802 insert. Lane 1, E9 brain; lane 2, E9 gizzard; lane 3, E15 brain; lane 4, E15 gizzard; lane 5, E15 liver. (*B*) Proteins extracted in phosphate-buffered saline from the equivalent of 5 μl of packed E15 brain tissue (lane 1) or extracted in 4 M guanidine hydrochloride/0.1 M sodium phosphate, pH 7.4, from the equivalent of 100 μl of packed E15 gizzard (lane 2) or liver (lane 3) tissue were resolved in a $NaDodSO_4$/6% polyacrylamide gel, transferred to nitrocellulose, and detected by immunoblotting using the monoclonal anti-cytotactin antibody 1D8. Migration and $M_r \times 10^{-3}$ of standard proteins are indicated. (*C*) Genomic DNA prepared from embryonic chicken livers was digested with *Eco*RI (lane 1), *Xba* I (lane 2), *Pst* I (lane 3), *Hin*dIII (lane 4), or *Sst* I (lane 5), resolved in a 1.0% agarose gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled pEC803 insert DNA. The positions and lengths (in kb) of *Hin*dIII fragments of phage λ DNA are indicated in *A* and *C*.

residues at the 5′ end of the clone and four potential asparagine-glycosylation sequences (Asn-Xaa-Thr or Asn-Xaa-Ser). The pEC803 cDNA sequence also had a single continuous open reading frame (261 amino acids) that includes two potential asparagine-glycosylation sequences and two cysteine residues.

Five amino acids at the 5′ end of pEC802 and five at the 3′ end of pEC803 are probably not in the cytotactin sequence but are most likely translations of DNA from *Eco*RI linkers used to make the library; because they are in-frame, we include them until further studies justify their removal. We also assume that pEC802 and -803 are contiguous in the λC801 sequence. The sizes and restriction maps of the clones support this assumption, but we have not yet sequenced an overlapping segment, so they are shown as separate units.

The polypeptide epitope of the 1D8 monoclonal antibody used to detect λC801 was localized to a single CNBr fragment (M_r 22,000), which was purified by NaDodSO$_4$/PAGE. Its amino-terminal sequence was identical to that deduced from pEC802 residues 396–410, and in the deduced sequence it was preceded by a methionine. This result substantiates that λC801 is an authentic cytotactin cDNA. Another methionine appears at residue 544, suggesting that the 1D8 epitope is contained within residues 396–544.

Immediately preceding methionine-395, the deduced amino acid sequence of pEC802 contains the tripeptide sequence Arg-Gly-Asp, which has been implicated as a recognition sequence for the attachment of extracellular matrix proteins to cellular receptors (20). Moreover, computer searches revealed that the amino acid sequences of cytotactin encoded

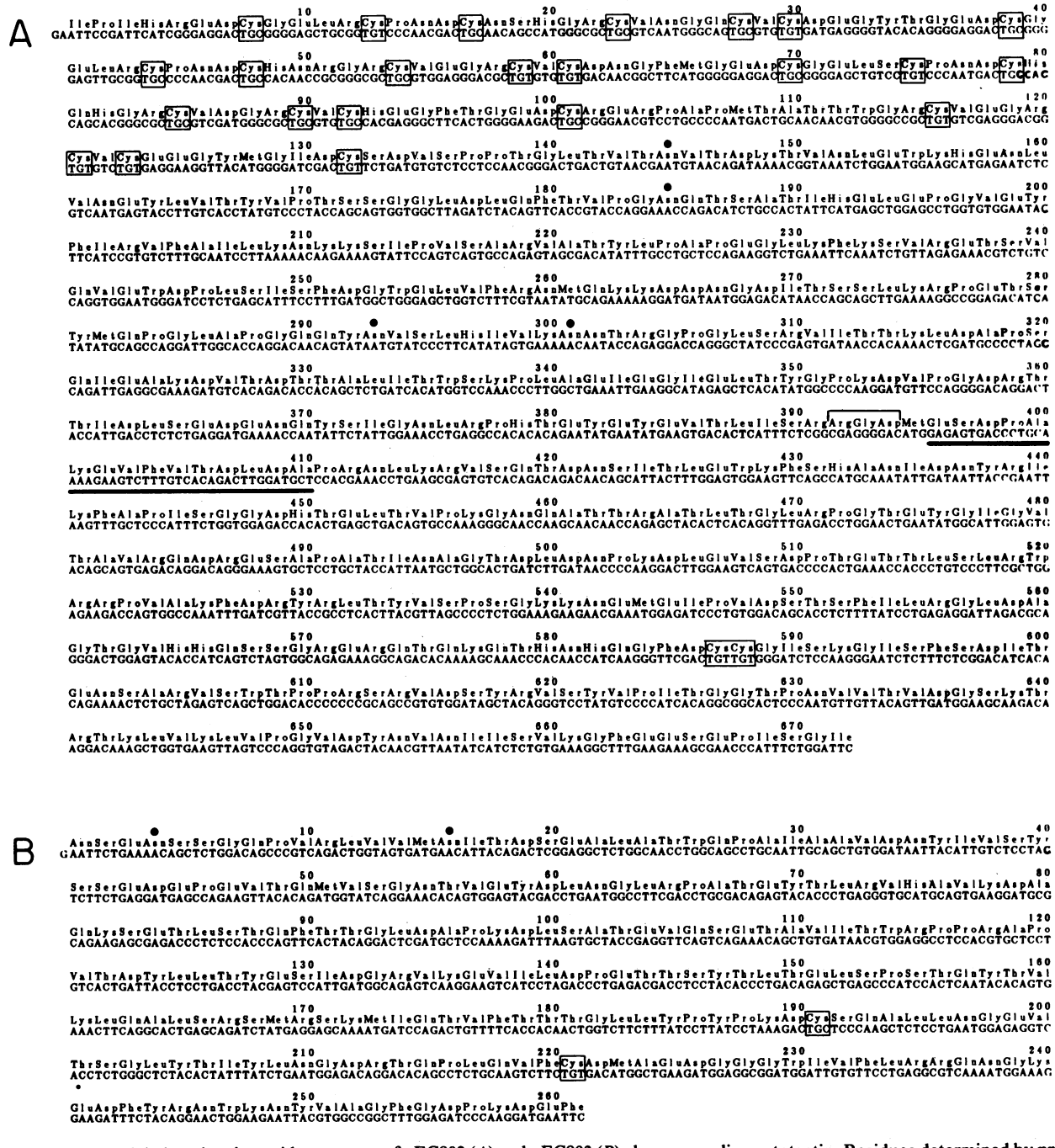

FIG. 2. DNA and deduced amino acid sequences of pEC802 (*A*) and pEC803 (*B*) clones encoding cytotactin. Residues determined by protein sequence analysis of the 1D8-immunoreactive CNBr fragment of cytotactin are underlined with a dark bar. Potential asparagine-glycosylation sites are indicated with dots and cysteines by boxes. The sequence Arg-Gly-Asp is marked by a half-box.

by pEC802 and pEC803 contain three distinct regions of similarity with (*i*) cysteine-rich EGF-like repeats, (*ii*) the type III repeats of fibronectin, and (*iii*) a segment of the β and γ chains of fibrinogen. These similarities are summarized in Fig. 3 and shown schematically in Fig. 4.

Beginning at the 5′ end of λC801, the homologous segments are contiguous. Amino acids 6–132 encoded by pEC802 can be depicted as four cysteine-rich repeats of 31 amino acids (Fig. 3*A*). The first three repeats are 67% identical at the amino acid level and follow a pattern of $CX_4CX_3CX_5CX_4CXCX_8$, where C is cysteine and X_n designates amino acids between cysteines. The fourth repeat has the pattern of $CX_{14}CX_4CXCX_8$. The sequence that encodes all four repeats is 53% identical at the DNA level and 30% identical at the amino acid level to a comparable sequence derived from the region of EGF-like repeats encoded by the Notch gene product (13); an example of a part of this region of even greater similarity between the cytotactin and Notch sequences is shown in Fig. 3*B*.

The remaining 80% of pEC802 and the 5′ half of pEC803 encode units of 90 amino acids that are similar to each other and to the central block of type III repeats in fibronectin (21) (Fig. 3*C*); one of these repeats (repeat III, residues 313–405) in cytotactin contains the Arg-Gly-Asp sequence 10 residues from the end of the repeat, just as it is found in fibronectin (22). If pEC803 is immediately adjacent to pEC802 in λC801, the eight repeats would be consecutive in the cytotactin sequence. Two residues, tryptophan and leucine, are conserved in all of the cytotactin repeats (see Fig. 3*C*) and in the fibronectin type III array. A tyrosine near the conserved leucine is also present in all type III units of fibronectin and in all but one of the cytotactin type III repeats. Each cytotactin repeat has 26–40% identity with its most homologous counterpart in fibronectin. The greatest similarity is shown in Fig. 3*D*; no other pair is as similar (35 identities) either among the cytotactin type III repeats or among the 17 type III repeats in fibronectin. The continuous sequences are also similar; the first six cytotactin repeats (residues 155–667) are 26% identical to residues 623–1133 of human fibronectin.

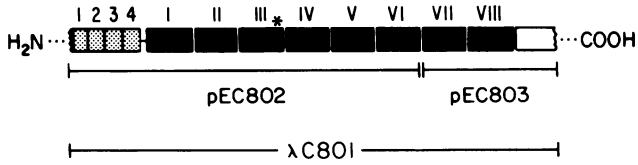

FIG. 4. Schematic drawing of the portion of cytotactin represented by the λgt11 clone λC801 and its subclones pEC802 and pEC803. Stippled boxes denote cysteine-rich EGF-like internal repeats (numbered 1–4), shaded boxes denote fibronectin type III-like repeats (I–VIII), and the open box denotes a region that is homologous to segments in the β and γ chains of fibrinogen. The asterisk denotes the position of an Arg-Gly-Asp sequence. The location of this 933 amino acid sequence within the overall cytotactin sequence is unknown, but it does represent about half of a cytotactin polypeptide.

The remaining half of pEC803 encodes a sequence (amino acids 189–255) that resembles a number of vertebrate fibrinogens. The greatest similarity is with amino acids 239–304 in the β chain of human fibrinogen and amino acids 177–242 in the γ chain of human fibrinogen, segments that are known to be highly homologous to each other (23) and that include two conserved cysteine residues. The cytotactin sequence contains comparable cysteines and is as similar to each fibrinogen chain as the fibrinogen chains are to each other.

DISCUSSION

We have described the sequence of a cDNA that encodes approximately half of the extracellular glycoprotein cytotactin. The cDNA, designated λC801, codes for a polypeptide epitope (1D8) recognized by a monoclonal anti-cytotactin antibody and has identical amino acid sequence for 15 residues with a CNBr fragment of cytotactin that reacts with the same antibody, supporting its authenticity as a cDNA encoding cytotactin.

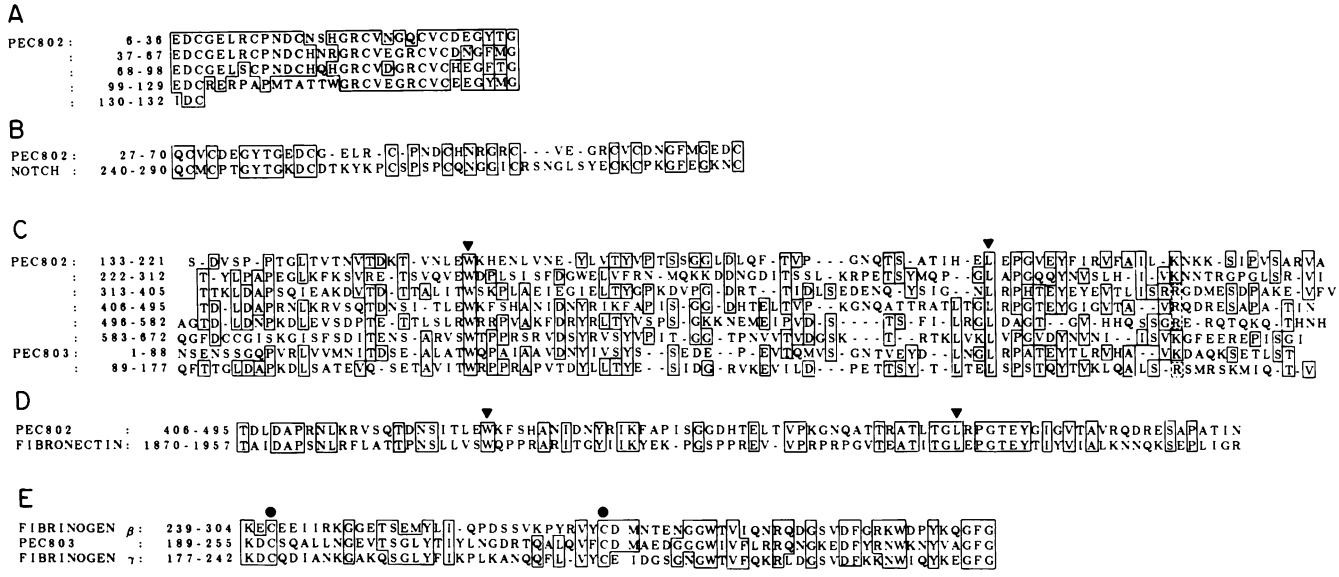

FIG. 3 Similarities of amino acid sequences encoded by pEC802 and pEC803 with known proteins. Amino acids are represented by standard one-letter symbols. Identical amino acids are boxed. (*A*) Cysteine-rich repeats from pEC802. (*B*) Comparison of 44 amino acids from the cysteine-rich repeats with a sequence from the EGF-like repeats in the Notch gene product of *D. melanogaster*. (*C*) Alignment of similar segments within the sequence encoded by pEC802 and pEC803 that resemble type III repeats in fibronectin. (Only residues appearing in four or more repeats are boxed.) Triangles indicate the tryptophan and leucine common to all repeats. (*D*) Comparison of a cytotactin repeat (from *C*) with the most closely related type III repeat from human fibronectin. (*E*) Comparison of the region encoded by the 3′ end of pEC803 with segments within the β and γ chains of human fibrinogen. Identities between cytotactin and fibrinogen are boxed. The two cysteine residues conserved in all sequences are indicated by black circles.

The outstanding feature of this portion of cytotactin is its extensive similarity to portions of three different proteins. These homologous units are arranged in a contiguous array. The most amino-terminal region has four cysteine-rich EGF-like repeats (13). These repeats are followed by a short segment with no known similarity to other proteins and then by eight consecutive similar segments that closely resemble the type III repeats of fibronectin (21). The remainder of the sequence is similar to regions in the β chain and γ chain of human fibrinogen that are known to be similar to each other (23). Additional cysteine-rich repeats may be encoded by the segment of cDNA that is 5′ to the λC801 clone, and additional amino acid sequence homologous to fibrinogen may be encoded by cytotactin cDNA 3′ to λC801.

The similarities between cytotactin and fibronectin may reflect their roles as SAMs. Both contain the Arg-Gly-Asp cellular-recognition sequence in comparable locations in a type III repeat (22). The surrounding sequence in cytotactin (Arg-Arg-Gly-Asp-Met) differs, however, from that in fibronectin (Gly-Arg-Gly-Asp-Ser). The Arg-Gly-Asp-containing repeat and flanking units shown in Fig. 3*C* are likely to represent a cell-binding domain of cytotactin. This notion is supported by experiments showing that Arg-Gly-Asp peptides and Arg-Gly-Asp-containing fragments of cytotactin inhibit the binding of cytotactin to cells (unpublished observations). Of the other fibronectin-like type III repeats, the two most similar segments in the two proteins are repeat IV of cytotactin and the heparin-binding repeat of fibronectin (22); so far, however, we have detected no heparin-binding activity in cytotactin isolated from brain.

Overall, the observations suggest that both cytotactin and fibronectin inherited a large type III repeat array from a common ancestral precursor. In fibronectin, tissue-specific splicing occurs within the array of type III repeats, yielding molecules with different functional properties and solubilities (23). Similar alternative splicing may occur within the type III array of cytotactin to yield the differentially extractable cytotactin polypeptides (5, 7). Hybridization experiments suggest that the cytotactin polypeptides are encoded by a single gene, but identify several RNA species. Moreover, both cytotactin polypeptides and mRNAs are differentially expressed in gizzard and in brain. These data suggest that multiple cytotactin mRNAs may arise from alternative splicing of primary transcripts from a single gene in a tissue- and time-specific manner during development.

EGF-like repeats appear in a wide variety of extracellular and developmentally regulated proteins (see ref. 13). The role of such tightly folded, disulfide-bonded elements is unknown. Of the known proteins with EGF-like repeats, those in cytotactin most closely resemble those of the Notch gene product in *D. melanogaster*, which has 32 such units. It has been proposed that the Notch gene product is involved in cell–cell interactions during neural development (13). In view of the role of cytotactin in cell migration and adhesion (6, 11, 12), it will be of interest to compare the functions of the EGF-like repeats in these two developmentally regulated proteins.

The resemblance among the cytotactin sequence and the β and γ chains of fibrinogen is extensive, suggesting a common evolutionary origin. The conserved element would appear to include an intrachain disulfide loop of about 20 amino acids (24). In fibrinogen, this small loop is on the arm of the β and γ chains well away from the area where the three chains are interconnected by interchain disulfide bonds. By analogy, we anticipate that the loop will appear in cytotactin in one or more of the arms, away from the node of the hexabrachion, which probably is the region where the multiple cytotactin polypeptides are joined by interchain disulfide bonds.

The similarities of cytotactin to elements in three distinct proteins support notions that metazoan proteins evolve in part by combining genetic elements that specify existing protein structures with differing functions. This is becoming apparent in a variety of molecules, such as the immunoglobulin superfamily (25), regulatory proteases (26), and extracellular matrix proteins (27). In each case, the evolutionary precursors from which such combinations are made are unknown, but it is likely that the process involves genes that have undergone significant duplication.

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