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Glycosylphosphatidylinositol-Anchored Cell Surface Proteins Regulate Position-Specific Cell Affinity in the Limb Bud

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Although regional differences in mesenchymal cell affinity in the limb bud represent positional identity, the molecular basis for cell affinity is poorly understood. We found that treatment of the cell surface with bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) could change cell affinity in culture. When PI-PLC was added to the culture medium, segregation of the progress zone (PZ) cells from different stage limb buds was inhibited. Similarly, sorting out of the cells from different positions along the proximodistal (PD) axis of the same stage limb buds was disturbed. Since PI-PLC can remove glycosylphosphatidylinositol (GPI)-anchored membrane bound proteins from the cell surface, the GPI-anchored cell surface proteins may be involved in sorting out. To define the GPI-anchored molecules that determine the segregation of limb mesenchymal cells, we examined the effect of neutralizing antibody on the EphA4 receptor that binds to GPI-anchored cell surface ligands, called ephrin-A. Sorting out of the PZ cells at different stages could be inhibited by the neutralizing antibody to EphA4. These results suggest that EphA4 and its GPI-anchored ligands are, at least in part, involved in sorting out of limb mesenchymal cells with different proximal–distal positional values, and that GPI-anchored cell surface proteins play important roles in determining cell affinity in the limb bud.

Key Words: **limb pattern formation; cell sorting out; positional identity; GPI-anchored molecule; EphA4; ephrin-A.**

INTRODUCTION

The vertebrate limb bud is composed of undifferentiated mesenchymal cells and the ectodermal jacket surrounding them at the early stage of limb development. These mesenchymal cells differentiate into chondrogenic cells in the central region of limb bud, or into fibroblastic cells in the peripheral region. Limb cartilage is formed in the specific structure along three axes: the proximal–distal (PD), anterior–posterior (AP), and dorsal–ventral axes. Various molecules are implicated in cell–cell interactions during cartilage pattern formation.

There are distinct signaling regions in a limb bud that control limb patterning: the apical ectodermal ridge (AER), zone of polarizing activity (ZPA), and dorsal ectoderm. Several signaling molecules produced in these regions regulate pattern formation of the limb bud (reviewed by Cohn and Tickle, 1996; Johnson and Tabin, 1997). These molecules influence the position-dependent expression patterns of the homeobox-containing genes (*Hox* genes). The *Hox* genes are expressed in the limb bud with regional specificity, and their expression patterns represent the regional differences in the mesenchyme (Nohno *et al.*, 1991; Yokouchi *et al.*, 1991; Nelson *et al.*, 1996). In fact, disruption of *Hox* gene expression causes position-specific transformation of the limb cartilage structure (Rijli and Chambon, 1997). Although the *Hox* genes

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influence mesenchymal condensation by regulating cell adhesivity (Yokouchi *et al.*, 1995; Goff and Tabin, 1997), the downstream factors of the *Hox* genes are still unknown.

Cytological and histological studies suggest roles for cell adhesion in mesenchymal condensation (Hall and Miyake, 1992). Since modulation of cell adhesion properties affects cell migration and condensation processes, it has been proposed that the differential affinities of mesenchymal cells influence the cartilage pattern (Oster *et al.*, 1985; Newman, 1996). Therefore, it is important to investigate the spatial and temporal differences in the cell–cell affinities of the limb bud mesenchyme in pattern formation.

We have previously reported on regional and temporal differences in the cell affinities of the limb bud mesenchyme (Wada and Ide, 1994; Ide *et al.*, 1994). When cells derived from the progress zone (PZ) of chick limb buds at different stages were mixed and cultured, these cells segregated from each other and formed cell clusters. Sorting out is also observed when the cells from different regions along the PD and AP axes are mixed, and we showed that the stage-specific affinity of PZ cells is progressively allotted to the proximal region according to limb development (Wada and Ide, 1994; Ide *et al.*, 1994). Similar results were obtained *in vivo*, using recombinant limb bud (Wada *et al.*, 1993). These results indicate that the spatial and temporal differences in the cell surface properties of the mesenchyme are formed sequentially during limb development. It is supposed that the molecules contributing to these sorting out processes are reflected in the positional values of the mesenchyme and play important roles in limb development, although we have not been able to identify them so far.

To elucidate the molecules that are involved in the sorting out process, we examined the effect of treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) on cell sorting out. PI-PLC treatment inhibited segregation of PZ cells at the different stages, or cells from different positions along the PD axis in a monolayer culture. Since PI-PLC is an enzyme that removes glycosylphosphatidylinositol (GPI)-anchored membrane-bound proteins from the cell surface, the GPI-anchored cell surface proteins may be involved in the cell sorting out process along the PD axis of the limb bud. Furthermore, we showed here the possible involvement of EphA4 (Cek-8) receptor and its ligands in the cell sorting out process using the neutralizing antibody to EphA4. EphA4 can bind to GPI-anchored cell surface ligands, called the ephrin-A family, and act as cell–cell recognition molecules during morphogenesis (Orioli and Klein, 1997). These results suggest that GPI-anchored cell surface proteins have important roles in determining regional differences in cell affinity in the limb bud.

MATERIALS AND METHODS

Animals

Fertile White Leghorn chicken eggs were used. Eggs were incubated at 38°C, and embryos were staged according to Hamburger and Hamilton (1951).

Medium

To culture mesenchymal cells of the limb bud, we used F-12 medium (Nissui) containing 1% FBS (ICN) as the basal medium. To treat the cells with PI-PLC, we prepared a medium containing 0.2 unit/ml PI-PLC from *Bacillus thuringiensis* (Funakoshi) and cultured the cells in the medium during the incubation period. As a control, a medium containing 2.0% glycerol was used. In some experiments, 0.5 unit/ml phospholipase B (PLB; Sigma) or 0.5 unit/ml phospholipase D (PLD; Sigma) was used instead of PI-PLC.

Antibodies

Monoclonal antibodies to EphA4 were prepared as previously reported (Ohta *et al.*, 1996). Briefly, a Balb/c female mouse was immunized with fusion protein constructed with the extracellular domain of EphA4 and the Fc region of human immunoglobulin (EphA4-Fc), and cell fusion was performed according to standard methods (Kohler and Milstein, 1975). Positive clones were screened by ELISA. A neutralizing antibody to EphA4 was selected from the EphA4-positive antibodies. The neutralizing antibody blocked ephrin-A2 and ephrin-A5 binding to EphA4-expressing 293 cells. Neutralizing activity was checked using the stripe assay method. In the antibody treatment experiment, 0.1 mg/ml of purified neutralizing antibody was added to the culture medium. As a control, 0.1 mg/ml of nonneutralizing antibody was used.

Monolayer Culture of Mesenchymal Cells

Limb buds were dissected out from the embryos, and mesenchymal cells from various regions of the limb bud were prepared as previously described (Wada and Ide, 1994; Ide *et al.*, 1994). The cells were suspended in the F-12 basal medium. Vital dye-labeled cells were mixed with nonlabeled cells and were seeded into penicillin cups (6 mm diameter) in a 24-well plate (Falcon 3047) to a final density of 2.2×10^5 cells/well. A fluorescent dye PKH26 (ZYNAXIS Cell Science, Inc.) was used to label cells. To more clearly visualize cell sorting out, the ratio of labeled cells to nonlabeled cells was 1:2.5 to 1:3. The penicillin cups were removed 3 h after incubation and filled with a culture medium containing enzyme or antibody. After 20-h incubation, the distribution of dye-labeled cells was observed under a fluorescent microscope.

In some experiments, we used chick–quail mixed cultures. Quail mesenchymal cells were prepared in the same manner as the chick cells. Chick cells and quail cells were mixed, plated, and incubated. In this experiment, the ratio of chick cells to quail cells was 1:2.5. After 20-h incubation, cultures were fixed and stained with A223, a chick-specific mouse monoclonal antibody. After treatment with FITC-labeled anti-mouse IgG (Tago), the distribution of chick cells was observed (Wada and Ide, 1994; Ide *et al.*, 1994). An independent experiment was repeated at least three times for each combination, and typical results are shown in this paper.

RESULTS

PI-PLC Treatment Inhibits Sorting Out of PZ Cells at Different Developmental Stages

We first investigated the effect of PI-PLC on the sorting out of PZ cells at different developmental stages. When PKH26 dye-labeled stage 25 PZ cells were mixed with nonlabeled

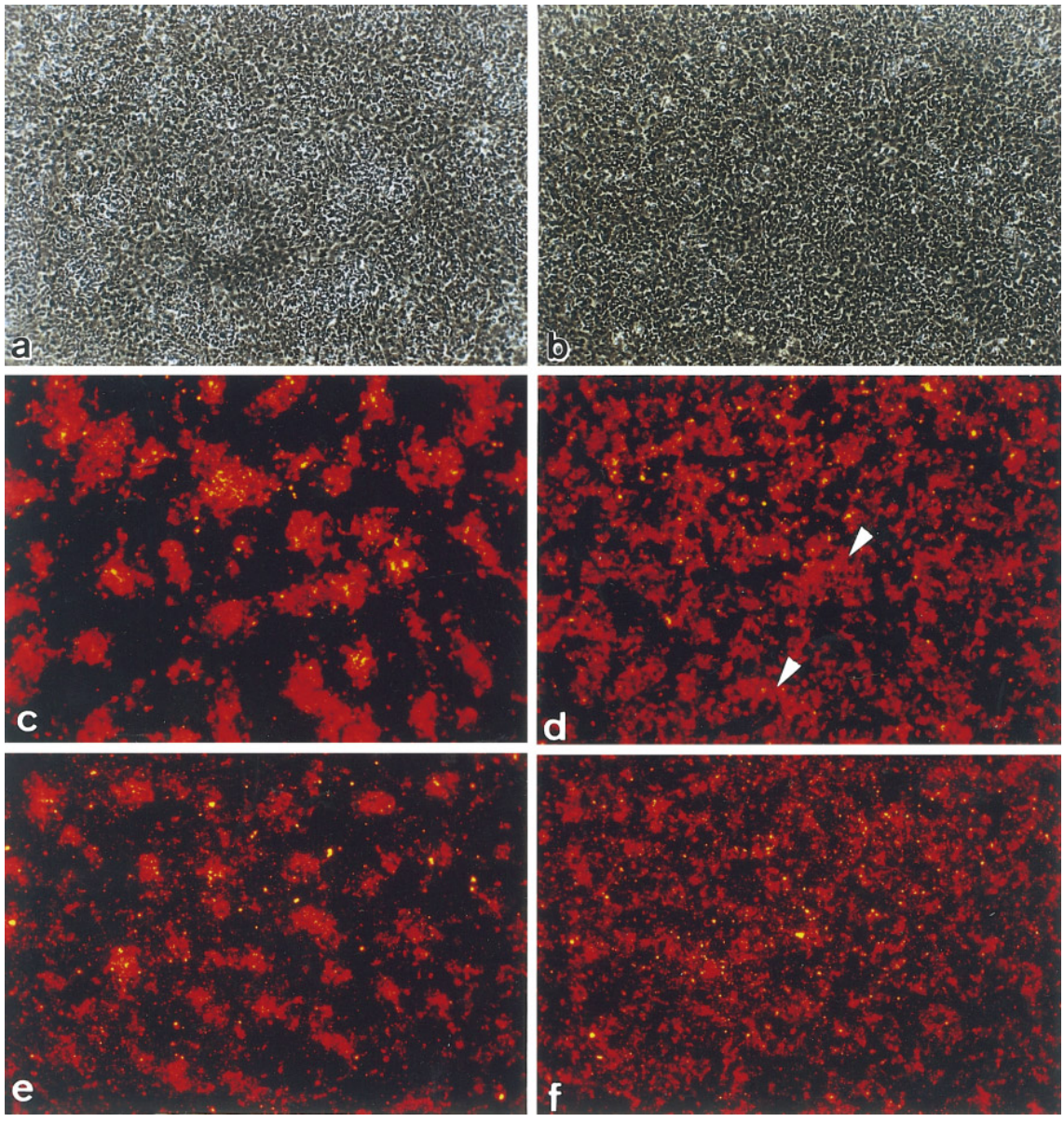

FIG. 1. Inhibition of sorting out of PZ cells from different developmental stage limb buds. (a–d) PKH26-labeled stage 25 PZ cells were mixed with nonlabeled stage 20 PZ cells and cultured for 20 h. (a) and (b) are phase-contrast views of (c) and (d), respectively. (a, c) Control. In this case, labeled cells were segregated from nonlabeled cells and formed cell clusters (c). (b, d) PI-PLC treated. In (d), the sorting out of labeled cells was inhibited, and the labeled cells were distributed evenly compared to the control culture (c), although some small loosened cell clusters were formed (d, arrowheads). (e, f) Labeled stage 20 PZ cells were mixed with nonlabeled stage 25 PZ cells. (e) Control. (f) PI-PLC treated. Cell clusters were not observed after PI-PLC treatment, although many clusters formed in the control culture.

stage 20 PZ cells, stage 25 cells were segregated from stage 20 cells, and formed cellular clusters on the culture plate after 20-h incubation (Figs. 1a and 1c). In contrast, in the cultures using a medium containing 0.2 unit/ml PI-PLC, the sorting out of cells observed as cellular clusters was markedly inhibited (Figs. 1b and 1d). Many of the labeled cells were evenly distributed, and the aggregates composed of labeled stage 25 cells were small and less packed than those observed in the control culture (Fig. 1d, arrowheads). PI-PLC-treated cells adhered to the culture plate over the whole of the culture area, and no obvious differences from the control culture were observed as judged from a phase-contrast view (Figs. 1a and 1b). Therefore, we concluded that the PI-PLC treatment of cultured cells had no toxic or inhibitory effects on cell-to-

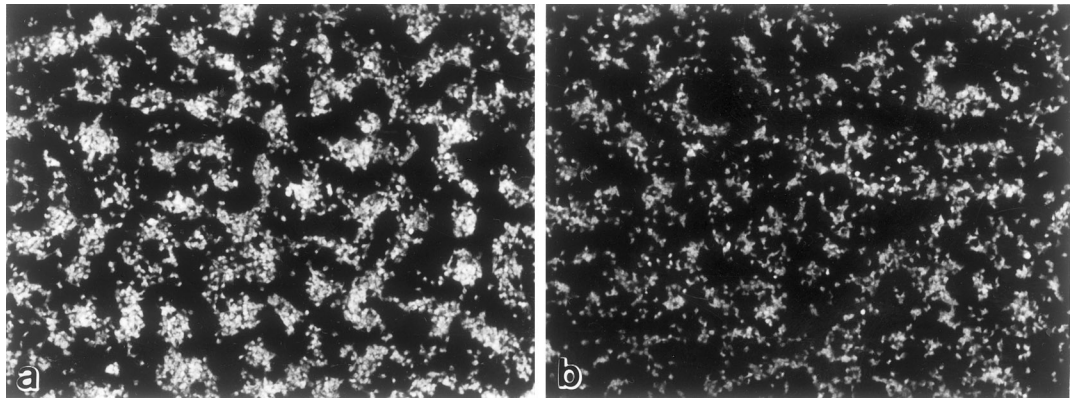

FIG. 2. Inhibition of cell sorting out detected by chick–quail chimera culture. Chick stage 20 PZ cells were mixed with quail stage 25 PZ cells and cultured. Cultures were fixed and stained with A223 chick-specific monoclonal antibody (Ide *et al.*, 1994). (a) Control. (b) PI-PLC treated. Stage 20 chick cells (A223 positive) were segregated from stage 25 quail cells in the control medium (a), but they did not sort out in the medium containing PI-PLC (b).

culture plate adhesion at the concentrations used in this study. Cell sorting out was also observed when stage 20 PZ cells were labeled and mixed with nonlabeled stage 25 PZ cells (Fig. 1e). This sorting out was also inhibited by PI-PLC treatment (Fig. 1f). In this experiment, a few cell clusters of stage 20 cells were observed, but almost all stage 20 cells were distributed as single cells (Fig. 1f).

Similar results were obtained using mixed cultures of chick stage 20 PZ cells and quail stage 25 PZ cells. In these experiments, we observed the distribution of chick cells using A223 chick-specific monoclonal antibody (Ide *et al.*, 1994). The same results as with PKH26 cell surface staining were obtained (Figs. 2a and 2b). Chick cells were evenly distributed in PI-PLC-treated cultures (Fig. 2b), although they were segregated from quail cells in the control medium (Fig. 2a). This result indicates that inhibition of the sorting out of PZ cells with PI-PLC treatment is independent of PKH26 staining.

We examined the effect of pretreatment with PI-PLC on both of mesenchymal cells for 1 h before plating, but detected very weak inhibition of sorting out (data not shown). This indicates that PI-PLC treatment is most effective to inhibit the sorting out after cell adhesion on the culture plate.

To test the specificity of the effects of PI-PLC, we next examined the effects of other phospholipases, phospholipase B (PLB) and phospholipase D (PLD), with higher doses (0.5 unit/ml) than PI-PLC (0.2 unit/ml). These enzymes do not remove GPI-anchored proteins from the cell surface (Chang *et al.*, 1992). No inhibition of cell sorting out was observed in these cultures (data not shown). In addition, the inhibitory effect of PI-PLC treatment on cell sorting out is concentration dependent, and less evident at low concentrations of PI-PLC (below 0.02 unit/ml) (data not shown). These results indicate that the inhibitory effect on sorting out of PZ cells is specific to PI-PLC treatment.

PI-PLC Treatment Inhibits Segregation along the PD Axis in the Stage 25 Limb Bud

Segregation of limb bud mesenchymal cells is observed not only in the combination of two PZ cells at different stages, but also in the combination of cells prepared from different regions of limb bud (Wada and Ide, 1994; Ide *et al.*, 1994). We tested the inhibitory effect of PI-PLC on sorting out of distal cells and proximal cells from stage 25 limb buds. In these experiments, labeled distal cells were mixed with nonlabeled proximal cells, or vice versa. Results are shown in Fig. 3. In each combination, the labeled cells were distributed as single cells or small clusters of cells in the culture with PI-PLC (Figs. 3b and 3d). In contrast, labeled cells were segregated from nonlabeled cells in the control cultures (Figs. 3a and 3c).

PI-PLC Treatment Could Not Inhibit Segregation along the AP Axis

We tested the effect of PI-PLC on the cell sorting out observed in mixed cultures of anterior cells and posterior cells from stage 25 limb buds. In this experiment, labeled cells from the anterior one-third of stage 25 PZ were mixed with the nonlabeled cells from posterior one-third of PZ. No inhibition was observed in the PI-PLC-treated culture, and many cell clusters formed (Fig. 4b). Similar results were obtained when PI-PLC was used at higher concentration (0.4 unit/ml) (data not shown).

Neutralizing Monoclonal Antibody to EphA4 Inhibits the Segregation of PZ Cells

Since PI-PLC treatment removes GPI-anchored proteins from the cell surface, some GPI-anchored proteins are concerned with sorting out. Ephrin-A families are members of the GPI-anchored molecules that are expressed with regional and temporal specificity in the limb buds (Flenniken *et al.*, 1996;

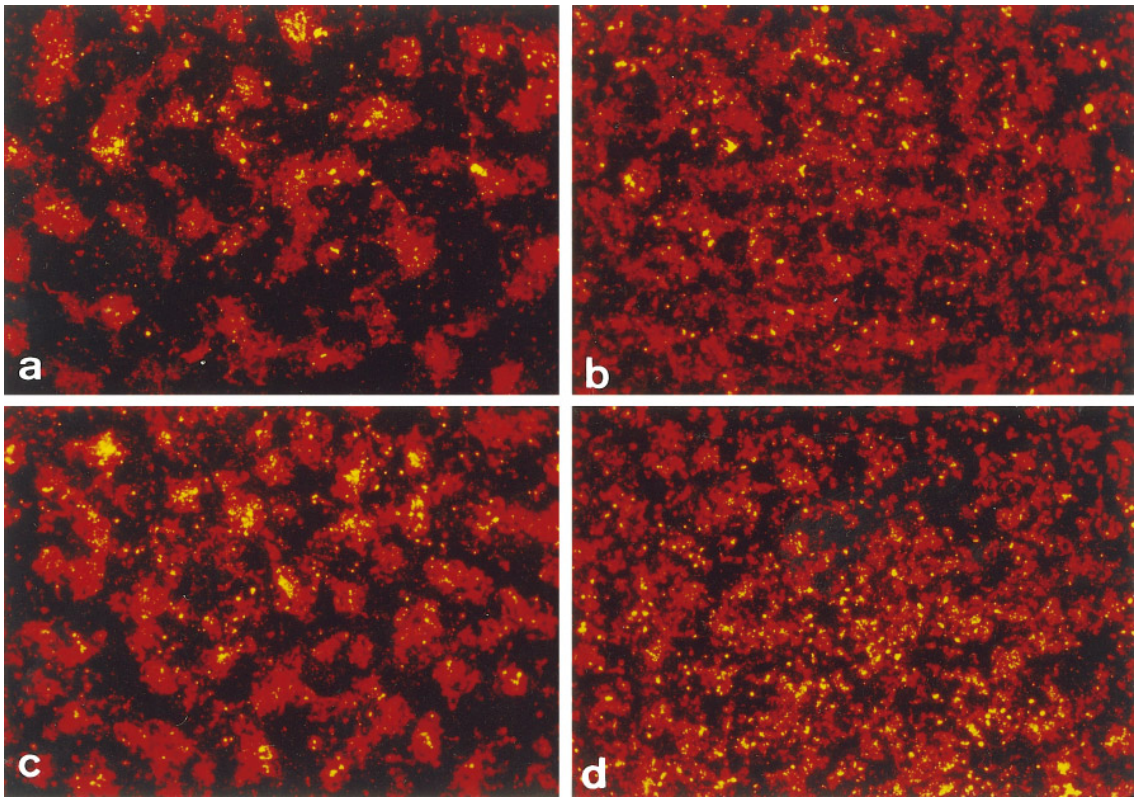

FIG. 3. Inhibition of sorting out between cells derived from the distal and proximal regions of a stage 25 limb bud. PKH26-labeled distal cells were mixed with nonlabeled proximal cells (a, b), or vice versa (c, d). (a, c) Control. (b, d) PI-PLC treated. In each experiment, labeled cells were distributed more evenly after PI-PLC treatment (b, d) than in the control culture (a, c).

Gale *et al.*, 1996; Ohta *et al.*, 1997). Therefore, we investigated the correlation of these molecules with the sorting out of limb bud cells. Since ephrin-A ligands are known to bind to various EphA receptors (Gale *et al.*, 1996; Orioli and Klein, 1997), we attempted to inhibit the binding of receptors to their ligands. We used the neutralizing antibody to the EphA4 receptor

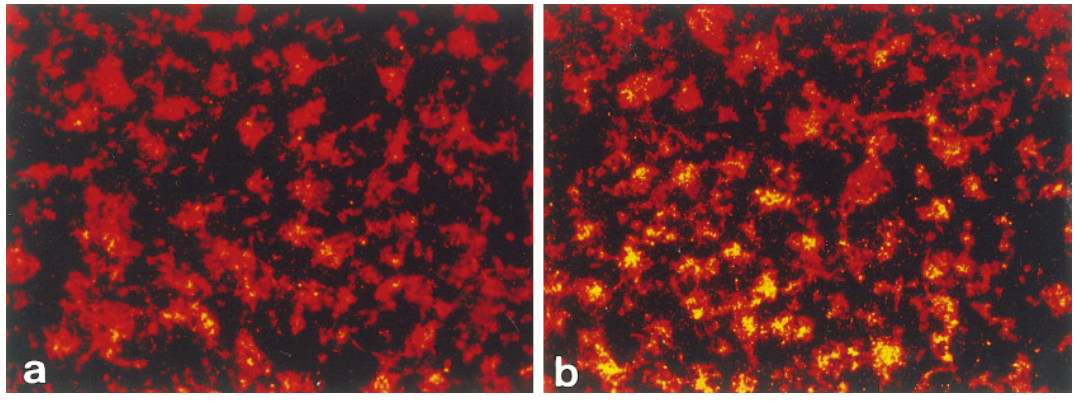

FIG. 4. Effect of PI-PLC treatment on sorting out between the cells from different positions along the AP axis of the stage 25 limb bud. PKH-26-labeled cells of the anterior one-third of stage 25 PZ were mixed with nonlabeled cells of the posterior one-third of stage 25 PZ. (a) Control. (b) PI-PLC treated. No inhibition was observed even after PI-PLC treatment (b), and many cell clusters were formed.

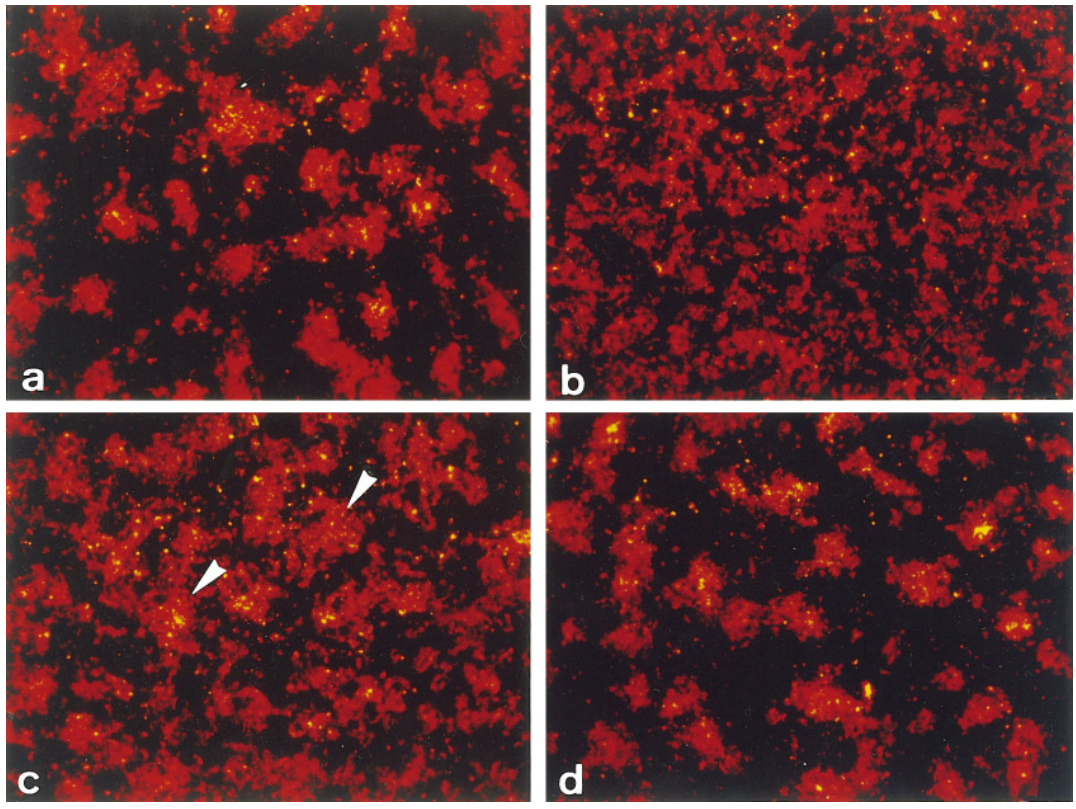

FIG. 5. Effects of neutralizing antibody to EphA4 on cell sorting out. PKH26-labeled cells of stage 25 PZ were mixed with nonlabeled cells of stage 20 PZ, and cultured. (a) Control. (b) PI-PLC treatment. (c) Neutralizing EphA4 antibody treatment. (d) Nonneutralizing EphA4 antibody treatment. (a) and (b) are identical to Figs. 1c and 1d, respectively. In (c), incompletely, loosened cell clusters were observed (arrowheads), and more single cells were detected than in the control culture (a), although the inhibition was less effective as compared to PI-PLC treatment (b). No inhibition occurred with nonneutralizing antibody treatment (d).

because EphA4 is expressed in the distal mesenchyme of the limb bud (Patel *et al.*, 1996; Ohta *et al.*, 1996).

Segregation of labeled stage 25 PZ cells from nonlabeled stage 20 PZ cells was partially inhibited with the medium containing the neutralizing antibody to EphA4 (Fig. 5c). In this culture, cellular clusters of stage 25 PZ cells were formed incompletely and were looser compared to the clusters formed in the control medium (Fig. 5a), and a large number of labeled cells were distributed as single cells (Fig. 5c). Such an inhibitory effect on cell sorting out was not observed when a nonneutralizing antibody to EphA4 was used instead (Fig. 5d). This suggests that the inhibition of sorting out is the result of a blocking effect on EphA4, and is not the result of cross-linking of anti-EphA4 immnoglobulin to EphA4.

DISCUSSION

Inhibition of Cell Sorting Out with PI-PLC Treatment

The mesenchymal cells of developing chick limb buds have spatial and temporal differences in cell affinities as determined by cell sorting out assays in mixed cultures (Wada and Ide, 1994; Ide *et al.*, 1994). Regional differences in cell affinity may influence the segmentation and bifurcation of precartilaginous condensations by regulating local cell movement (Hall and Miyake, 1992). We showed here the inhibitory effect of PI-PLC treatment on the sorting out of PZ cells derived from different stage limb buds, and of cells from different regions along the PD axis of stage 25 limb buds. The inhibitory effect of PI-PLC treatment on cell sorting out is concentration-dependent. On the other hand, the sorting out of cells prepared from different regions along AP axis was not inhibited with PI-PLC treatment. Since we used the stage 25 PZ region, the most distal region within 250 μm from the AER, to prepare the cells for the AP sorting out experiment, we considered that the mesenchymal cells derived from the anterior region and those from the posterior region had the same property with respect to the PD axis. These results indicate that the mechanisms of cell sorting out along the AP axis are different from those along the PD axis. Furthermore, since cell sorting out along the AP axis was observed even after PI-PLC treatment, it

can be concluded that PI-PLC treatment does not affect cell motility.

It is known that bacterial PI-PLC removes GPI-anchored proteins from the cell surface. The other phospholipases used in this study do not release GPI-anchored proteins from the cell surface (Chang *et al.*, 1992). Therefore, GPI-anchored protein(s) on the mesenchymal cell surface may contribute to the sorting out of limb bud cells *in vitro*. PI-PLC treatment has been shown to disturb various morphogenetic events. For example, myoblast adhesion and myotube formation were inhibited by PI-PLC treatment *in vitro* (Knudsen *et al.*, 1989). Similarly, migration of peripheral pioneer growth cones of the grasshopper embryo is affected by PI-PLC (Chang *et al.*, 1992). These results suggest the importance of GPI-anchored cell surface proteins in cell–cell adhesion and recognition during morphogenensis. Some cell adhesion molecules, such as NCAM-120 and T-cadherin, are known to be GPI-anchored proteins (Cunningham, 1995; Suzuki, 1996). In the developing nervous system, several GPI-anchored cell surface molecules play important roles in positional recognition (Tessier-Lavigne and Goodman, 1996; Chiba and Keshishian, 1996). The GPI-anchored proteins expressed in mesenchymal cells of the limb bud are probably involved in cell sorting out. Since differential cell affinity determined by the cell sorting assay reflects their original position in the limb bud (Wada and Ide, 1994; Ide *et al.*, 1994), the GPI-anchored proteins involved in cell sorting out may have a role in defining the positional identities in the limb bud. It is important to investigate whether the sorting out occurs also if only one of the two cell population is treated with PI-PLC. Unfortunately, we could detect very weak inhibition of sorting out by PI-PLC pretreatment, and therefore we must find out an alternative method to clarify this problem.

In the mixed culture of PZ cells, sorting out of labeled stage 20 cells from nonlabeled stage 25 PZ cells was completely inhibited by PI-PLC treatment (Figs. 1f and 2b). On the other hand, in the opposite combination, some small cell clusters of labeled stage 25 cells were formed even after PI-PLC treatment (Fig. 1d), although the sizes of the cell clusters were smaller than those of the control culture. These results indicate that other cell surface molecules, which resisted PI-PLC treatment, also contributed to the process of segregation of stage 25 PZ cells. Stage 25 PZ cells can adhere to each other more tightly than those of stage 20 PZ in rotated cultures (unpublished data). Furthermore, many types of cadherins are differentially expressed in stage 25 PZ cells compared with stage 20 PZ cells (Tamura *et al.*, submitted). Therefore, other cell adhesion molecules (not GPI-anchored) may also contribute to the segregation of stage 25 PZ cells from stage 20 PZ cells. These differential adhesive potentials along the PD axis may contribute to differential cartilage structure formation of the limb.

Roles of Eph Receptors and Their Ligands in the Sorting Out of Limb Bud Cells

At present, only a few molecules have been reported to be GPI-anchored proteins expressed on cell surface of chick limb bud mesenchyme, and the ephrin-A family is one of them. Ephrin-A proteins are the ligands of EphA receptors, and these GPI-anchored ligands are expressed in dynamic and distinct patterns in the mouse limb bud (Gale *et al.*, 1996; Flenniken *et al.*, 1996) and chick limb bud (Ohta *et al.*, 1997). In addition, the expression patterns of EphA receptors in the limb bud have also been shown in the mouse (Ganju *et al.*, 1994; Gale *et al.*, 1996) and chick (Ohta *et al.*, 1996; Patel *et al.*, 1996). Since Eph receptors and ephrins are known to be repulsive factors in axonal guidance (Tessier-Lavigne and Goodman, 1996; Ohta *et al.*, 1997; Orioli and Klein, 1997), and have roles as regulator of neural crest cell migration (Krull *et al.*, 1997; Smith *et al.*, 1997), these receptors and ligands are in general involved in contact-mediated cell–cell recognition during morphogenesis.

We demonstrated here that the anti-EphA4-neutralizing antibody could inhibit sorting out of limb bud cells, although its effect was weaker than that of PI-PLC treatment (Fig. 5). This suggests that the interaction between EphA4 and ephrin-A ligands is partially involved in cell sorting out. EphA4 is expressed in the distal region of the chick limb bud at early stages (Ohta *et al.*, 1996; Patel *et al.*, 1996), and its expression is regulated by the AER factor (Patel *et al.*, 1996). Since ephrin-A ligands are GPI-anchored proteins, inhibition of cell sorting out with PI-PLC treatment may result from the removal of these ligands from the cell surface. Since ephrin-A2 and -A5 are the ligands for EphA4 shown to be expressed in the chick limb bud (Ohta *et al.*, 1997), these molecules are probably involved in the cell sorting out process. Furthermore, other ephrin-A ligands which can bind to EphA4, such as ephrin-A1, -A3, and -A4 (Gale *et al.*, 1996; Orioli and Klein, 1997), may also be implicated in sorting out, although the chicken cognates are not identified yet.

However, PI-PLC treatment could inhibit cell sorting out more effectively than the treatment with the anti-EphA4 antibody. This suggests that other GPI-anchored molecules that act independently of the EphA4 receptor are involved in cell sorting out. Interactions between other EphA receptors and ephrin-A ligands may also be involved in this process, since many other receptors and ligands are expressed in the limb bud (Cheng and Franagan, 1994; Ganju *et al.*, 1994; Gale *et al.*, 1996).

Differential Cell Affinities along the Proximal–Distal Axis of the Limb

Our results suggest that differential cell affinity along the PD axis of the limb bud is mediated at least by two mechanisms of contact-dependent cell–cell interaction. One mechanism is selective cell adhesion between cells having the same positional value. It has been shown that

cell sorting out is regulated by quantitative and qualitative differences in cell adhesion molecules (Friedlander *et al.*, 1989; Steinberg and Takeichi, 1994). In the limb bud, mesenchymal cells having the same positional value may express the same types and equal amounts of cell adhesion molecules, and hence they show the same adhesive properties and segregate each other in mixed culture. Some cell adhesion molecules are involved in this process, including GPI-anchored cell surface proteins.

The other mechanism is cell–cell repulsion between cells having different positional values. EphA receptors and ephrin-A ligands may be involved in this mechanism, since they are expressed in different regions in the limb bud (Cheng and Flanagan, 1994; Ganju *et al.*, 1994; Gale *et al.*, 1996; Flenniken *et al.*, 1996). Moreover, other members of the Eph and ephrin family, EphB and ephrin-B, are also expressed in the limb bud (Gale *et al.*, 1996; Flenniken *et al.*, 1996). When cells derived from different regions are mixed, receptor–ligand interaction occurs between the two types of cell, and they migrate away from each other. These position-specific cell–cell adhesions and repulsions may regulate cell migration and influence precartilaginous condensation, eventually leading to region-specific cartilage pattern formation *in vivo.*

Hox genes are thought to define the positional specificities of the limb bud mesenchyme. The *AbdB*-related *HoxA* genes, such as *HoxA10, HoxA11,* and *HoxA13,* are expressed along the PD axis, and correspond well to the presumptive structure (Yokouchi *et al.*, 1991; Nelson *et al.*, 1996). It has been shown that the position-specific cell affinity along the PD axis of the limb bud mesenchyme is affected by misexpression of the *HoxA13* gene, which is normally expressed in the presumptive autopod region (Yokouchi *et al.*, 1995). Furthermore, the *Hox* genes have been found to regulate the expression of cell adhesion molecules *in vitro* (Jones *et al.*, 1992). Therefore, the expressions of some GPI-anchored types of cell recognition molecules are possibly regulated by the *Hox* genes expressed in the limb bud. It is important to investigate the correlation between the *Hox* genes, GPI-anchored molecules and PD axial pattern formation of the limb.

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