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Lysosomal Acid Phosphatase in Regenerating Salamander Limbs Studied with Monoclonal Antibodies

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In previous studies, we have shown that lysosomal acid phosphatase (LAP) activity increases at the dedifferentiation stage in the regenerating larval limbs of salamander, *Hynobius leechii*. Monoclonal antibodies against LAP were generated to determine the spatial and temporal distribution of the protein in the regenerates. A total of 22 monoclonal antibodies recognizing different epitopes of the protein were obtained, of which five strongly stained the regenerating limb by immunohistochemistry. In LAP immunohistochemical examination, LAP showed distribution coincident with the state of dedifferentiation, both spatially and temporally, in the limb regenerates. When unfractioned protein of regenerating salamander limbs were separated by gel electrophoresis and immunoblotted, the antibodies recognized a single protein band of 53 kDa, which comigrates with a monomeric subunit of LAP. Using the anti-LAP antibodies as probe, we investigated the cross-reactivities of LAPs from other sources. The immunoreactive bands on Western blots appeared to be the same in molecular mass-53 kDa in axolotl and *Xenopus*, but no protein band was detected in mouse, *Drosophila*, or *C. elegans*. These results show that the antibodies generated in this study specifically recognize *Hynobius leechii* LAP and that LAPs may be highly conserved among amphibians. Furthermore, the distribution of the protein is consistent with a role for LAP in the dedifferentiation process of limb regeneration.

KEY WORDS: Limb Regeneration, Dedifferentiation, Lysosomal Acid Phosphatase Pattern Formation

Lysosomal enzymes are known to play important roles in many biological processes; for example, tissue demolition during tadpole and insect metamorphosis, regression of chick embryo Müllerian duct, and dedifferentiation in regenerating planarian tissue (Weber, 1963; Rasch and Gawlik, 1964; Robinson, 1970, 1972; Coward *et al.*, 1973). Generally, lysosomal acid hydrolases are believed to be involved in the process of intra- and intercellular digestions (Holtzman, 1989). More than 50 lysosomal enzymes have been described, and those enzymes were classified into 6 groups, i. e., glycosidase, phosphatase, sulphatase, lipase, protease, and nuclease.

In the regressing tail of *Xenopus* tadpole, activities of lysosomal acid hydrolases such as acid phosphatase, cathepsin, and collagenase were detected (Weber, 1957; Eisen and Gross, 1965; Robinson, 1970, 1972; Filburn, 1973). Among the lysosomal acid hydrolases, acid phosphatase has been considered to be the most convincing marker of lysosomal enzymes (de Duve, 1959; Bertolini and Hassan, 1967). Lysosomal acid

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phosphatase (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. However, a natural substrate for LAP has not been found. LAP has been found in yeast, *C. elegans*, *Drosophila*, rat, and human with similar molecular weight and characteristics (Igarashi and Hollander, 1968; Saini and Van Etten, 1978; Feigen *et al.*, 1980; Pavlovic *et al.*, 1985; Beh *et al.*, 1991).

In the regenerating salamander limb, lysosomal enzymes are believed to play a key role especially at the early phase of regeneration process. Once salamander limb is amputated, the stump tissues of the limb, such as muscle, bone, cartilage, dermis, and nerve lose their characteristics and become dedifferentiated. Varied enzyme activities such as proteases and acid hydrolases including serine proteases, collagenases, cathepsin, and acid phosphatase have been found in the dedifferentiating limbs of urodele (Schmidt and Weidman, 1964; Grillo *et al.*, 1968; Schmidt, 1968; Slattery and Schmidt, 1975; Ju and Kim, 1994).

In previous studies, we have shown that retinoic acid (RA), an inducer of pattern duplication, causes increased level of dedifferentiation in the regenerating larval limbs of salamander, *Hynobius leechii* (Ju and Kim, 1994). Furthermore, increase of lysosomal acid phosphatase (LAP) activity during dedifferentiation was also noted after RA treatment.

Here, we produced a panel of monoclonal antibodies recognizing different epitopes of the LAP. Using the antibodies as probe we determined the distribution of the protein in the regenerating limb of salamander and some of its characteristics, and discuss its role in regeneration.

Materials and Methods

Experimental Animals

The larvae of Korean salamander (*Hynobius leechii*) and Mexican axolotl (*Ambystoma mexicanum*) were used in this study. Newly hatched larvae were kept in dechlorinated tap water and were fed freshly hatched brineshrimp or finely chopped beef heart. At the time of experimentation, the animals were approximately 30 mm in length.

Purification of salamander LAP

Unless otherwise specified, all the experimental procedures were carried out at 4°C. To obtain pure lysosomal acid phosphatase (LAP) of Korean salamander, whole bodies were minced into small pieces and were homogenized in 0.1 M sodium acetate buffer, pH 4.8, containing 1 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, and 1 mM PMSF using a Potter-Elvehjem type glass homogenizer. To obtain crude extract, the homogenate was centrifuged twice at 12,000 $\times$ g for 30 minutes and the supernatant was collected. Then, the supernatant was adjusted to pH 4.8 with 1 M cold acetic acid, the precipitate formed was discarded. Solid $(NH_4)_2SO_4$ was added to supernatant to 30% saturation and the precipitate was removed by centrifugation. $(NH_4)_2SO_4$ was then added to the supernatant to 70% saturation. The precipitate after centrifugation was dissolved in 10 mM sodium acetate buffer (pH 4.8) containing 1 mM EDTA and dialyzed against 10 mM sodium acetate buffer (pH 4.8) for overnight. The enzyme preparation was subjected to CM-cellulose ion-exchange chromatography with a linear gradient of 10 - 500 mM NaCl. Acid phosphatase activity in each fraction was measured using p-nitrophenyl phosphate (Sigma) as a substrate after Robinson's procedure (1970). In each fraction, protein concentration was determined spectrophotometrically by measuring absorbance at 280 nm. The fractions which showed high level of acid phosphatase activity were pooled and concentrated with a Centricon-30 concentrator (Amicon). For further purification of LAP, the concentrated filtrate was subjected to native polyacrylamide gel electrophoresis (ND-PAGE) according to Davis (1964), and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970).

Activity staining

To confirm LAP activity in the ND-polyacrylamide gel, activity staining was performed according to the procedure of Beh *et al.* (1991). Briefly, staining solution was prepared by mixing 5 ml of 4% pararosaniline in 2.4 M HCl with 5 ml of freshly prepared 4% $NaNO_2$, 85 ml of 0.15 M sodium acetate buffer (pH 8.0), 4.75 ml of 1 N NaOH, and 1 mg/ml α-naphthyl phosphate (Sigma) in sodium acetate buffer. The gel was immersed in staining solution for 3-4 hours and washed in distilled water. One dark brown band appeared at the site of LAP activity. Molecular weight of the native LAP was determined using Nondenatured protein molecular weight marker kit (Sigma) according to manufactuer's instruction.

Production of monoclonal antibody (mAbs)

After SDS-PAGE of the purified enzyme preparation, the gel was stained with Coomassie Brilliant blue R and the 53 kDa band corresponding to monomeric subunit of lysosomal acid phosphatase was excised. To immunize mice with salamander LAP, the excised band was minced into small pieces. The emulsified antigen in complete Freund's adjuvant was injected into 8-10 week old female BALB/c mice intraperitoneally. The first injection was followed by three booster injections by 3- to 4- week intervals. The final injection was given 3 or 4 days before the cell fusion without adjuvant. To prepare feeder layer cells, a 12 week old mouse was killed by cervical dislocation, its abdominal skin was carefully removed and peritonial cells were collected by centrifugation. The cells were suspended in 60 ml HAT medium and 0.5 ml of the cell suspension was placed into each well of five 24-well plates. Splenocytes from LAP immunized mouse and SP2/o Ag-14 cell suspension were combined and washed with incomplete DME by centrifugation for 3 minutes at 650 $\times$ g. The cell pellet was mixed and 1 ml of 50% PEG in incomplete DME was added slowly to the tube over a period of 1 minute with constant swirling at 37°C. The fusion process was allowed to continue for another 90 second at 37°C (Goding, 1985; Choi and Jeon, 1989). About two weeks after cell fusion, culture supernatants were screened for the reactivity to salamander LAP by western blot analysis. Positive clones selected were transferred to 6-well plates and finally grown in tissue culture flasks (75 cm^2) and frozen in liquid nitrogen. All hybridomas showing positive reactions were frozen and cloned after thawing.

Western blot

Protein that had been electrophoretically fractionated on 10% SDS-gel was blotted to nitrocellulose membrane as described previously (Towbin *et al.*, 1979). The blots were exposed to mAbs and then to anti-mouse IgG-horseradish peroxidase conjugate (Sigma). Bands were visualized by color reaction with 4-chloro-1-naphtol. For chemiluminescent detection, blots were sequentially exposed to mAb, anti-mouse IgG-biotin conjugate, and Avidin-alkaline phosphatase streptavidin conjugate, and disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tri-cyclo[3.3.1.13,7]decan}-4 -yl) phenyl phosphate (CSPD) was used as a substrate. The bands were detected by direct contact with x-ray film. Molecular weight of the LAP subunit was determined using High molecular weight standard kit (Sigma). After transfer of molecular weight standard proteins into nitrocellulose membrane, the blot was stained with Ponceaus S.

Peptide mapping

One-dimensional peptide mapping was carried out according to the procedure of Cleveland et. al. (1977). Briefly, purified LAP were mixed with V8 protease (10 μg /ml) and digested at 37°C for 30 minutes. Following addition of β-mercaptoethanol and SDS to the final concentration of 10% and 2%, respectively, proteolysis was stopped by boiling the samples for 10 minutes. Proteolyzed LAP was separated by SDS-PAGE in 10 % gel and Western blotting was carried out as described above.

Deglycosylation of salamander LAP

Purified salamander LAP was denatured in 1% SDS by boiling for 10 minutes. To avoid inactivation of N-glycosidase F by SDS, Nonidet P-40 was added into denatured sample for the final

concentration of 0.5%. After addition of 4 unit of N-glycosidase F (Boehringer Manheim), digestion was performed at 37°C for 16 hours. Western blotting was carried out as described above.

Lysosome fractionation

Mexican axolotl livers were homogenized in a 10 mM Tris buffer (pH 7.0) containing 0.25 M sucrose and 1 mM EDTA using a Plotter-Elvehjem type glass homogenizer. The homogenate was diluted to a protein concentration of 25 mg/ml with the homogenizing buffer and centrifuged twice for 10 minutes at 750 × g and once at 20,000 × g. The pellet was resuspended in 20 mM Hepes (pH 7.0) containing 0.25 M sucrose. The pellet solution was mixed with isotonic Percoll (55:45; v/v) and centrifuged at 35,000 × g for 90 minutes. The resulting gradient was divided into 20 fractions. To confirm the lysosomal fraction, β-hexosaminidase activity was measured according to Lippincott-Schwart and Fambrough (1986). The protein concentration was determined by fluorescarmine method (Bohlen *et al.*, 1973).

Immunohistochemistry

Larval limb regenerates of Korean salamander for immunohistochemistry were amputated from the limb stumps and were embedded in O.C.T. compound (Miles) for cryosection. Tissue was sectioned serially at 10 μm, and the sections were mounted on gelatin-coated slide and stored at −70°C until use. For immunohistochemical detection of LAP, tissue sections were fixed with 4% paraformaldehyde for 15 minutes and were rinsed with TBS containing 0.3% Triton X-100, followed by rinsing in TBS 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 (Sigma) for 2 hours. After thorough washing in TBS, the sections were mounted in Gelvatol. The sections were viewed and photographed with Kodak Ektapress 1600 film.

Results and Discussion

Production and characterization of mAbs to Korean salamander LAP

LAP from Korean salamander larvae, *Hynobius leechii*, was purified to a single 53 kDa band in SDS-PAGE. This corresponds to LAP monomeric subunit. The gel-purified LAP was used as an immunogen to prepare monoclonal antibodies. Twenty two out of 144 hybrodoma clones were initially found to recognize the 53 kDa Korean salamaner LAP subunit in Western blots analysis (Table 1). Further screening was carried out to select clones with strong immunoreactivities among those positive clones by Western blot analysis and immunohistochemistry. Five monoclonal antibodies (mAbs; HlAcp 10, 36, 59, 62, 76) showed strong reactivity, and were finally selected for further study. The immunoreactivities of 5 mAbs with purified Korean salamander LAP are shown in Fig. 1. When the total protein of whole salamander homogenate was separated by SDS-PAGE and immunoblotted, all of the mAbs showed specific reactivity with a protein band of 53 kDa. In order to confirm the 53 kDa protein to be the LAP, we undertook a series of experiments as follows. First, when the pure form of LAP was subjected to ND-PAGE followed by activity staining, only one protein band with M.W. of 110 kDa was stained (Fig. 2, lane 1). Second, in the Western blot analysis with mAb HlAcp62, immunoreactivity appeared at the same band (Fig. 2, lane 2). Third, when the 110 kDa band was excised and immunoblotted after SDS-PAGE, 53 kDa protein band was stained (Fig. 2, lane 3). These results also suggests that LAP is a dimeric protein composed of 53 kDa monomers.

To ascertain that the monoclonal antibodies selected in this study are specific to lysosomal acid phosphatase, Mexican axolotl lysosome, an alternative for the Korean salamander lysosome, was fractionated by differential centrifugation. Lysosomal fraction could be identified by measuring the fluorescence of 4-methyl-umbeliferrone which was known to be produced by lysosomal β-hexosaminidase action on 4-methyl-umbeliferryl β-D-galactoside (Lippincot-Schwart and Fambrough, 1986). The specific

Table 1. The summary of screening of 22 mAbs against salamander LAP by Western blot analysis and immunohistochemistry.

N0. of mAb	Western blot analysis		Immunohistochemistry		Peptide mapping type
	ND-PAGE	SDS-PAGE	Normal limb	Dedifferentiaiting limb	
HlAcp 1	−	+	+	+	III
HlAcp 10	+	++	+	++	III
HlAcp 14	−	+	++	+	IV
HlAcp 15	++	+++	++	+	II
HlAcp 18	−	++	+	−	IV
HlAcp 32	−	+	+	+	II
HlAcp 36	++	+++	+	+++	I
HlAcp 38	−	+	−	+	IV
HlAcp 45	+++	+++	++	+	I
HlAcp 52	+	+++	+	+	II
HlAcp 53	−	+	+	+	III
HlAcp 54	++	+++	+++	+++	I
HlAcp 59	+++	+++	+	+++	I
HlAcp 62	++	++	+	+++	I
HlAcp 76	+	+	+	+++	I
HlAcp 85	++	++	++	+	I
HlAcp 86	−	+	+	+	II
HlAcp 97	−	+	+	+	IV
HlAcp 99	+	++	+	+	I
HlAcp 122	−	+	++	−	II
HlAcp 126	+	+++	+	−	I
HlAcp 143	+++	+++	+++	+++	I

+++, highly reactive; ++, moderately reactive; +, slightly reactive; −, non-reactive

activity of β-hexosaminidase in lysosomal fraction was about 7 fold higher than that in cytoplasmic fraction (data not shown). When the protein in the lysosomal fraction was analyzed by Western blot, a strong immunoreactive band appeared as a protein band of 53 kDa and a minor 67 kDa protein band was also detected which was presumed to be the precursor form of LAP subunit contaminated in the process of lysosome fractionation (Fig. 3). However, no immunoreactive band was detected in the cytoplasmic fraction (Fig. 3, lane 5). Therefore, these results clearly indicate that the prepared mAbs are specific to lysosomal acid phosphatase. Similar molecular weight and dimeric structure have been reported for lysosomal acid phosphatase from human, rat, *Drosophila* and *C. elegans* (Igarashi and Hollander, 1968; Saini and Van Etten, 1978, Feigen *et al.*, 1980; Beh *et al.*, 1991).

To examine further the reactivities of mAbs, we digested LAP with V-8 protease and immunostained the protein digest. Among the mAbs tested, four subgroups recognizing different peptide fragments were identified. All types of antibodies recognized a main peptide fragment in common (Fig. 4, arrow). In type I and II, several peptide fragments were recognized weakly (Fig. 4, lane 1 and 2, arrow head). However, type III antibodies recognized only a main peptide fragment and type III antibodies recognized it weakly (Fig. 4, lane 3 and 4). The antibodies showing a similar staining pattern are presumed to recognize the same epitope. Interestingly, it was found that four out of five mAbs that showed strong reactivity in the Western blots and immunohistochemistry belong to type I. Mole (1988) suggested that conformational epitopes

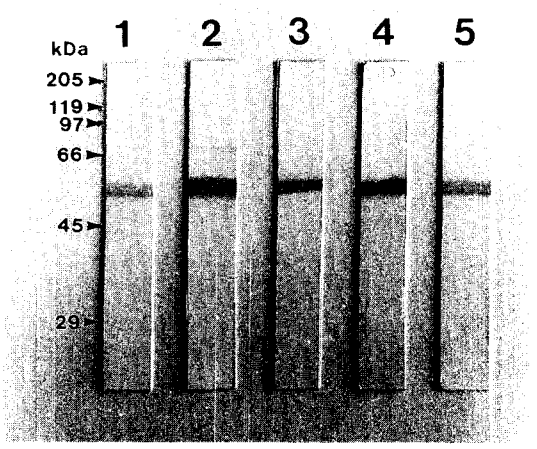

Fig. 1. Immunoreactivities of five representative mAbs after SDS-PAGE of purified salamander LAP. 1; mAb HlAcp 10, 2; mAb HlAcp 36, 3; mAb HlAcp 59, 4; mAb HlAcp 62, 5; mAb HlAcp 76.The relative mobility of molecular weight standard proteins are marked in the left: myosin (205 kDa), β-galactosidase (119 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa).

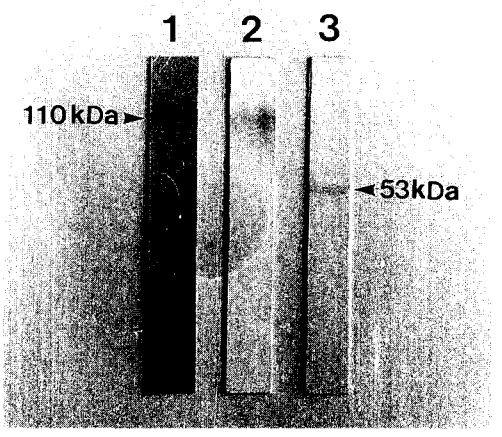

Fig. 2. Comparison of the activity staining pattern and the Western blot profies of salamander LAP Activity staining of LAP after ND-PAGE (lane 1). Western blot profile of LAP after ND-PAGE (lane 2). Western blot profile of LAP after SDS-PAGE (lane 3).

were more susceptible to denaturing agents, such as SDS, than linear epitopes, and conformational epitopes were prone to disruption in the process of Western blotting. Thus, the strong reactivity of

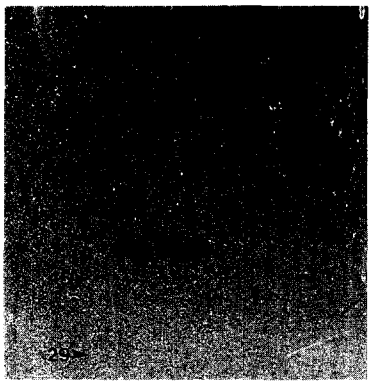

Fig. 3. Subcellular localization of salamander LAP by immunostaining with mAb HlAcp 62. 1; total protein from axolotl liver, 2; lysosomal fraction of axolotl liver, 3; nuclear fraction of axolotl liver 4; microsomal fraction of axolotl liver, 5;cytoplasmic fraction of axolotl liver.

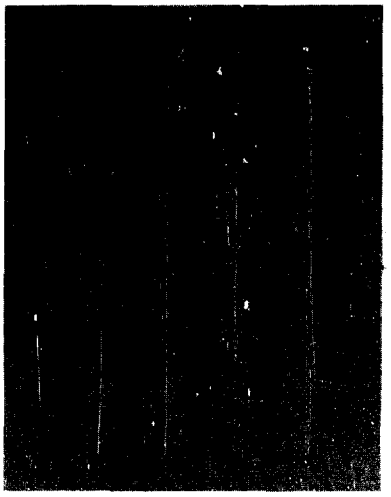

Fig. 4. Immunoreactivity of four types of mAbs with LAP digested with V8 protease and separated by SDS-PAGE. 1; type I (immunostaining with mAb HlAcp 62), 2; type II (immunostaining with mAb HlAcp 52), 3; type III (immunostaining with mAb HlAcp 38), 4; type IV (immunostaining with mAb HlAcp 1).

those mAb's both in immunohistochemistry and Western blot suggests that the epitope(s) of type I antibodies might be linear one(s).

In addition, we have checked the possibility of the mAbs recognizing glycosylated part of LAP. However, even after complete deglycosylation of LAP, immunoreactivity on the Western blot was

detected as a 50 kDa band (Fig. 5, lane 2) which indicates that the mAbs recognize the peptide epitope(s) of salamander LAP.

Localization of LAP in the regenerating limb of salamander

Among 22 mAbs, five showed very strong reactivity in the regenerating limbs of Korean salamander, especially at the dedifferentiation stage when the LAP activity would be peak level (Ju and Kim, 1994). As shown in Fig. 6A, the intensity of immunoreactivity at the wound healing stage of limb regeneration was somewhat strong in epidermis, and very weak in dermis and muscles. However, at dedifferentiation stage, LAP signal became very strong in the epidermis, tips of cartilages and muscles (Fig. 6B). Other mAbs (HlAcp 36, 59, 62, 76) showed similar reactivity (data not shown).

Localization of LAP 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 most intense in macrophages, less intense in epidermis and cartilage matrix at early phase of regeneration (Weiss and Rosenbaum, 1968). In the regenerating limbs of *Nothopthalmus viridescence*, LAP activity was detected in epidermis, subepidermal glands, skeletal muscle, and nerve tissue at the dedifferentiation stage (Miller and Wolfe, 1968). However, sometimes, histochemical methods are known to produce some artifacts. Artificial substrates for LAP have been known to be hydrolyzed by other phosphatases such as the cytosolic form of acid phosphatase, red blood cell acid phosphatase, and protein-tyrosine phosphatase (Filburn, 1973; Boivin and Galand, 1986; Zhang, 1995). Thus, the LAP activity visualized by artificial substrates in regenerates might not faithfully reflect the LAP activity *in situ*. We have minimized these potential inaccuracies in

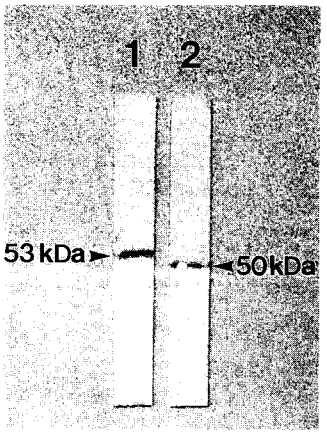

Fig. 5. Comparison of immunoreactivities between (1) salamander LAP and (2) deglycosylated salamander LAP after treatment after N-glycosidase F.

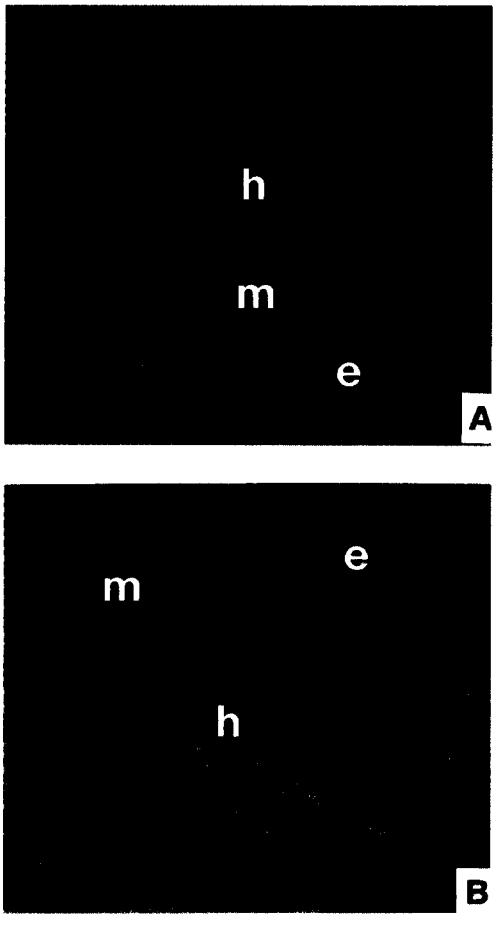

Fig. 6. Immunohistochemical localization of LAP (A) in the limb stump immediately after amputation and (B) in the limb at 6 days after amputation. amputation level; distal stylopodium probe; mAb HlAcp 62 *e*, epidermis; h, humerus; m,muscle.

localization of LAP by generating monoclonal antibodies against LAP and examined LAP distribution during limb regeneration.

Cross-reactivities of antibodies with LAP's of other species

In order to determine if mAb selected in the present study recognizes LAP from different species, Western blot analysis was performed. With HlAcp62 mAb, strong immunoreactivity appeared at 53 kDa bands in axolotl and a weak immunoreactivity appeared at the similar position in *Xenopus* (Fig. 7). However, the HlAcp62 did not show any immunoreactivity with LAP's from mouse, *Drosophila*, and *C. elegans* (data not shown). These results suggest that LAP's among amphibians, especially, urodeles, share a similar epitope and the epitope might be at the region which is not well conserved among various animal species.

In general, prior to the formation of a blastema, lysosomal enzymes are thought to be required in the dedifferentiating tissues of the stump to catalyze the breakdown of proteins and extracellular matrix. Collagenase and several acid hydrolases including acid phosphatase have been detected in the limb blastema of the newt, *Notophthalamus viridescens* (Schmidt and Weidman, 1964; Grillo *et al.*, 1968; Miller and Wolfe, 1968; Weiss and Rosenbaum, 1968). Acid phosphatase belongs to the group of acid hydrolases which characterize the lysosome in several types of vertebrate tissue and has been widely accepted as a primary marker of lysosomal activity. Coward *et al.* (1973) showed that, in the regeneration of planaria (*Dugesia dortocephala*), acid phosphatase activity increased during the early phase of regeneration and the enzyme activity was mainly localized in the dedifferentiating cells of regenerates. Robinson (1970, 1972) also found increased acid phosphatase activity during the late stage of tail regression in metamorphosing *Xenopus laevis* tadpoles.

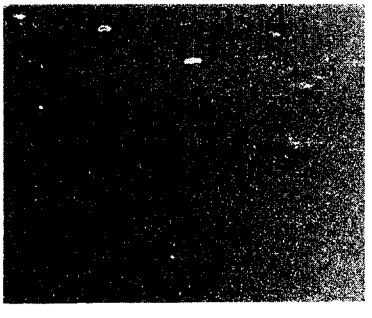

Fig. 7. Cross reactivities of LAP enzymes from amphibian liver with mAb HlAcp 62. Animal livers were removed and the total protein of liver homogenates were immunoblotted. 1;purified LAP from Korean salamander, 2;total protein of korean salamander 3;total protein from axolotl liver, 4; total protein from Xenopus liver.

Little is known about the natural substrate(s) of LAP and its physiological functions. Recently, it has been suggested that many of acid phosphatase would act on phosphotyrosyl residues of proteins. Acid phosphatases from frog liver, bovine heart, human red blood cell and human prostate gland are known to exhibit tyr-p hydrolysing activity (Boivin and Galand, 1985; Chernoff and Li, 1985; Pavlovic *et al.*, 1985; Janska *et al.*, 1988). Collectively, previous results suggest that all acid phosphatases, regardless of molecular weight and subcellular location, possess dephosphorylation activity on the phosphotyrosine moiety of target protein. Furthermore, those findings suggest that LAP may play a role in determining the phosphorylation state of phosphotyrosine-proteins which regulate the cell growth and differentiation. Interestingly, protein phosphorylation pattern coincides well with the trends of acid phosphatase activity in the regenerating limbs of salamander and some proteins are dephosphorylated in response to the addition of purified LAP (unpublished data). Many kinds of protein appeared underphosphorylated during dedifferentiation stage when acid phosphatase activity increased at maximum level. These results indicate that LAP might be a key component in mediating the dedifferentiation process of regenerating salamander limb which might be causally related to the phosphorylation status of many molecules.

Geiger *et al.* (1992) showed that mouse LAP mRNA is highly expressed in mouse hippocampal neuron and Purkinje cell where PDGF-A chain, C/EBP, and G-protein subunit are expressed at high level. Furthermore, it has been reported that cytosolic low molecular weight acid phosphatase

from bovine brain dephosphorylated a Mr 170 kDa phosphotyrosine protein that is believed to be the epidermal growth factor receptor (Ramponi *et al.*, 1989; Shimohana *et al.*, 1994). Those studies indirectly support the idea that LAP might be involved in the regulation of phosphorylation status during salamander limb regeneration.

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리소솜 Acid Phosphatase에 대한 단일 항체를 이용한 도롱뇽 다리 재생 연구

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앞서 본 연구실에서는 도롱뇽(*Hynobius leechii*) 다리 재생 과정중 기존의 조직들이 와해되고 조직 및 그 구성 세포의 분화 양상이 소멸되는 탈분화 과정중 리소솜 acid phosphatase의 활성도가 급격히 증가함을 보고한 바 있다. 본 연구에서는 다리 재생 과정에서 이 효소의 시간적, 공간적 분포 및 발현 양상을 알아보기 위해 단일 항체를 만들었다. 리소솜 acid phosphatase에 대한 22 단일 항체군중 5 항체군이 탈분화 조직과 강한 면역반응을 보였으며 이들의 시간적, 공간적 반응 양상은 조직의 탈분화 상태와 일치하였다. 이 결과는 탈분화 과정시 증가하는 리소솜 acid phosphatase의 활성도가 이 효소의 시간적, 공간적 분포 및 발현 양상과 밀접히 연관되어 있음을 반영하며 탈분화 과정중 이 효소의 역할이 매우 중요함을 시사하고 있다. Immunoblotting 결과 이들 단일 항체군은 리소솜 acid phosphatase의 monomer인 53 kDa 밴드를 인식하였다. 한편 도롱뇽의 리소솜 acid phosphatase에 대한 단일 항체와 타종의 LAP에 대한 cross-reactivity를 immunoblot으로 조사한 결과 양서류인 axolotl(*Ambystoma mexicanum*)과 *Xenopus laevis*에서는 유사한 분자량 band에서 반응이 나타났으나 그외 생쥐, 초파리, *C. elegans*에서는 cross-reactivity가 없는 것으로 조사되었다. 이러한 결과들은 본 연구에서 만들어진 단일 항체가 한국산 도롱뇽의 리소솜 acid phosphatase를 특이적으로 인식하며 나아가 양서류내에서는 이 효소의 상동성이 높음을 시사하고 있다.