Screening for Drosophila Proteins with Distinct Expression Patterns during Development by use of Monoclonal Antibodies

Takeshi Kumagai, Hiroaki Yokoyama, Akira Goto, Junko Hirose,* Tatsuhiko Kadowaki, Hiroshi Narita,* and Yasuo Kitagawa†

Graduate Program for Regulation of Biological Signals, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan
*Department of Food and Nutrition, Kyoto Women’s University, Higashiyamaku, Kyoto 605-8501, Japan

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Kc 167 is a cell line established from Drosophila embryonic hemocytes and has been shown to express many extracellular matrix (ECM) and other proteins important during development. We have screened monoclonal antibodies (mAbs) raised against heparin affinity purified proteins from conditioned medium of Kc 167 cells to identify novel proteins with important roles for development. One mAb recognized a protein expressed with temporary and tissue specific patterns during Drosophila embryogenesis and larval development. This approach is an alternative to screening of Expression Sequence Tag (EST) clones by in situ hybridization to initiate reverse genetics. In addition, a number of mAbs recognizing ECM proteins were also identified. These mAbs will be useful for biochemical and cell biological analyses of Drosophila ECM proteins.

Key words: monoclonal antibody; Drosophila cell line; development; extracellular matrix proteins; reverse genetics

A large number of genes involved in development of the fruit fly, Drosophila melanogaster have been identified by a forward genetics. In this approach, mutants with specific phenotypes are identified by large-scale screen of progenies (F1 or F2) generated from mutagenized males. The genes identified in Drosophila are well conserved in other organisms (for example, Caenorhabditis, elegans, mouse, and human) and have important roles during embryogenesis and organogenesis. Reverse genetics is the opposite of forward genetics. This approach is initiated by identification of cDNAs and proteins with specific expression patterns during development followed by generating the mutants with lesions in the genes encoding those cDNAs. Analysis of their phenotypes will reveal the functions of the proteins during development. The recent increase of the number of EST clones makes this approach very powerful and this information will be integrated into the Drosophila genome sequencing project.

We have taken the approach to raise mAbs against protein fraction enriched from conditioned medium of Drosophila Kc 167 cells by heparin affinity chromatography and then screen them by their expression patterns in embryos. This cell line was used as a source of variety of Drosophila ECM proteins and expresses other cellular proteins known to play a key role during development. One of major ECM components is heparan sulfate proteoglycans, which have large glycosaminoglycan chains similar to heparin. Thus, other ECM proteins were shown to have high affinity to heparin. In addition, many secreted signaling factors were known to bind with heparin. Therefore we expected most of the mAbs were directed against ECM proteins and signaling factors. One mAb recognizing protein with novel expression pattern was successfully isolated by this approach. The mAbs against ECM proteins were isolated as well. The use of mAbs for identifying developmentally important genes to start reverse genetics will be discussed.

Materials and Methods

Preparation of mAbs. For preparation of antigens, 4 ml of wet heparin sepharose CL-6B was added to 200 ml of conditioned medium of Kc 167 cells and the mixture was gently stirred at 4°C overnight. The gel was collected by centrifugation, packed into a small column, and washed with PBS. About 0.5 mg of protein bound to the gel was eluted with PBS containing 1.5 M NaCl. Seven female

† To whom correspondence should be addressed, Yasuo Kitagawa, FAX: 81-52-789-5228; E-mail: i45073a@nucc.cc.nagoya-u.ac.jp

Abbreviations: MAb, monoclonal antibody; ECM, extracellular matrix; HRP, horseradish peroxidase; EST, Expression Sequence Tag; PBS, phosphate buffered saline; ELISA, enzyme linked immuno sorbent assay; PT, PBS + 0.1% TX-100; NGS, normal goat serum; PBST, PBS + 0.1% Tween 20; DAB, 3,3′-diaminobenzidine; TCA, Trichloro acetic acid
Balb/c mice (9 weeks old) were injected intraperitoneally with 200 µg of this protein fraction in Freund's complete adjuvant. They were boosted twice with 100 µg of the protein in Freund's incomplete adjuvant at two-week intervals. Their spleens were removed 3 days after the third boost with 100 µg of the protein. Spleen cells from one mouse were fused with 2 × 10^7 NS1/1-Ag4-1 myeloma cells with 50% polyethylene glycol 1500. Hybridomas producing mAbs were screened by ELISA and hybridomas identified in the second screening were cloned by the limiting dilution method until the percentage of antibody-producing cells exceeded 95%. For mass preparation, ascitic fluids were generated by the injection of 1 × 10^7 hybridoma cells into pristane-primed mice and mAbs were obtained from them. The isotypes were identified with a mouse mAb isotyping kit.

Immunostaining. Dechorionated Drosophila embryos (0–21 h after egg laying) were fixed and then vitelline membrane was removed by vortexing for 30 second in a mixture of methanol and heptane. The embryos were washed with methanol, stored at −20°C, and rehydrated by washing three times with PT (PBS containing 0.1% Triton X-100) before being used. The embryos were first blocked with PT containing 5% normal goat serum (NGS) for 30 min then incubated overnight with appropriately diluted mAbs followed by washing with PT six times. The embryos were incubated with 200-fold-diluted horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin antibody for 1 h and then washed as above. The color development was done by incubation of embryos with a reaction mixture containing 3,3′-diaminobenzidine (DAB) and hydrogen peroxide then stopped by washing with PT.

For immunostaining of Drosophila third instar larvae, they were dissected under Ringer solution (130 mM NaCl, 5 mM KCl, 1.5 mM CaCl2, 2 mM Na2HPO4, and 0.37 mM KH2PO4) and then fixed for 30 min. After washing with PT three times, larvae were processed as above.

Western blotting. Conditioned medium of Kc 167 cells was treated with TCA and the precipitate was separated by 4% SDS-PAGE. The total proteins (100 µg) extracted from the embryos, third instar larvae, pupae, and adults were separated by 10% SDS-PAGE. The proteins in the gel were transferred to nitrocellulose membrane and then blocked with PBST (PBS with 0.1% Tween 20) containing 5% skim milk. The membrane was incubated with appropriately diluted mAbs for 2 h at room temperature and washed three times with PBST. It was then incubated with 1000-fold-diluted HRP-conjugated anti-mouse immunoglobulin for 1 h. After washing with PBST three times, signal was detected by an ECL system (Amersham).

Results and Discussion

The heparin affinity-purified fraction from the conditioned medium of Kc 167 cells was injected to mice and positive mAbs were obtained after screening by ELISA. The mAbs were then screened by whole-mount immunostaining of Drosophila embryos to identify the ones exhibiting distinct staining patterns. One mAb, TK2, was isolated by this procedure and characterized in detail.

As shown in Fig. 1A, both termini of stage 1 embryos are strongly stained by this mAb. This pattern of expression is similar to that of Torso (Tor) signaling components such as Torsolike (Tsl), Tailless (Tll), and Huckbein (Hkb). 5,7 Tor is one of the Drosophila receptor tyrosine kinases and is necessary

![Fig. 1. TK2 Expression in Embryos.](image_url)

TK2 is expressed at both termini of a stage 1 embryo (A). Head expression is lost but tail expression (arrow) remains in the pole cells at stage 5 (B). At stage 12, TK2 is found in the amnioserosa, at the most dorsal part of the embryo (C). The anterior of the embryos are on the left and the dorsal side is on the top.
for head and tail formation in embryos. The head expression immediately disappears at stage 2 but the tail expression remains in the pole cells until stage 5 (Fig. 1B). Thus, the protein recognized by TK2 mAb (referred as a TK2 hereafter) seems to be deposited in the oocytes during oogenesis and degraded by an active protein degradation pathway in the head. This later expression pattern is different from that of Tor signaling components, suggesting that TK2 is not the target of Tor activation. TK2 may be involved in the spatially restricted activation of Tor receptor tyrosine kinase signaling by, for example, regulating the presentation of Tor ligand. In stage 12 embryos, TK2 is highly expressed in amnioserosa that is the most dorsal part of the embryos and degenerates during dorsal closure (Fig. 1C). The dorsal closure involves multiple signaling events (for example, decapentaplegic and Jun kinase signaling pathways) and extensive cell movement. TK2 may have roles in these processes. In third instar larvae, TK2 is expressed in the specific cells of the trachea where branches form (Fig. 2A). In addition, it is expressed at the boundary between the midgut and hindgut where Malpighian tubules branch out (Fig. 2B). The trachea and gut were not, therefore, made of a population of homogeneous cells forming a tubular structure. Instead, there are the cells expressing TK2 at the origin of branching tubule but not other sites, suggesting that TK2 would be involved in branch formation. TK2 has a molecular weight of 40,000 and is expressed throughout development, in smaller amounts in larvae than in other phases (Fig. 3). Surprisingly, TK2 was found in the cytoplasmic fraction but not in the conditioned medium of Kc 167 cells. It was therefore released into the medium by, for example, cell lysis or cleavage at the plasma membrane during antigen preparation.

As we and others previously reported, Kc 167 cells synthesize many ECM proteins and most have been identified by their fractionation patterns in sucrose density gradient. In addition, some novel large secreted proteins were identified. To isolate mAbs directed against ECM proteins, the positive clones by ELISA screening were tested on their reactivity to large proteins in Kc 167 conditioned medium by Western blot. The ECM protein recognized by each mAb was identified by its molecular mass in SDS-PAGE (Fig. 4), nonreducing and reducing two-
dimensional gel electrophoresis, and sucrose density gradient fractionation. The mAbs specifically recognize tiggrin,\(^{12}\) glutactin,\(^{12}\) the \(\alpha,^{13}\) \(\beta,^{14}\) and \(\gamma^{15}\) chains of laminin. The 3-3 1B mAb appears to recognize the protein with a molecular weight of approximately 400,000. Based on its partial amino acid sequence, it is found to be a novel secreted protein highly expressed in this cell line. The mAbs against laminin chains were used for immunostaining of embryos at different stages. Large amounts of laminin are found at the midline on the mesectodermal strand at the border between central nervous system segments and glial cells (Fig. 5A). They are also expressed around peripheral sensory neurons and their support cells in the peripheral nerve system (Fig. 5A), suggesting possible roles on the pathfinding of neurons or maintaining the structures of these nervous systems. Other basement membrane structures (for example, visceral mesoderm and the fat body) also contain laminin in stage 14 and 15 embryos (Fig. 5B). These are the first mAbs against native Drosophila ECM proteins. Since their epitopes probably are on the surface of the proteins, epitope mapping will help to determine their three dimensional-structures. In addition, these mAbs can be used for immunoprecipitation thus useful for biochemical and cell biological analysis of Drosophila ECM proteins.

As one of approaches to identify Drosophila proteins expressed with specific patterns during development, we raised mAbs using the heparin-affinity purified fraction from the conditioned medium of Drosophila Kc 167 cells as antigen. We then screened them by immunostaining of embryos and ones giving specific staining patterns were collected. The Kc 167 cell line was chosen because it is easily grown on a large scale with serum-free medium. In addition to the screen of ELISA positive clones by immunostaining, we also tested their reactivity to ECM proteins by Western blot. Heparin affinity purification enabled us to concentrate the ECM proteins from Kc 167 cells conditioned medium and we successfully raised mAbs recognizing various Drosophila ECM proteins.

In addition to screening of EST clones by in situ hybridization, raising and screening of mAbs directed against a particular set of antigens by immunostaining should be useful for the identification of proteins with specific expression patterns during development. Since not all mRNA could be represented in the cDNA libraries, the screening of EST alone will not survey all gene products in the organism. Thus, this approach makes it possible to
identify novel genes difficult to be isolated by screening of EST clones. Furthermore, it will be expected that, for example, TK2 homologs in the other species also exist and their functions can be tested by reverse genetics.

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References