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# Lysosomal acid phosphatase mediates dedifferentiation in the regenerating salamander limb

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In this study, monoclonal antibodies against lysosomal acid phosphatase (LAP) of a salamander, *Hynobius leechii*, were used to determine the spatial and temporal expression of the LAP in the regenerating limbs. The Western blot and immunohistochemical analysis in the limb regeneration revealed that LAP was highly expressed at the dedifferentiation stage, especially in the wound epidermis and dedifferentiating limb tissues such as muscle and cartilage. With RA treatment, the LAP expression became upregulated in terms of both level and duration in the wound epidermis, blastemal cell and dedifferentiating limb tissues. In addition, in situ activity staining of LAP showed a similar result to that of immunohistochemistry. Thus, the activity profile of LAP activity coincides well with the expression profile of LAP during the dedifferentiation period. Furthermore, to examine the effects of lysosomal enzymes including LAP on salamander limb regeneration, lysosome extract was microinjected into limb regenerates. Interestingly, when the lysosome extract was microinjected into limb regenerates with a low dose of RA (50 μg/g body wt.), skeletal pattern duplication occurred frequently in the proximodistal and transverse axes. Therefore, lysosomal enzymes might cause the regenerative environment and RA plays dual roles in the modification of positional value as well as evocation of extensive dedifferentiation for pattern duplication. In conclusion, these results support the hypothesis that dedifferentiation is a crucial event in the process of limb regeneration and RA-evoked pattern duplication, and lysosomal enzymes may play important role(s) in this process.

**Keywords:** limb regeneration; dedifferentiation; lysosomal acid phosphatase; retinoic acid; pattern formation

## Introduction

Lysosomal enzymes play important roles in many tissue remodeling processes such as tissue demolition during metamorphoses of frog tadpoles and insect larva (Weber 1957, 1963; Schmidt 1968; Robinson 1970, 1972; Jones and Bowen 1993; Mahapatra et al. 2001; Rabossi et al. 2004). In the dedifferentiating limbs of urodele, various lysosomal acid hydrolases including acid phosphatase, cathepsins, carboxylic ester hydrolase and β-gluconidase have been found (Dukiet and Niwelinski 1960; Schmidt and Weidman 1964; Schmidt and Norman 1965; Grillo et al. 1968; Miller and Wolfe 1968; Schmidt 1968; Weiss and Rosenbaum 1968; Ju and Kim 1994; Ju and Kim 2000).

Generally, lysosomal acid hydrolases are known to be involved in the process of intra- and intercellular digestions (Holtzman 1989). Among the lysosomal acid hydrolases, acid phosphatase (LAP) is regarded as the most convincing marker of lysosomal enzymes (De Duve 1983). LAP (orthophosphoric monoester phosphohydrolase, acid optimum, EC 3.1.3.2) is a glycoprotein having carbohydrate in the form of mannose and glycosamine. It catalyzes the hydrolysis of a variety of artificial phosphomonoesters, i.e., *p*-nitrophenyl phosphate, α-naphthyl phosphate, β-glycerophosphate, etc., in an acidic environment. LAPs have been found in yeast, *C. elegans*, *Drosophila*, rat, and human with similar molecular weight and characteristics. However, a natural substrate for LAP has not been discovered yet but many kinds of acid phosphatases are considered to be important in the regulation of phosphotyrosine levels since acid phosphatase from frog liver and bovine heart are known to exhibit phosphotyrosine hydrolysing activity (Chernoff and Li 1985; Janska et al. 1988).

In planaria (*Dugesia dortocephala*), Coward et al. (1973) showed that LAP activity increased during the early phase of regeneration and the enzyme activity was mainly localized in the lysosomes in the regenerating tissue. It was also found that LAP activity increased in the regressing tail of metamorphosing *Xenopus* tadpole and regenerating tail of lizard (Robinson 1970, 1972; Alibardi 1998). Localization of LAP activity in the regenerating limbs of salamander by histochemical methods using several kinds of substrates had been reported previously. In the regenerating larval limbs of spotted salamander (*Ambystoma maculatum*), LAP activity was found to be most intense in macrophages, andless intense in epidermis and cartilage matrix at the early phase of regeneration (Weiss and Rosenbaum 1968). In the regenerating limbs of *Nothopthalmus viridescence*, LAP activity

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was detected in epidermis, subepidermal glands, skeletal muscle and nerve tissue at the dedifferentiation stage (Miller and Wolfe 1968). From the above study, it has been suggested that LAP might be involved in the process of dedifferentiation which is a prerequisite condition for urodele limb regeneration.

Still, little is known about the function and regulation of LAP activity and its expression in urodele limb regeneration. However, the elevation of LAP activity during the early phase of limb regeneration suggests that there is de novo synthesis of LAP as well as conversion of the latent, inactive form of LAP into the non-latent, active form (Ju and Kim 1994). It is possible that some physiological changes brought in by amputation cause the modification of LAP activity in the regenerating salamander limbs. Previously, retinoic acid (RA), an inducer of pattern duplication, has been found to cause increased level of dedifferentiation in the regenerating limbs of *Hynobius leechii* (Ju and Kim 1994). Furthermore, increase of LAP activity during dedifferentiation was also noted after RA treatment (Ju and Kim 1994). However, the spatial profile of LAP distribution and its role in the regenerating salamander limbs have not been studied yet.

In the present study, we studied the temporal and spatial expression profile of LAP using monoclonal antibodies (mAbs) against *Hynobius* LAP to understand the role of LAP in the regeneration process.

## Materials and methods

### *Experimental animals*

In this study, the larvae of a native salamander species in Korea (*Hynobius leechii*) were used and limb amputation and administration of retinoic acid were carried out as described previously (Ju and Kim 1994).

### *Western blot analysis*

Protein that had been electrophoretically fractionated on 10% SDS-PAGE was blotted to a nitrocellulose membrane according to the standard procedure. The blots were incubated in mAb (HlAcp62) solution developed against *Hynobius* LAP (Ju et al. 1996) and the bound mAb on the blots was detected chemiluminescently.

### *Immunohistochemistry and LAP in situ activity staining*

Larval salamander limb regenerates for immunohistochemistry and in situ activity staining were collected and embedded in O.C.T. compound (Miles, USA) for cryosection. The regenerates were sectioned serially at 10 μm, and the sections were mounted on gelatin-coated slide. The slides were kept at −70°C until use.

For immunohistochemical detection of LAP, tissue sections were fixed with 4% paraformaldehyde for 15 minutes and were rinsed with PBS containing 0.3% Triton X-100 followed by blocking in PBS containing 0.5% BSA for 15 minutes. After incubation of sections with mAb for 2 hours, sections were treated with FITC-conjugated anti-mouse IgG for 2 hours. Then, the sections were mounted in Gelvatol after washing in PBS. The sections were viewed and photographed in the Orthoplan fluorescence microscope (Leitz, Germany).

For in situ activity staining, the cryosectioned tissues were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 hour at 4°C. After washing in 0.1 M phosphate buffer for 15 minutes twice, the sections were incubated in the reaction solution for 30 minutes at 37°C. The reaction solution was prepared according to the original procedure of Burstone (1958) with minor modification. Briefly, the staining solution was made up with 4% pararosaniline and 4% sodium nitrite in 2 M HCl, and it was adjusted to pH 5.0 and filtered with Whatmann No. 1 paper. Then, 3.2 ml of staining solution was mixed with 36 ml of substrate solution containing 4 mg/ml sodium α-naphthyl phosphate in Michaelis buffer (1 mM sodium barbital, 2 mM sodium acetate, 0.85% sodium chloride). After washing in distilled water, the sections were mounted in Gelvatol. The reaction product in sections were viewed and photographed in the Orthoplan phase contrast microscope (Leitz, Germany).

### *Lysosome fractionation*

Lysosome fractionation was performed according to Symons and Jonas's method (1987) with minor modification. Tissues were homogenized in 10 mM Tris buffer (pH 7.0) containing 0.25 M sucrose and 1 mM EDTA using a Potter-Elvehjem-type glass homogenizer. Then, the protein concentration in the homogenate was adjusted to 25 mg/ml and centrifuged twice for 10 minutes at 750 × *g* and once at 20,000 × *g*. The pellet was suspended in 20 mM Hepes (pH 7.0) containing 0.25 M sucrose and it was mixed with isotonic Percoll (55:45; v/v) followed by centrifugation at 35,000 × *g* for 90 minutes. The resulting gradient was divided into 20 fractions.

To select the lysosome fraction, β-hexosaminidase activity was measured according to Lippincott-Schwart and Fambrough (1986). Briefly, 100 μl of Percoll gradient fraction was added to 0.3 ml of substrate solution containing 0.1 ml of 0.4 M sodium acetate buffer (pH 4.4), 0.1 ml of 0.4% Triton X-100, 0.1 ml of 5 mM 4-methyl-umbelliferyl-β-D-galactoside and

incubated for 30 minutes at 37°C. The incubation was stopped by adding 2 ml of 0.5 M glycine and 0.5 M sodium carbonate (pH 10). The liberated 4-methylumbelliferone was measured in a fluorometer (Sequoia-Turner, USA) at 365 nm for excitation and 450 nm for emission. Since Percoll is known to interfere with the Lowry protein assay, protein concentration was determined by the fluorescamine method (Bohlen et al. 1973). Briefly, 250 µl of fractions was transferred to a tube containing 1.5 ml of 50 mM sodium phosphate buffer (pH 8.0). While vortexing, 0.5 ml of fluorescamine in acetone (0.3 mg/ml) was added rapidly to the tube, and the fluorescence was measured with excitation at 390 nm and emission at 475 nm. Bovine serum albumin was used as a standard protein.

### *Microinjection of lysosome extract*

To test whether the lysosomal enzymes affect salamander limb regeneration, lysosome extract was prepared as follows. The lysosome fraction described as previously was placed in 0.1% Triton X-100 for 2 hours at 4°C to release membrane-bound lysosomal enzymes. The final lysosome extract was prepared by ultracentrifugation at 100,000 × *g* for 2 hours.

At 4 days and 6 days after amputation, 70 nl of lysosome extract or 20 mM Hepes buffer (pH 7.0) containing 0.25 M sucrose and 0.1% Triton X-100 was microinjected into limb regenerates using Nanoject (Drummond, USA). To test whether a low dose of RA might evoke pattern duplication in the dedifferentiated limb regenerates by microinjection of lysosome extract, a subthreshold level of RA (50 µg/g body wt.) for pattern duplication was also injected intraperitoneally into animals at 4 days after limb amputation. As controls, lysosome extract alone or 20 mM Hepes buffer (pH 7.0) containing 0.25 M sucrose and 0.1% Triton X-100 was microinjected twice into limb regenerates at 4 days and 6 days after amputation.

### *Staining and analysis of limb skeleton*

When the regeneration was completed at 6–7 weeks, limbs were fixed in Gregg's fixative and soaked in 10% $H_2O_2$ to remove skin pigment. Depigmented limbs were stained in Victoria blue B and cleared in methyl salicylate to analyse stained skeletons (Bryant and Iten 1974). Since the degree and the type of duplication in each regenerate were variable, the level of duplication was determined according to the previous scheme and characteristics in each specimen was also recorded (Thoms and Stocum 1984; Kim and Stocum 1986). From the raw data, the duplication index (DI) in each experimental group was calculated using the formula; $DI = \Sigma(DD \times f/n)$ (DD, degree of duplication; *f*, frequency; *n*, total number of cases) to reveal the tendency of proximalization by RA treatment (Kim and Stocum 1986).

## Results

### *Expression profile of LAP in the regenerating limbs of* H. leechii

To examine the LAP expression level during regeneration of *Hynobius* limbs, Western blot analysis was performed using mAb HlAcp62, which recognizes the 53 kDa of LAP (Ju et al. 1996). As shown in Figure 1A, LAP was not detected yet immediately after amputation (day 0). However, an increased level of LAP was detected from 2 days after amputation and a peak level was achieved at 8 days after amputation. Thereafter, the expression level declined gradually to the basal level. In RA-treated limb regenerates, the elevation of LAP expression was remarkable and the expression level was much higher than that in the normal limb regenerates (Figure 1B). In detail, the LAP expression level started to increase from 2 days after RA treatment (6 days after amputation) and reached peak level at 6–10 days after RA treatment (10–14 days after amputation). A high expression level was maintained until 12–14 days after RA treatment (16–18 days after amputation).

In addition to Western blot analysis, immunohistochemistry was performed to reveal the spatial and temporal expression pattern of LAP in the regenerating limbs. After simple amputation, the expression profile of LAP changed remarkably with the progression of dedifferentiation. A basal level of immunoreactivity was noted in all tissues including muscle, cartilage,

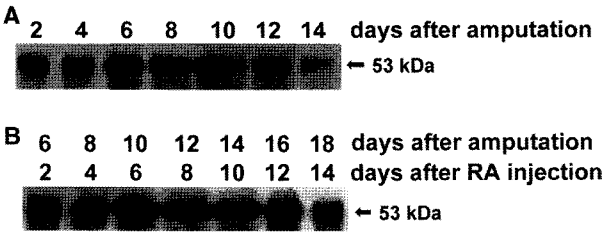


Figure 1. Expression profiles of LAP in normal (A) and RA-treated (B) limb regenerates of *H. leechii*. After SDS-PAGE of total proteins from limb regenerates at various regeneration stage, immunoblots were performed with mAb HlAcp62.

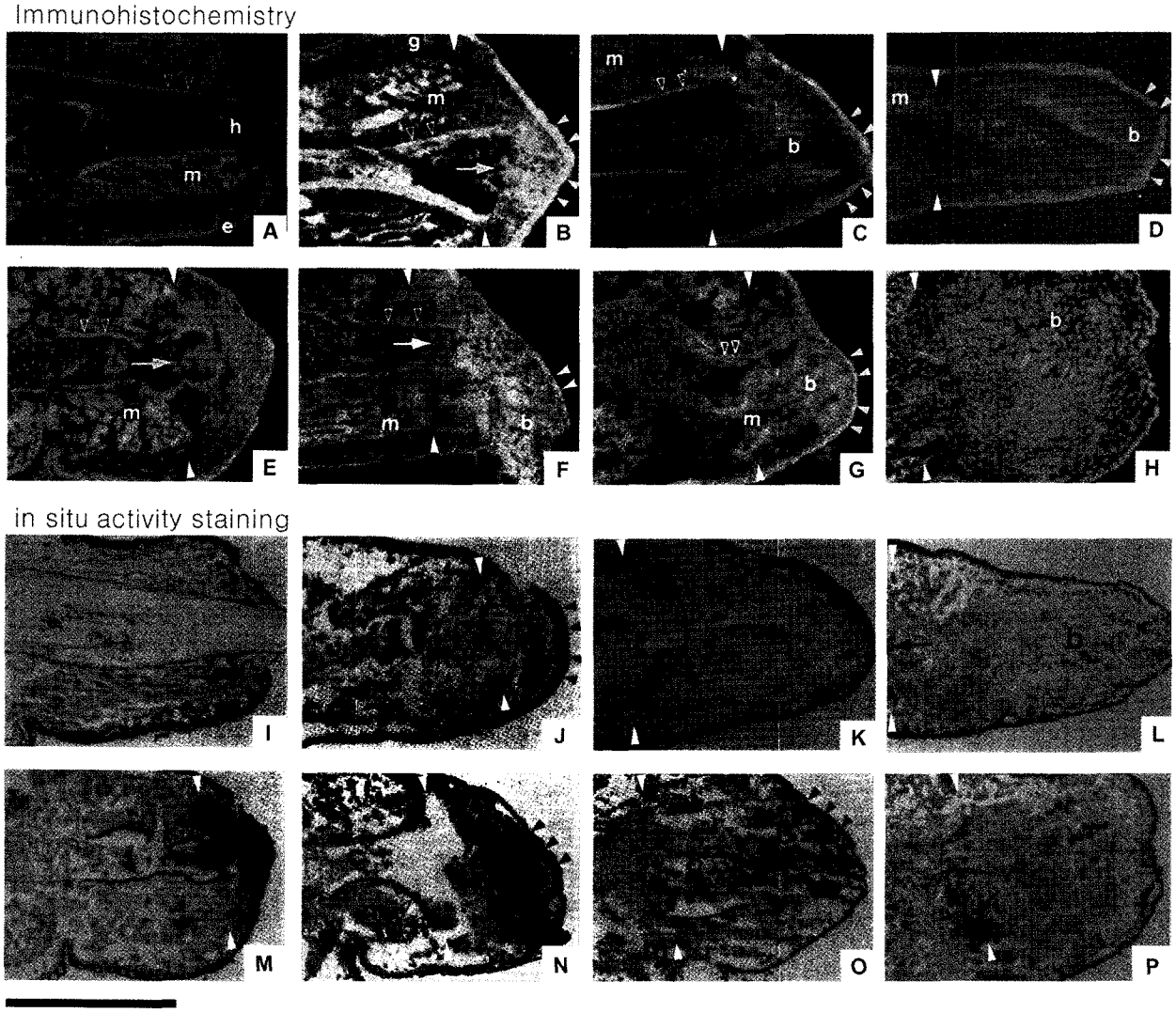


Figure 2. Immunohistochemical localization of LAP and histochemical profile of LAP activity in normal and RA-treated limb regenerates of *H. leechii*. After amputation of forelimb through the distal stylopodium, RA was injected intraperitoneally at 4 days after amputation. The normal and RA-treated limb regenerates were cryosectioned and subjected to immunohistochemistry with anti-LAP mAb HlAcp62 or LAP in situ activity staining. b, blastema cell; c, cartilage; e, epidermis; g, Leydig cell; h, humerus; m, muscle; open arrowhead, perichondrium; arrowhead, wound epidermis; yellow arrowhead, amputation plane. Scale bar; 1 mm. (A) At 0 day after amputation (immediately after amputation). The tip of the humerus is somewhat protruded due to soft tissue retraction. Immunoreactivity is weak in epidermis, muscle, perichondrium and humerus. (B) At 6 days after amputation. Note that immunoreactivity is strong at the distal tip of cartilage (arrow), wound epidermis, Leydig cell, perichondrium and disintegrating muscle. (C) At 10 days after amputation. Immunoreactivity decreased clearly in muscle and blastema cells. However, somewhat strong immunoreactivity can be observed in wound epidermis and perichondrium. (D) At 14 days after amputation. The immunoreactivity is very weak in wound epidermis, blastema cells and muscle. (E) At 4 days after RA injection (8 days after amputation). Note strong immunoreactivity in the distal tip of regressing cartilage (arrow), muscle and perichondrium. (F) At 8 days after RA injection (12 days after amputation). Note that immunoreactivity is still strong at the distal tip of cartilage (arrow), wound epidermis, perichondrium, muscle and ectopic blastema. (G) At 10 days after RA injection (14 days after amputation). Immunoreactivity is again still strong in condensing blastema, wound epidermis, perichondrium and muscle. (H) At 16 days after RA injection (20 days after amputation). Note decreased immunoreactivity in blastema cells. (I) At 0 day after amputation (immediately after amputation). LAP activity is high in the distal part of internal tissues close to amputation plane (arrow). (J) At 6 days after amputation. LAP activity is very high in tissues undergoing histolysis including cartilage and muscle. Also, note the intense LAP activity in wound epidermis and accumulating blastema cell. (K) At 10 days after amputation. Note the decreased LAP activity in blastema cells. However, high LAP activity can be observed at the distal tip of stump cartilage (arrow). (L) At 14 days after amputation. Although LAP activity is very low in blastema cells, somewhat high LAP activity is present in the newly formed cartilage (arrow). (M) At 4 days after RA injection (8 days after amputation). Limited extent of LAP activity is visible in the distal tip

epidermis and the Leydig (mucus gland) cells in skin, which reflects LAP being a ubiquitous lysosomal enzyme in all tissues. Immediately after amputation through the upper arm (distal stylopodium), immunoreactivity was weak in epidermis, muscle, Leydig cells, perichondrium and cartilage (Figure 2A). By 2 days after amputation, immunoreactivity started to increase in wound epidermis and muscle (data not shown). As shown in Figure 2B, immunoreactivity became very high both in the dedifferentiating internal tissues of stump and in the wound epidermis at 6 days after amputation. During the dedifferentiation stage (2–6 days after amputation), inner stump tissues such as muscle, cartilage, dermis and other connective tissues began to disintegrate and start to liberate stump cells which had lost their differentiated-state characteristics (Figure 2B). Soon the dedifferentiated cells start to divide and accumulate under the wound epidermis. Strong immunoreactivity continued to exist in wound epidermis, blastema cells, perichondrium and muscle at 8 days after amputation (data not shown). As the blastema grow, immunoreactivity started to decline in wound epidermis and the dedifferentiating distal region of the stump close to the amputation plane at 10 days after amputation (Figure 2C). At the redifferentiation stage, the blastema flattened and the dedifferentiated blastema cells differentiated to form cartilage, muscle and other connective tissues (Figure 2D). The decrease of immunoreactivity was accelerated even further with the onset of redifferentiation by 14 days after amputation, as shown in Figure 2D. As shown in Figure 2E–H, enhanced expression of LAP was remarkable in RA-treated limb regenerates. Compared with the normal limb regenerates, immunoreactivity was especially intense in wound epidermis and the distal tip of disintegrating cartilage between 4 and 10 days after RA injection (8–14 days after amputation; Figure 2E–G). Strong immunoreactivity was still observable in growing blastema cells at 12 days after RA injection (16 days after amputation; data not shown), but decreased immunoreactivity was evident in uniformly distributed blastema cells at 16 days after RA injection (20 days after amputation; Figure 2H).

To relate the increase of LAP expression to the LAP activity, the LAP activity in the limb regenerate of *H. leechii* was examined histochemically by in situ activity staining. Immediately after amputation through the upper arm, LAP activity was very low in all tissues except in epidermis and distal part of internal tissues close to the amputation plane (Figure 2I). However, increased LAP activity was detected thereafter, and the peak level was achieved at 4–6 days after amputation (Figure 2J). At this period, the LAP activity was mainly localized in the wound epidermis and dedifferentiating internal tissues. At 8 days after amputation, a high level of activity was still observable at the distal tip of disintegrating cartilage, wound epidermis and blastema cells (data not shown). As redifferentiation started, LAP activity decreased gradually in blastema cells and wound epidermis. However, a high level of activity was still present at the distal region of disintegrating cartilage at 10 days after amputation (Figure 2K). As shown in Figure 2L, LAP activity decreased noticeably in the blastema cells at later stages. Interestingly, somewhat patchy high LAP activity was observed in the redifferentiating chondrocytes at 14 days after amputation.

In RA-treated limb regenerates, LAP activity increased remarkably in the dedifferentiating tissues of limb stump close to the amputation plane and wound epidermis. High LAP activity was observed in dedifferentiating muscle, cartilage, perichondrium and wound epidermis at 2 days after RA injection (6 days after amputation). As dedifferentiation proceeded further, LAP activity increased even further at the distal region of disintegrating cartilage and in the condensing blastema cells, perichondrium, muscle and wound epidermis between 4 and 10 days after injection (8–14 days after amputation; Figure 2M–P). The LAP activity appears to be somewhat higher than that in the normal regenerates at the dedifferentiation stage. Also, the area showing strong LAP activity was noted to expand more proximally. Although LAP activity decreased slightly in blastema cells, high activity was still observable in wound epidermis at 12–14 days after injection (16–18 days after amputation; data not shown). Thereafter, LAP activity decreased noticeably in blastema cells and wound epidermis. However, somewhat high activity remained in the distal tip of regressed cartilage (Figure 2P).

### *Effects of lysosomal extracts on the salamander limb regeneration*

Considering that many kinds of lysosomal enzymes are involved in the dedifferentiation and pattern

of cartilage (arrow) and perichondrium. (N) At 8 days after RA injection (12 days after amputation). The LAP activity is very high in the disintegrating muscle, cartilage, perichondrium, ectopic blastema and wound epidermis. (O) At 10 days after RA injection (14 days after amputation). Still, LAP activity is high in the distal part of internal tissues including cartilage and muscle. Also, note the intense LAP activity in wound epidermis and accumulating blastema cell. (P) At 16 days after RA injection (20 days after amputation). Although somewhat high activity can be observed in the distal region of cartilage (arrow), LAP activity is low elsewhere.

Table 1. Effects of lysosome extract on salamander limb regeneration.

| Group | Total | Hypomorphic or inhibition | Normal | Duplication | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Total class | Degree of duplication | | | DI ($\Sigma DD \times f/n$) |
| | | | | | 0.5 | 1.0 | 1.5 | |
| Lysosome extract | 26 | 4 (15.4) | 20 (76.9) | 2 (7.7) | 0 | 1 | 1 | 0.10 |
| Hepes | 14 | – | 14 (100) | – | – | – | – | 0 |
| Lysosome extract+RA | 86 | 25 (29.1) | 25 (29.1) | 24 (39.5) | 1 | 12 | 24* | 0.69 |
| Hepes+ RA | 22 | 4 (18.2) | 14 (63.6) | 4 (18.2) | 1 | 2 | 1 | 0.18 |
| RA | 40 | 4 (10.0) | 29 (72.5) | 7 (17.5) | 0 | 5 | 2 | 0.20 |

Lysosome extract: injected twice (70 nl/injection) at 4 days after amputation.
RA: intraperitoneal injection of retinoic acid (50 μg/g body wt) at 4 days after amputation.
Hepes: 20 mM Hepes buffer (pH 7.0) containing 0.25 M sucrose and 0.1% Triton X-100.
*Three cases showed duplication both in proximodistal and transverse axes. Numbers in parentheses are percentages.

restoration processes during salamander limb regeneration, the effects of lysosomal enzymes in limb regeneration were examined. When lysosome extract was microinjected alone into limb regenerates at 4 and 6 days after amputation, the skeletal pattern was normal even though limb regeneration was delayed due to the extended dedifferentiation period for 7–12 days (Table 1 and Figure 3A). When a low dose of RA (50 μg/g body wt) was injected, some duplicating effect was observed but the duplication index (DI) was very low. Interestingly, when the lysosome extract was microinjected into limb regenerates with a low dose of RA, skeletal duplication occurred frequently in the proximodistal axis, and in a few cases both in proximodistal and in transverse axes (Figure 3B–D). The DI in this group was about 3.5-times higher than that in the control group (Table 1).

## Discussion

In this study, expression of LAP was found to be dependent on the regeneration stages. At dedifferentiation and early bud stages, the expression level of LAP increased remarkably, as shown by Western blot analysis and immunohistochemistry. Especially, a high level of LAP expression was found in the dedifferentiating distal limb tissues such as the tip of cartilage, the distal end of muscle fragments and in the perichondrium and wound epidermis. In fact, it is well known that histolysis of differentiated tissues takes place in the region adjacent to the amputation plane (Carlson 1974; Stocum 1979). Interestingly, acid phosphatase activity is known to be high in the apical ectodermal ridge (AER) of chick, mouse and rat (Milaire 1997). Considering that AER is probably an equivalent structure to wound epidermis for the proliferation of underlying mesenchymal cells, it would be interesting to know whether the wound epidermis plays similar role in the regeneration process (Globus and Vethamany-Globus 1976; Stocum 1995).

Previous and present studies show clearly that LAP expression and activity was increased in the dedifferentiating salamander limb regenerates (Ju and Kim 1994). Especially, high levels of expression and activity were noted in the disintegrating cartilage and muscle as well as in the wound epidermis, perichondrium and blastema cells of the limb regenerates. The mechanism for the increase of LAP activity in the dedifferentiating limb tissues may be due to either the increased synthesis of LAP and/or increased conversion of the latent form of LAP into the non-latent form. For example, LAP neosynthesis is responsible for the increase of LAP activity in the regressing oral sucker of *Xenopus* tadpole at metamorphosis (Ling and Lyerla 1976). At present, de novo synthesis of LAP appears to be partially, at least, responsible for the increased LAP activity since the conversion of the latent form of LAP to the active form alone cannot explain the difference between the basal level and the increased levels of LAP (data not shown). Our results also show clearly that RA treatment causes an increase of LAP expression and activity in salamander limb regenerates for an extended dedifferentiation period (Ju and Kim 1994). In chicken osteoclast, RA elevates LAP activity, probably due to the increased enzyme synthesis, and accelerates bone resorption (Oreffo et al. 1988). Another possible mechanism for the increased enzyme activity by RA might be related to the destabilization of lysosomal membrane (Roles 1969; Wang et al. 1976; Kumar et al. 2008).

So far, little is known about the natural substrate(s) of LAP and its physiological roles. Interestingly, many kinds of acid phosphatases are considered to be important in the regulation of the phosphotyrosine

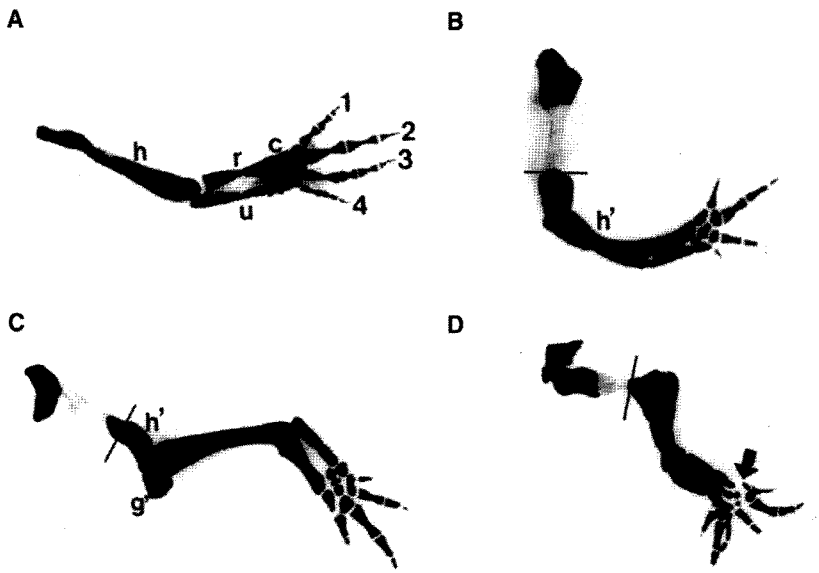


Figure 3. Various types of skeletal pattern obtained from microinjection of lysosome extract and low dose of RA (50 μg/g body wt) into limb regenerates of *H. leechii*. The lines in B, C and D indicate junctions between stump and regenerate. ×10. (A) Normal limb regenerate; h, humerus; r, radius; u, ulna; c, carpals; 1~4, anterior to posterior sequence of digits. (B) Partial duplication of humerus (h′). Due to the direct connection of the duplicated humerus with the stump humerus, the overall length of the stylopodial segment is abnormally long. (C) Duplicated girdle (g′) in addition to duplicated humerus (h′). (D) In some cases, duplication occurred in the proximodistal and transverse axes. Microscopic observation revealed that two limbs fused at the posterior regions (arrow).

level since acid phosphatase from frog liver, bovine heart, human red blood cell and human prostate gland are known to exhibit phosphotyrosine hydrolysing activity (Chernoff and Li 1985; Pavlovic et al. 1985; Boivin and Galand 1986; Janska et al. 1988). Milaire (1997) suggested that the dephosphorylating activity of acid phosphatase coincides with the inductive signalling process before tissue differentiation in the AER of chick, mouse and rat. Therefore, it appears that the phosphorylation state of various cellular components is correlated with the activities of the enzymes for modifying phosphorylation such as LAP in urodele limb regeneration. Furthermore, data obtained from the present study suggest that lysosomal enzymes including LAP have an important role(s) in the dedifferentiation process and RA-evoked pattern duplication even though the mechanism itself is still obscure.

When the lysosome extract was microinjected into limb regenerates, limb regeneration was delayed by 7–12 days due to the extended dedifferentiation period. Interestingly, when lysosome extract was co-microinjected into limb regenerate with a subthreshold level of RA (50 μg/g body wt) for pattern duplication, skeletal pattern duplication was evoked frequently in the proximodistal and transverse axes. In fact, a high activity of lysosomal enzymes has been reported in disintegration of intact stump tissues such as cartilage, muscle, nerve and other connective tissues and formation of blastema cells during dedifferentiation (Stocum 1995). Furthermore, it has been reported that lysosomal enzymes such as cathepsin could release growth factors for the blastema cell growth via extracellular matrix degradation (Stocum 1995; Takei et al. 1997). Considering the high level of lysosomal enzyme activities during the dedifferentiation stage of urodele limb regenerates (Stocum 1995), lysosome extracts might make the regenerative environment more favorable for pattern duplication at a low dose of RA. This interpretation implies that pattern duplication with a high dose of RA in the regenerating salamander limbs is accomplished through not only the modification of the positional value in the blastema cells but also the enhancement of dedifferentiation. Previously, it has been reported that the regenerative ability of limbs of *Rana temporaria* can be extended by repeated amputation or deep pricking with a needle, which would have induced some degree of dedifferentiation (Stocum 1995). Infusion of blastemal extract or alkaline phosphatase into irradiated limb stumps of the newt promotes the growth of conical structures that contain amorphous cartilage (Deck and Dent 1970). Similarly, high NaCl treatment which results in the release of growth factors induces partial limb regeneration in anurans and mice (Neufeld 1980). These results suggest that induction of dedifferentiation is a key process for

limb regeneration in non-regenerating animals. Therefore, the study of dedifferentiation will help to understand the pattern formation, and offer insights into how we might stimulate regeneration where it does not naturally occur.

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