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Identification of a Homolog of Actin-Binding Protein, ABP-280, Localized at Epithelial Cell-Cell Boundaries in Hydra

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ABSTRACT—ABP-280 cross-links actin filaments and connects membrane glycoproteins and actin filaments in mammalian blood cells. We isolated cDNA clones for ABP-280 from hydra by screening a cDNA expression library with an antiserum against a cell membrane-enriched crude fraction. The amino acid sequences deduced from the cDNA clones showed significant similarity to human ABP-280, including intramolecular repetition of a unique sequence motif, suggesting similar tertiary structure and molecular activity. Monospecific antibodies were purified from the antiserum, by affinity to fusion proteins produced from the cDNA clones, and used for immunohistochemistry. Specific staining was detected at epithelial cell-cell boundaries in the hydra. The signals were restricted to the subapico-lateral regions corresponding to the locations of septate junctions. These results suggest that the identified molecule is a possible component of the septate junction, and presumably connects membrane cell adhesion molecules to actin filaments in the epithelial cells of hydra. This is the first report describing the subcellular distribution of ABP-280-related molecules in epithelial cells.

INTRODUCTION

The septate junction is a major cell-cell adhesion apparatus in invertebrate epithelial cells, characterized by electron microscopy as electron-dense septa between the cells associated with actin filaments. Septate junctions are found widely in invertebrates, including all the Protostomia and the Echinodermata (Green and Bergquist, 1982). Septate-like junctions were also identified at the cell-cell contact site between mating protozoans (Dallai and Talluri, 1988). Although the septate junction is prominent in epithelial cells, its functions and molecular organization are not nearly as well studied as those of the adherens junction.

Only five molecules associated with septate junctions have been identified in *Drosophila* (Woods and Bryant, 1991; Woods and Bryant, 1993; Fehon *et al.*, 1994; Baumgartner *et al.*, 1996; Takahisa *et al.*, 1996; Woods *et al.*, 1996). Strikingly, four of these molecules play essential roles in the development of cell polarity, cell-cell communication, or growth regulation in epithelial cells. The septate junction is not merely a permeability barrier, as formerly thought (Green and Bergquist, 1982), but a signaling center for maintaining and regulating multicellular organization in invertebrates (Woods and Bryant, 1993). To investigate the functions of the septate junction, further identification of septate junction molecules is important.

The septate junction was first identified in the hydra by electron microscopy. The hydra has a very simple body plan, consisting of an outer layer of ectodermal epithelium and an inner layer of endodermal epithelium. Its simple organization and high regenerative capacity provide model systems to investigate morphogenesis at the cellular level (Kishimoto *et al.*, 1996; Murate *et al.*, 1997). Identifying the molecules involved in cell adhesion is of interest and important for studying the morphogenesis of hydra. In this study, we applied the epitope selection method, which allows simultaneous rapid isolation of both cDNA clones and specific antibodies, and screened proteins localized at the epithelial cell-to-cell boundary, in order to identify septate junction molecules in hydra.

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MATERIALS AND METHODS

Animals

Hydra magnipapillata strain 105 was used for all the experiments.

The animals were maintained in standard cultures used for immunostaining as described (Takano and Sugiyama, 1983). A mass culture for RNA and antigen preparation was maintained in a 25×35 cm plastic tray. The animals were fed heavily and transferred to fresh culture water daily with a nylon net.

Antiserum generation

Live hydra were homogenized in water with a Dounce's homogenizer, and spun down at 1,000g for 5 min. The supernatant was pelletted by centrifugation at 10,000g for 30 min. The pellet was resuspended in PBS, mixed with Freund's adjuvant, and injected in rabbits following conventional immunization methods.

DNA manipulation

Standard procedures were used for DNA and RNA manipulation (Perbel, 1988; Sambrook *et al.*, 1989). RNA was extracted from whole polyps using the AGPC method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was purified with oligo-dT beads (Oligotex, Roche). A cDNA expression library was constructed using the methods described in the manufacturer's product manual (Stratagene); cDNA was produced with random primers, ligated to EcoRI adaptors, inserted in λZAPII, and packaged *in vitro* (Gigapack Gold, Stratagene).

Epitope selection screening

The screening strategy is shown schematically in Fig.1. As described above, antiserum was generated against a hydra cell membrane enriched fraction that was insoluble after homogenizing the tissues. The cDNA expression library was screened with the antiserum at a 1/1000 dilution according to standard procedures. Signals were detected with alkaline phosphatase-conjugated anti-rabbit IgG and the NBT/BCIP reaction. Affinity purification of monospecific antibodies was modified from Weinberger *et al.*, (1985). For each clone, 1×10^5 p.f.u. of phages were grown on a 10cm dish at 37°C for 2 hrs. A 5 × 5 cm nitrocellulose filter immersed in 0.1 M IPTG was placed on the culture, which was then kept at 37°C for 4 hr. The filter was blocked in 5% skim milk for 30 min, incubated with the serum at a 1/1000 dilution for 2 hr, washed with TBS, eluted with 1ml of 10 mM glycine-HCl pH 2.8 and immediately neutralized with 1 M Tris-base, and then 1/30 vol. of 5 M NaCl was added. This solution was used as a 1× antibody solution for whole mount immunostaining.

Immunostaining and immunoblotting

For whole mount staining, the animals were fixed in Lavdowsky's fixative for 30 min, washed with PBS, blocked with 5% skim milk in PBS for 1 hr, and incubated with the recovered monospecific antibodies overnight at 4°C. FITC-conjugated anti-rabbit IgG was used for detection.

Western blotting was carried out according to conventional methods (Sambrook *et al.*, 1989). Hydra polyps were dissolved in sample buffer, loaded on a 6% polyacryamide gel, and blotted on a nitrocellulose filter. The filter was blocked with 5% skim milk in Tris-buffered saline pH 7.5 (TBS), and incubated with the recovered monospecific antibodies for 2 hr at room temperature. Alkaline phosphatase-conjugated anti-rabbit IgG was used for detection.

RESULTS

Isolation of cDNA clones from hydra

A cDNA expression library with over 1×10^6 plaques was exhaustively screened with an antiserum against hydra-insoluble materials, and 200 positive clones were isolated. The fusion protein for each of the 200 clones was produced, transferred to a nitrocellulose filter, and incubated with the antiserum. Bound antibodies were recovered and assessed by whole mount immunostaining (Fig.1). The resulting staining patterns

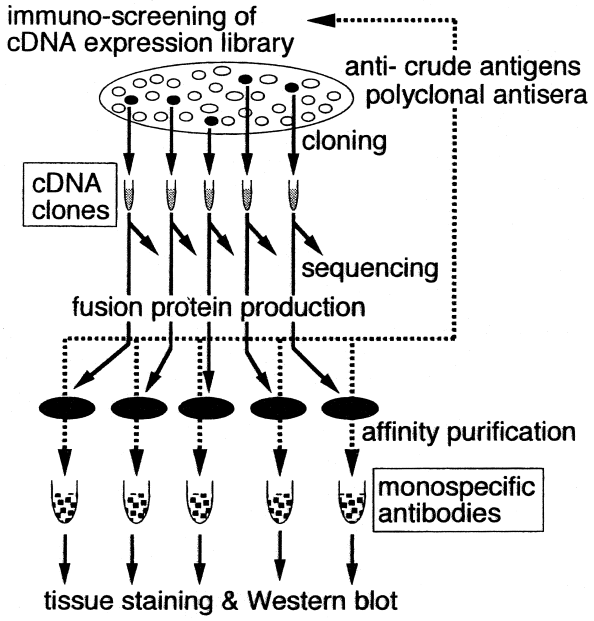

Fig. 1. Schematic diagram of epitope selection screening.

were classified into four groups: 1) fibrous structures positioned longitudinally along the body column and tentacles, 2) longitudinal and circumferential fibrous structures, 3) both ectodermal cell-cell boundaries and longitudinal fibrous structures, and 4) epithelial cell-cell boundaries (data not shown). We further characterized the last group according to the staining of the epithelial cell-cell boundary.

Sequence comparison

The isolated clones contained 2-4kbp insert fragments in most cases. Short DNA sequences of the cDNA clones were determined for the ends of the inserts, and an open reading frame (ORF) was identified in frame with the leading bacterial protein encoded in *lac*Z. The deduced amino acid sequences were subjected to database search, and ABP-280 (actin-binding protein, non-muscle filamin; Gorlin *et al.* 1990) was found significantly similar to the cDNA sequences. The nucleotide sequences of 3 overlapping clones were determined further for 660bp long, although only partially. The deduced amino acid sequences were aligned with part of the human ABP-280 sequence and are shown in Fig. 2. ABP-280 has intramolecular repeats with a unique motif presumed to form a β-sheet structure in its entire central region, and is thought to form a rigid structure resembling a leaf-spring (Gorlin *et al.* 1990). The hydra cDNA also has intramolecular repeats (Fig. 2, upper 3 lines) similar to the human ABP-280 repeating motif. Although the value of simple amino acid identity is not remarkable (23.7%), specific amino acid clusters appear regularly in the hydra cDNA as in human ABP-280 (Fig. 2, consensus). The repetition of apparently similar sequence motifs suggests that the clones encode a hydra homolog of human ABP-280.

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hydra                                                          KPAYGVQSAPDGSLVLDFTPTEVGKHLIDVKKSGRPVKGSAFEVIV
                          DHDDGDKPKVGSPCD  VNLAIDDVHLPE DLDKLSAELTRPSGKKEPIKCKMAPDGSLALDFTPTEVGKHLIEVKKNGRPVKGSPFEIFV
                          DHPDHEKPKVGSPCG LKKVIDDVQKLPE DLAKLTAELTKPSGKKEPIKCKEHLMAVWYWLHQLKLQSTLVDVKKSGGPSQKASPF

human repeat 18    TGDDSMRMSHLKVGSA       ADIPINISETDLSLLTATVVPPSGREEPCLLKRLRNGHVGISFVPKETGEHLVHVKKNGQHVASSPIPVVI
      repeat 19 SQSEIGDASRVRVSGQGLHEGHTFEPAEFIIDTRDAGYGGLSLSIEGPS  KVDINTEDLEDGTCRVTYCPTEPGNYIINIKFADQHVPGSPFSVKV
      repeat 20 TGEGRVKESITRRRRAPSV ANVGSHCDLSLKIPEISIQDMTAQVTSPSGKTHEAEIVEGENHTYCIRFVPAEMGTHTVSVKYKGQHVPGSPFQFTV

consensus            D       KVGS                                PSGKKEP       DG     F PTE G      K  G   V GSPF V V
```

Fig. 2. Alignment of the deduced amino acid sequences. The deduced amino acid sequences of the hydra clones are aligned in upper 3 lines, and compared with part of the human ABP-280 sequence (amino acid position 1953–2229, repeat 18–20, Gorlin *et al.* 1990). The conserved amino acids are shaded. Highly conserved amino acids are shown in the lowest line as consensus. The DNA sequences are available in the DDBJ/GenBank/EMBL databases under accession number AB022862.

Blotting analysis

Northern blot analysis was carried out to determine features of the transcript. A single band 12kb was detected (Fig. 3A), revealing that the mRNA is transcribed as a single molecular species. This size is similar to that of human ABP-280 (Gorlin *et al.*, 1990).

By Western blot analysis, a single band was detected (Fig. 3B). This result suggested that the purified antibodies were specific to recognize a single molecule. The molecular weight of the signal was estimated at about 300 kDa, similar to human ABP-280.

Localization of ABP-280-like molecule

Monospecific antibodies were prepared by affinity purification with the fusion protein produced by the cloned phages, and used for whole mount immunostaining (Fig. 4). Staining was specific for epithelial cell-cell boundaries. Ectodermal cell boundaries stained strongly, whereas endodermal only stained faintly (data not shown). Staining was weaker in the tentacles than in the body column, probably due to the flat shape of the tentacle epithelium. The staining intensity was uniform along the body axis (data not shown). The cell-cell boundaries between ectodermal cells and embedded nematocytes were not

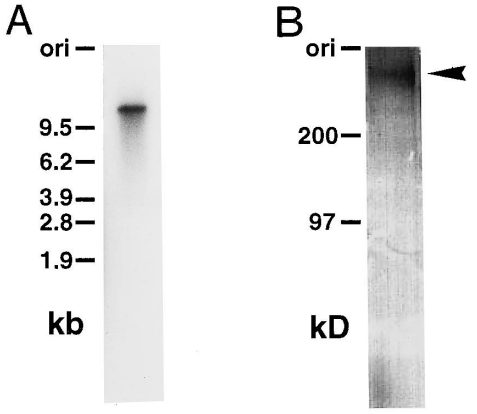

Fig. 3. Blotting analysis. (A) Northern blotting. 0.1 μg of poly(A)$^+$ RNA was applied and probed with a cDNA insert. *ori* on the left side of the figure marks the origin of electrophoresis. The length of the molecular size marker is also indicated in size (kb). (B) Western blotting. The signal is indicated by an arrowhead. The positions of molecular weight marker is indicated on the the left in size (kD), and the origin of electrophoretic separation is marked by ori.

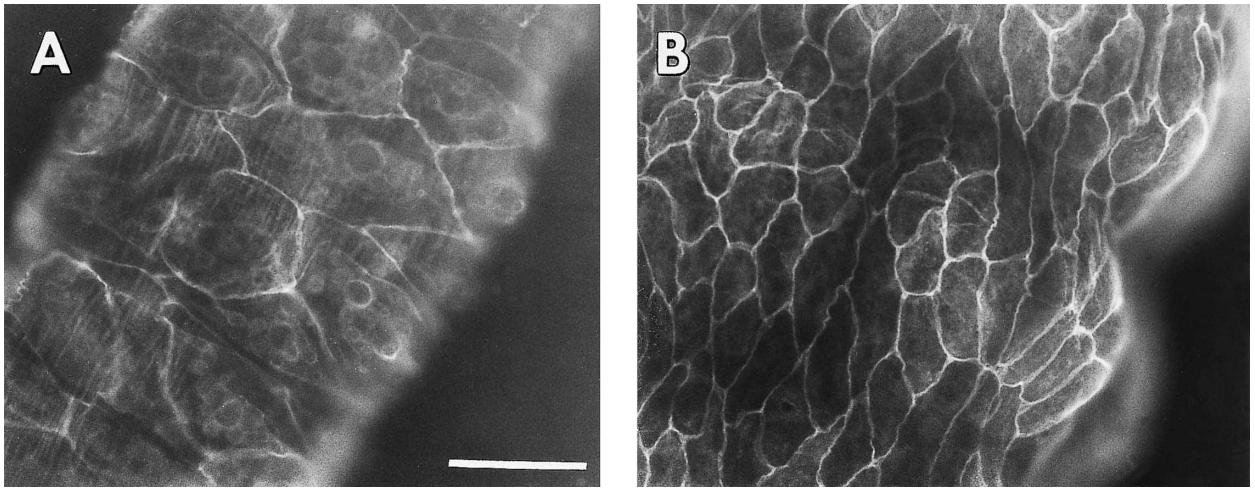

Fig. 4. Whole mount immunostaining of a tentacle (A) and the body column (B). The hydra was stained with monospecific antibodies purified by affinity with a cDNA clone. The signals were detected with FITC-conjugated secondary antibodies. In panel A, the fibrous and round signals are background. Scale bar: 50 μm.

stained.

The subcellular localization of the ABP-280-like protein was observed in detail using LASER scanning microscopy (LSM). Fig. 5 shows LSM images of sections of the body column prepared by whole mount staining. Signals were detected along the apical edges of ectodermal cells to a 4.5 μm depth. In this view, the staining intensity was strong at depths from 1.5 μm to 4.0 μm from the apical surface of the cells, while there was no staining below 5 μm. The basal limit of localization varied from 3.0 to 5.5 μm in depth according to the specimen (data not shown). These distributions correspond to septate junctions described in EM studies, in which septate junctions were well-developed in subapico-lateral stretches from the apical edge to a depth of around 5 mm in hydra ectodermal cells, but adherens junctions (zonura adherens) were difficult to observe (Wood, 1985).

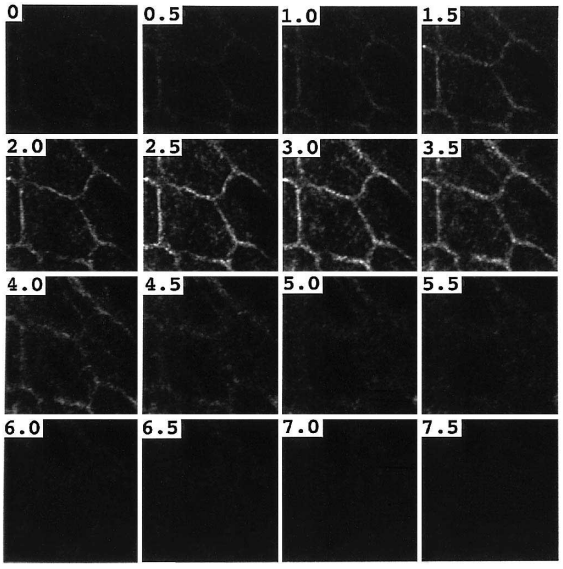

Fig. 5. Optical section of immunostaining. The optical section was made from a whole mount FITC-immuno-stained hydra body column region using LASER scanning microscopy. The values at the upper left of each panel represent the depth (μm) of the section from the apical surface of the sample.

DISCUSSION

We applied the epitope selection method to screen for molecules that are localized in particular cell types or subcellular structures. An advantage of this approach is its capacity to produce both cDNA clones and specific antibodies simultaneously. cDNA clones give information on the structural features of the gene products, while specific antibodies localize the proteins *in situ*, allowing speculation on their functions. Furthermore, this approach is very fast and does not require any special skills or facilities. Another advantage is that antisera can be generated against crude preparations of antigens, because specific antibodies are purified by affinity to the fusion proteins that are produced by each of the isolated cDNA clones. This feature permits application of the method to a variety of problems, such as isolating proteins that are difficult to purify by biochemical methods. We succeeded in identifying molecules localized in particular subcellular structures, such as the cell-cell boundary and cell processes. A cell type-specific molecule was also identified in the hydra (data not shown). Therefore, epitope selection screening seems to be an efficient way to isolate molecular markers quickly. It is a powerful method, especially in systems for which genetic approaches are not well developed.

In this study, we isolated cDNA clones very similar to human ABP-280. ABP-280 has a molecular size of about 280kDa and has been identified as an actin-binding protein in mammalian blood cells and other tissues (Hartwig and Stossel, 1975; Wang *et al.*, 1975). It forms a homodimer to cross-link actin filaments, and connects actin filaments to membrane glycoproteins. Its N-terminal domain has actin-binding activity and the C-terminal domain functions in self-association and binding to membrane glycoproteins. The central domain consists of 24 repeats of a predicted -sheet motif and forms a rod-like structure with a flexible hinge resembling a leaf spring (Gorlin *et al.*, 1990). The hydra clones contain intramolecular repeats of a sequence motif very similar to the repeating unit in the central domain of human ABP-280. Conservation of both primary amino acid sequences and repeating features suggest that the tertiary structures and activities of the molecules in human and hydra cells are similar.

The subcellular distribution of ABP-280 in epithelial cells has not been investigated and this is the first description of ABP-280-related molecules in invertebrates and in epithelial cells. In the hydra, monospecific antibodies prepared by affinity purification using the hydra ABP-280-like clones labeled epithelial cell-cell boundaries. LASER scanning microscopy localized the molecule at the cell-cell boundaries in stretches, between 0.5 and 5 μm in depth from the apical surface of the body column ectoderm in the hydra (Fig. 5). Electron microscopic studies described septate junctions along subapico-lateral stretches in hydra epithelial cells, while adherens junctions were rarely detected (Wood, 1985). Although immuno-electron microscopy is required to localize the protein precisely, it is very likely that the staining corresponds to septate junctions.

ABP-280 accelerates pseudopod extension and cell locomotion in human melanoma cells (Cunningham *et al.*, 1992). ABP-280 is thought to be involved in cell motility by regulating actin filament cross-linking and connecting actin filaments to membrane receptors. If ABP-280 is a component of the septate junction, its presumed roles are to maintain cell-cell adhesion and epithelial organization by connecting actin filaments to adhesion molecules in septate junctions and by forming an actin mesh underlying the junctions.

Very little is known of the functions and molecular organization of the septate junction, in spite of its wide existence in metazoans and prominent distribution in cells as the major cell-to-cell adhesion apparatus. Only five molecules have been identified as components of the septate junction, and four of

these play important roles in epithelial cell polarity formation, growth control, or tissue morphogenesis. Further investigation of the molecular organization of the septate junction may lead to an understanding of its functions in epithelial organization and morphogenesis. Identifying the components of the septate junction is the first step in investigating the functions of the septate junction at the molecular level. The identification of a homolog of ABP-280 as a candidate septate junction molecule in this study should contribute to this line of research.

The dynamics of the disappearance and reorganization of septate junctions during regeneration of hydra tissues have been described by electron microscopic observation (Bibb and Campbell, 1973; Murate *et al.*, 1997). Septate junctions were not detected soon after the cells were isolated and recombined. Adherens junctions seemed to be the major machinery responsible for cell migration and rearrangement in the regeneration processes. Adherens junctions disappeared and were replaced by septate junctions after cell movement was completed. With reorganization of the apical-basal cell polarity, septate junctions developed prominently where cells were in contact laterally in stretches from apical to basal. It is of future interest to describe the sequence of the processes of reorganization of septate junctions at the molecular level. ABP-280 is one of the molecules to be investigated.

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