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Pepstatin-Insensitive Carboxyl Proteinase: A Biochemical Marker for Late Lysosomes in *Amoeba proteus*

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In order to find a biochemical marker for late lysosomes, we characterized two cDNAs which were cloned by using a monoclonal antibody (mAb) against lysosomes in *Amoeba proteus* as a probe. The two cDNAs, a 1.3-kb cDNA in pBSK-lys45 and a 1.6-kb cDNA in pBSK-lys60, were found to encode proteins homologous to pepstatin-insensitive carboxyl proteinases (PICPs). *E. coli* transformed with pBSK-lys45 produced two immunopositive polypeptides (45 and 43 kDa) and the cDNA in 1274 bases encoded a 44,733-Da protein (Lys45) of 420 amino acids containing one site for a core oligosaccharide. On the other hand, *E. coli* transformed with pBSK-lys60 produced several polypeptides (64, 54, 45, 41, and 37 kDa) reacting with the mAb. The cDNA contained 1629 bases and encoded a 59,231-Da protein (Lys60) of 530 amino acids containing two sites for asparagine-linked core oligosaccharides. These two cDNAs showed identities of 60.3% in nucleotide sequences and 23.6% in amino acid sequences. Lys45 and Lys60 appeared to share XXEFQK as a common antigenic domain. The amino acid sequence of the Lys45 protein showed 17.4% identity and 40.9% similarity to that of PICP from *Pseudomonas* sp. 101. On the other hand, Lys60 showed a 24.3% identity and 51.9% similarity with human lysosomal PICP in the amino acid sequence. A putative active center for serine protease, GTS*xxxxxFxG, was found to be conserved among PICP homologues. The two PICPs are the first reported enzymatic markers for late lysosomes.

Lysosomes contain 50 or more different hydrolytic enzymes that are responsible for the degradation of macromolecules derived from the cell exterior by endocytosis or within the cell by autophagy (Barrett and Heath 1977). However, it is not known if lysosomes are homogeneous organelles with all these enzymes in each vesicle or if they are collections of heterogeneous vesicles, each containing different enzymes. As lysosomes are small organelles with similar density, the question could not be pursued experimentally in earlier microscopic studies of fixed cells or by enzymatic analysis of subcellular fractions (Choi et al., 1992).

The presence of subpopulations within the lysosomal compartments of animal cells has been presumed by many workers (Oliver, 1983; Storrie, 1988). In human myeloid leukemia cells, phagosomes formed during a 1 h pulse with latex beads acquired and lost various time-dependent markers during their transformation into phagolysosomes (Desjardins et al., 1994a; 1994b). Thus, phagolysosome formation was considered to be a highly dynamic process involving gradual and regulated acquisition of markers from heterogeneous endocytic organelles (Robinson et al., 1996). In rat liver endothelial cells, light and electron-lucent lysosomes fused rapidly (in 15 min) with newly formed phagosomes. On the other hand, heavy and electron-dense lysosomes fused after 1 h of phagosome formation (Kjeken et al., 1995). In these cells, a major degradation of labeled substrates began by fusion of early lysosomes, and it was completed by fusion with late lysosomes. Similar observations were also made in macrophages (Rabinowitz et al., 1992; Jahraus et al., 1994). These findings imply that phagosomes fuse with lysosomes containing different hydrolytic enzymes to complete digestion. However, an assay with a few marker enzymes could not clearly define different fractions as different lysosomal subpopulations. In order to demonstrate the heterogeneity of lysosomal subpopulations, it is necessary to differentiate subpopulations of lysosomes using unambiguous markers.

In *A. proteus*, the phagosomal pH decreases to 5.4 among 5-10 min after phagocytosis of *Tetrahymena* (McNeil et al., 1983). In newly formed phagosomes, enzyme activities of acid phosphatase and esterase are detected 8 min after phagosome formation and it reaches the maximum in the next 20 min (McNeil et al., 1983). Choi et al. (1992) produced several mAbs

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against lysosomal membrane proteins and confirmed the fusion between early lysosomes and newly formed phagosomes. In latex-bead containing phagosomes, acid phosphatase activity persists until 2 h after phagosome formation (Ahn and Jeon, 1982). In one study on the phagosome-lysosome fusion using mAbs as probes, four or more lysosomal subpopulations were found in the amoebae (Kim and Jeon, 1993).

In a previous study, we produced two mAbs, LCA45 against lysosomal content and LYA64 against lysosomal membranes of amoebae (Yoo et al., 1996). LYA64-stained lysosomes began to fuse with newly formed phagosomes containing *Tetrahymena* 10 min after the phagosome formation. The intensity of immunofluorescence on membranes of phagolysosomes reached maximum in 2 h and persisted until phagolysosomes were no longer detectable with a light microscope. On the other hand, LCA45-stained lysosomes began to fuse with phagolysosomes in 3 h and the immunofluorescence of phagolysosomes was most intense 8 h after phagocytosis. Thereafter, LCA45 stained-components were recycled to lysosomes. We also noted that lysosomes identified by each mAb fused among themselves and that they enlarged before fusing with phagosomes as in the case of the macrophage (Ward et al., 1997). When amoebae were stained with both LCA45 and LYA64, a fraction of LYA64-stained lysosomes was double stained with LCA45. With this observation, it became apparent that LCA45-stained late lysosomes form a subpopulation of LYA64-stained lysosomes sharing the same membrane antigen. Thus, the heterogeneity of lysosomal subpopulations in terms of their fusion with newly formed phagosomes has been well demonstrated in the amoebae. However, the content of lysosomal subpopulations is not known except that esterase and acid phosphatases are present in early lysosomes (Choi et al., 1992; McNeil et al., 1983).

Most of the mAbs produced against lysosomes of amoeba react with lysosomal membrane proteins (Choi et al., 1992; Kim and Jeon, 1993). Thus, enzyme characteristics of each subpopulation have not been defined yet. LCA45 mAb is the only one to recognize the content of lysosomes. In order to characterize the content of LCA45-stained late lysosomes, we screened the cDNA library of amoebae using LCA45 mAb as a probe and cloned two cDNAs. Both cDNAs were found to encode proteins homologous to the pepstatin-insensitive carboxyl proteinase (PICP: EC 3.4.23.33).

Materials and Methods

Cell culture

A. proteus were cultured in a modified Chalkley's solution (Jeon and Jeon, 1975) in Pyrex baking dishes (35 x 22 x 4 cm) at 24℃ and fed daily with axenically cultured and washed *Tetrahymena pyriformis* as the food organism (Goldstein and Ko, 1976).

Gel electrophoresis and immunoblotting

Proteins of amoebae or cloned *E. coli* were quantitated by the method of Bradford (Bradford, 1976) and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). Molecular mass markers (Sigma) were run in parallel for the estimation of M_r of resolved proteins. After electrophoresis, gels were stained with 0.25% Coomassie Brilliant Blue or processed for immunoblotting. For immunoblotting, proteins separated by SDS-PAGE were transferred to nitrocellulose membranes and immunostained (Ahn and Jeon, 1990) using ascites fluid containing the LCA45 mAb diluted in PBS (1 : 500) as the primary antibody and horseradish-peroxidase-conjugated goat anti-mouse IgG (Jackson Immuno Research Laboratories) as the secondary antibody. Immunoblots were developed with 4-chloro-1-naphthol as the enzyme substrate. Ascites fluid containing the LCA45 mAb was produced as described previously (Yoo et al., 1996).

cDNA cloning and nucleotide sequence analysis

Using the LCA45 mAb as a probe, we immunoscreened the cDNA library of the D strain of *A. proteus* constructed in an Uni-ZAP expression vector as previously described (Choi et al., 1997). In brief, pure phage clones were autoexcised into a pBluescript vector using R408 helper phage, and the clones were maintained as plasmids in *E. coli*. Synthesis of the protein encoded by the cDNA was induced by growing *E. coli* in the presence of 1 mM IPTG. The results were analyzed by SDS-PAGE and immunoblotting. For nucleotide sequencing, plasmid DNAs were isolated by the alkaline lysis method (Lee and Racheed, 1990). Deletion series from both DNA strands were generated using ExoIII/mung bean nuclease combination, and nucleotides were sequenced by the dideoxy chain termination method using ^{35}S-dATP. For comparisons of nucleotide and amino acid sequences, GenBank, EMBL and SwissProt databases were searched.

Results

Characteristics of two different cDNAs

Using LCA45 mAb as a probe, we screened the cDNA library of *A. proteus* and obtained two clones of phagemids, pBSK-lys45 and pBSK-lys60, that contained 1.3-kb and 1.6-kb cDNA, respectively. Restriction analysis showed that the two cDNAs differed in fragmentation pattern and they appeared to originate from different genes. *E. coli* transformed with pBSK-lys45 produced a protein of 45 kDa strongly stained and a 43-kDa protein less stained by the LCA45 mAb (Fig. 1). On the other hand, *E. coli* transformed with pBSK-lys60 was unusual in that they produced several immunopositive proteins, 64, 54, 45, 41 and 37 kDa. Since *E. coli* transformed with pBSKII plasmid as a control did

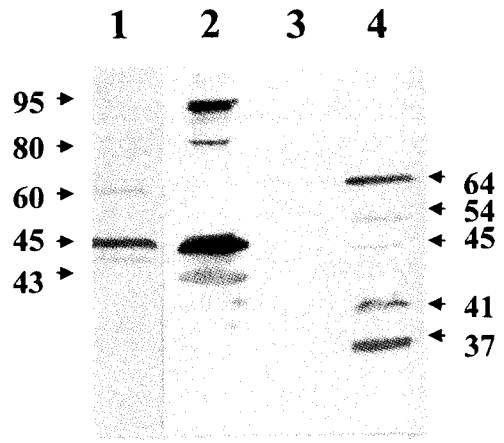

Fig. 1. Immunoblots of total proteins from *Amoeba proteus* and *E. coli* transformed with cloned cDNAs stained with the mAb LCA45. Lane 1, *A. proteus*. Lanes 2 and 4, *E. coli* transformed with pBSK-lys45 and pBSK-lys60, respectively. Lane 3, *E. coli* transformed with pBSK II⁻ as a control. Expression of the cloned gene in *E. coli* was induced with 1 mM IPTG. Note immunopositive bands in lanes 2 and 4. Molecular masses of the protein bands were determined from a gel run with molecular markers and stained with Coomassie Brilliant Blue. The resolving gel was 8% polyacrylamide.

not produce any immunopositive proteins, proteins recognized by the LCA45 mAb were apparently produced from the cDNAs inserted into the two plasmids.

When nucleotide sequences of the two cDNAs were analyzed (Fig. 2 and 3; GenBank Acc. No. AF142414 and AF142415, respectively), both cDNAs were found to contain poly-A tails and AATAA, the typical polyadenylation signal found in amoebae and other eukaryotic mRNAs upstream from the poly-A tails (Choi et al., 1997). The two cDNAs showed identities of 60.3% in nucleotide sequences and 23.6% in deduced amino acid sequences. The similarity was 46.4% when conserved amino acid exchanges were allowed.

The pBSK-lys45 cDNA contained 1,274 bp and encoded a protein (Lys45) of 420 amino acids (Fig. 2). The deduced M_r of Lys45 was 44,733 Da close to the observed mass of the major protein (45 kDa) by immunoblot of proteins from *A. proteus* (Fig. 1) (Yoo et al., 1996). The deduced amino acid sequence contained a site for the addition of asparagine-linked core oligosaccharides at ^{9}NSS and a site for O-glycosylation at ^{195}S. The pBSK-lys60 cDNA contained 1,629 bp and encoded a protein (Lys60) of 530 amino acids (Fig. 3). The deduced M_r (59,231 Da) of the protein was a little smaller than one of the observed mass of a major protein (64 kDa) in the immunoblot of proteins from *A. proteus* (Fig. 1). In the Lys60, ^{125}NSS and ^{383}NAT appeared to be sites for asparagine-linked glycosylation and several potential O-glycosylation sites were predicted.

A search for global amino acid sequence homologies with other proteins was carried out by screening Swiss-Prot and GenBank databases with the BLAST program (Altschul et al., 1990), but no extensive homologies with the deposited proteins were found for both proteins. Meanwhile, Lys45 showed 24.3% identity and 43.5% similarity with V4-7 vegetative stage-specific protein of *Dictyostelium discoideum* (GenBank Acc. No. U27540) and 17.4% identity and 40.9% similarity with pepstatin-insensitive carboxyl proteinase precursor of *Pseudomonas* sp. 101 (PPICP; GenBank Acc. No. A55368). Lys60 showed the highest 24.3% identity and 51.9% similarity with human CLN2 protein that is a precursor for the lysosomal PICP (GenBank Acc. No. AF 017456) (Sleat et al., 1997). A region of the protein extending from residues 356 - 412 has a 45% identity and 70% similarity with a portion of CLN2.

Prediction of antigenic domains

On the bases of sequence data, the two proteins were homologous to PICPs. Although identities between the two proteins were not significant they shared cross reactivity to LCA45 mAb. Since products of the two cDNAs in *E. coli* reacted with the same mAb, antigenic determinant did not appear to be carbohydrate moieties of the proteins. In order to identify common antigenic determinants, we analyzed hydropathy and antigenicity from the deduced amino acid sequences using the PC/Gene program. The most possible candidates predicted for antigenicity in Lys45 and Lys60 were ^{415}KDKKLK^{420} and ^{57}DRKSQD^{62}, respectively. These domains were hydrophilic as they contained several charged groups in 6 amino acids. However, these domains were not common to the two proteins of amoebae. The second probable candidate for antigenicity was ^{138}NTEFQK^{143} for Lys45 and ^{266}DVEFQK^{271} for Lys60, respectively. These portions had 4 identical pairs of amino acids, and were unique to amoebae among their homologues and the lysosomal proteins compared (Viitala et al., 1988; Holness and Simmons, 1993).

PICP homologues with a serine protease active center

For detailed comparisons of Lys45 and Lys60 with their homologues, we aligned amino acid sequences of these proteins with those of human CLN2 (Sleat et al., 1997) and two bacterial PICPs (Oda et al., 1996, GenBank Acc. No. A55368) using the Clustal Multiple Sequence Alignment Program (Genome Center, Baylor College of Medicine). When we aligned Lys45 with homologues from three other organisms, 18 amino acid residues were identical. On the other hand, 28 amino acids were found identical when Lys60 was aligned (Fig. 4). Residues 345-488 of Lys60 showed high homology with amino acids of CLN2. Among identical amino acids in both comparisons, glycine and proline were the most prevalent. In these alignments we found that a signal motif for a putative serine active center, GTSXXXXFXG, for serine protease (Siezen et al., 1991) was all conserved. Lys45 and Lys60 had the motif at ^{295}GTSASSPIFAG^{305} and ^{422}GTSPSATIFAG^{432}, respectively. It was noted that the motif for Lys45 was located at a different place

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1 GCACGAGCCAAGCCTGTCACTGGTAATTCCTCGGTTGGTGTTATCGAATTTGAAGATCAGAATTTTGCTCCAAGTGATCTCTCAGACTTT
  A R A K P V T G N S S V G V I E F E D Q N F A P S D L S D F 30

91 GCAACTTCTTTCTCAGTTCCCATTACACCTTTGACCGACAACCATATTATTGGATCGAATGATCCAACTAGTCCTCAGATAGAGGCAACT
  A T S F S V P I T P L T D N H I I G S N D P T S P Q I E A T 60

181 TTGGATATTCAGTACATTTTGGGTGTTCTCTTGGTACAACTGGATGGTTCTGGCTTGAAGGTGATAGTGTATCGGTTGTACGGCTTTCAT
  L D I Q Y I L G V L L V Q L D G S G L K V I V Y R L Y G F H 90

271 CATCTTTTTGCAACCAAAGACGTACCTCTCGTCAATTCTATTTCATATGGATGGAATGAGGAAGACCAATGTGAGAATGGAATTGGTGGA
  H L F A T K D V P L V N S I S Y G W N E E D Q C E N G I G G 120

361 GCTGAATGCCAACAGTTAGGTGTCACCTCAGCACAATACGTGGCAAGAGTAAACACAGAATTTCAGAAGATTGGCCTTCGTGGTATTACT
  A E C Q Q L G V T S A Q Y V A R V <N T E F Q K> I G L R G I T 150

451 CTCTTCGCTGCTTCTGGTGATTCTGGTGCCAATGGACGTACCGATCCAGATTGCTCTGAAAGCAATCTCAACCCAGCATATCCTGCCGCT
  L F A A S G D S G A N G R T D P D C S E S N L N P A Y P A A 180

541 TCTCCCTACATCACCTCTGTAGGAGCCACACAGATCTCTCAATCATCTGGTGTTGCCAAACTTCCTAATCCTCCTCCTGGTTGTGCAGGA
  S P Y I T S V G A T Q I S Q S S G V A K L P N P P P G C A G 210

631 GCAATCTTGTGCATCGGACTGGGACTGAGGAAGCGGTCAGCTATGATCAATCCAATTTTGCATCAGGTGGAGGATTTTCTGTGGTTGCCT
  A I L C I G L G L R K R S A M I N P I L H Q V E D F L W L P 240

721 CAGCACTGCCTTTCAGAAGGCAGCCATTGCAGCATACTTCAAGTCAGGTGTAACCTTGCCTCCAAGCTCTTATTACAATGCTGCTGCAAG
  Q H C L S E G S H C S I L Q V R C N L A S K L L L Q C C C K 270

811 AGAATTTCCGATGTCTCGGCCTTGGGTTCTGCCATTCTGATTGAAACAGGAGGAAATATTCAAACTGTAGGTGGAACCTCTGCTTCCTCT
  R I S D V S A L G S A I L I E T G G N I Q T V G G T S A S S 300

901 CCCATTTTTGCTGGTGTTGTTGGTCTGTTGAATGACTACGTAAACAGCAAGACTGGTAAACCTCTTGGTTTTGTTTCTCCTCTCTTGTAC
  P I F A G V V G L L N D Y V N S K T G K P L G F V S P L L Y 330

991 AAAATGGCTGCTGAAAGACCTGCTGCCTTCTTTGATGTTATCAAAGGGGACAATATTTGTACCGAGGATGGCTGTTCATCGGGATGCCAG
  K M A A E R P A A F F D V I K G D N I C T E D G C S S G C Q 360

1081 GCTTTTATGCTACAAAAGGATGGGACCCTGTCACTGGTCTTGGTACCCCTGTTTATCCTGAAATGTTGGCTTATGTTCAATCTTTGTTCT
  A F M L Q K D G T L S L V L V P L F I L K C W L M F N L C S 390

1171 AAGGCTAATAGTCCCAATCAAATCACTTATGAGGGAATTATTCACGAAATTGCTTGCTTCATGAAAGGAAATAAAGATAAGAAATTGAAA
  K A N S P N Q I T Y E G I I H E I A C F M K G N <K D K K L K> 420

1261 TAACAAAAAAAAAA
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Fig. 2. Nucleotide and deduced amino acid sequences of the cloned 1.3 kb cDNA for Lys45 protein (GenBank Acc. No. AF142414). Underlined nucleotide sequences indicate the polyadenylated tail. ^{9}NSS are consensus amino acids for N-glycosylation. ^{195}S is the potential O-glycosylation site. Amino acids shown in bold characters within < > are predicted potential antigenic determinants. Underlined amino acids are the motif predicted as a putative active center for serine protease (cf. Fig. 4).

from those for homologues (data not shown).

Discussion

Under the immunofluorescence microscope, LCA45 mAb stained the granular content of vesicles of 2-5 μm in diameter in *A. proteus* (Yoo et al., 1996). In immunoblots of SDS-PAGE of purified lysosomal fractions and total proteins of *A. proteus*, the mAb reacted with both 60 and 45-kDa proteins. The stain intensity and thickness of the 45-kDa band were much greater than those of the 60-kDa band (Fig. 1, lane 1). A similar result was obtained with mAbs from hybridoma cells that had been cloned twice by limiting dilution. This is not unusual for mAbs against lysosomal proteins. For example, CJL53 mAb produced against the lysosomal membrane also reacted with proteins of two different molecular masses, 150 kDa and 100 kDa (Choi et al., 1992). This could be due to a common antigenic domain shared by two proteins. Another possibility is that the smaller protein is a mature end product of the larger protein, known to be characteristic of lysosomal proteins that undergo post-translational maturation within lysosomes or phagolysosomes. Most lysosomal hydrolytic enzymes are synthesized as precursors and are processed to mature enzymes by proteolytic cleavage (Galjart et al., 1990; Wisselaar et al., 1993; Gelb et al., 1996).

The two cDNAs cloned in this study encoded proteins homologous to PICPs. PICP enzymes do not show high homology in amino acid sequences. CLN2 and PPICP by pair-wise comparison showed identity

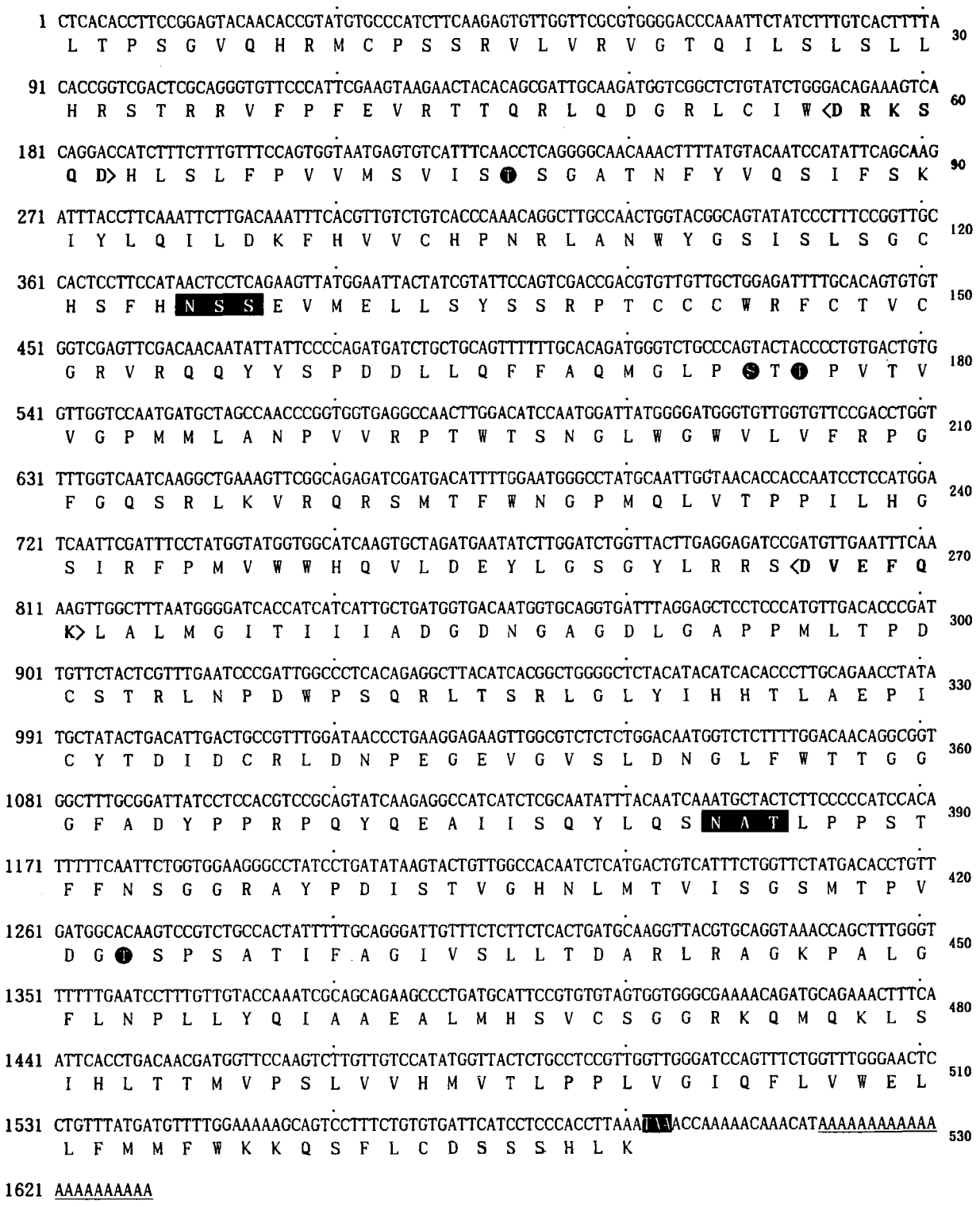

Fig. 3. Nucleotide and deduced amino acid sequences of the cloned 1.6 kb cDNA for Lys60 protein (GenBank Acc. No. AF142415). Underlined nucleotide sequences are the polyadenylated tail. ^{125}NSS and ^{383}NAT are consensus amino acid sequences for N-glycosylation. S and T amino acids in circles are potential O-glycosylation sites. Amino acids shown in bold characters within < > are predicted potential antigenic determinants.

and similarity scores of 25 and 46%, respectively (Sleat et al., 1997). The Lys45 protein had a 24.3% identity and 43.5% similarity with V4-7 vegetative-stage-specific protein of *Dictyostelium discoideum*, and 17.4% identity

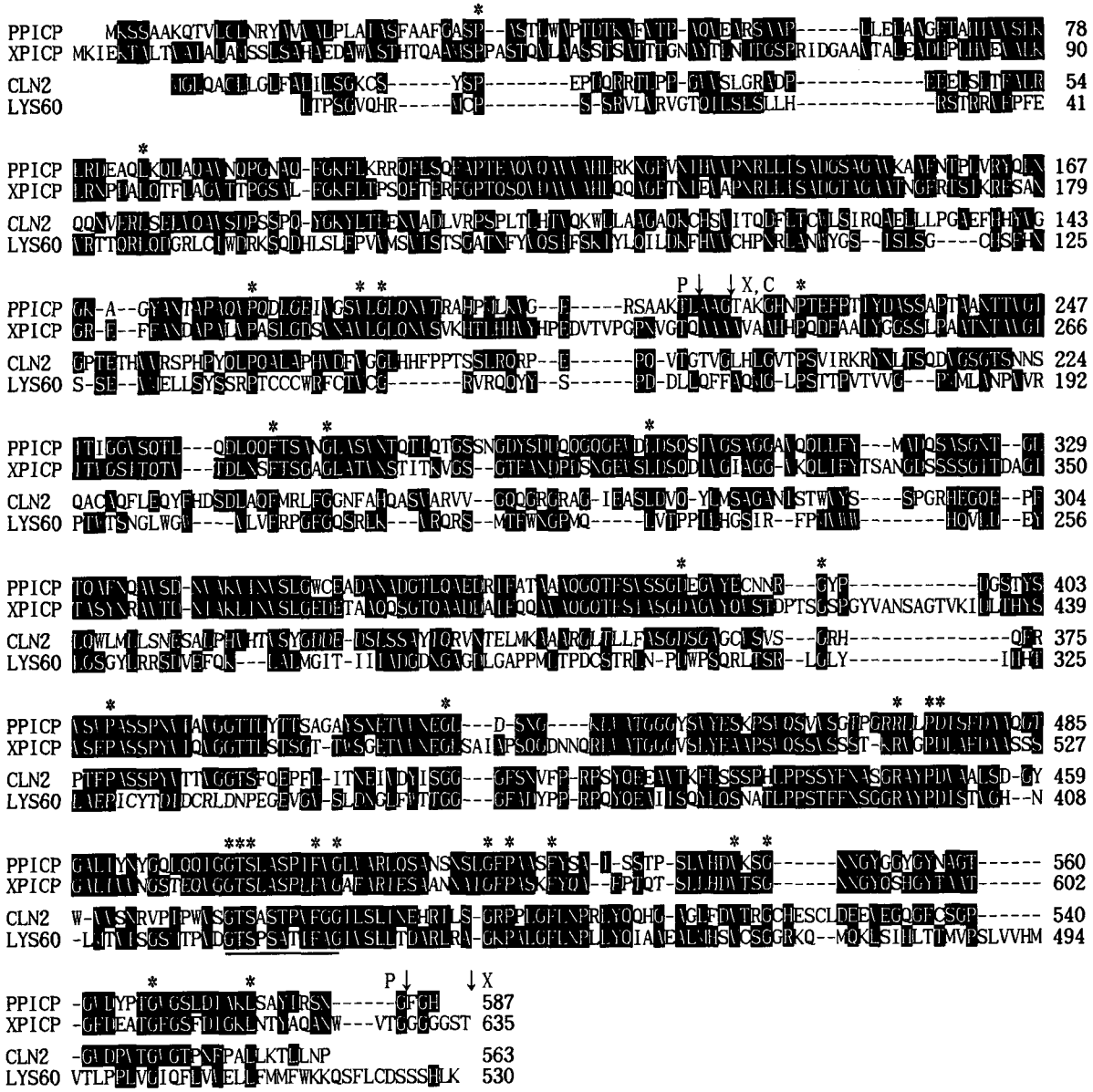

Fig. 4. Comparisons of deduced amino acid sequences of Lys60 with pepstatin-insensitive carboxyl proteinase of *Pseudomonas* sp. 101 (PPICP; GenBank Acc. No. A55368), *Xanthomonas* sp. T-22 (XPICP; Oda et al., 1996) and human (CLN2; Sleat et al., 1997). Amino acids in reverse characters indicate more than two identical or similar amino acids among the four amino acids aligned. Asterisks indicate amino acids that are all identical. Peptide cleavage sites during the maturation of CLN2, XPICP, and PPICP are indicated by arrows with C, X, and P, respectively. A consensus motif for the putative serine active center for serine protease is underlined.

and 40.9% similarity with PPICP. However, biochemical characteristics of the V4-7 protein are not known. On the other hand, the Lys60 protein showed the highest 24.3% identity and 51.9% similarity with human lysosomal PICP (Sleat et al., 1997). The Lys60 and Lys45 proteins showed a 23.6% identity and 46.4% similarity. Thus, Lys60 and Lys45 are closer to the eukaryotic lysosomal PICP than to prokaryotic homologues. Enzyme characteristics of PICPs were studied in several organisms (Oda and Murao, 1991; Prescott et al., 1995), but only three PICP genes have been characterized, one in human and two in prokaryotes.

All PICPs are known to be synthesized as precursors and processed to mature enzymes by proteolytic cleavage in acidic compartments (Oda et al., 1994; Oda et al., 1996; Sleat et al., 1997). The transcript of the CLN2 gene whose mutation is associated with classical late-infantile neuronal ceroid lipofuscinosis contained an open reading frame encoding a 563-residue protein (Sleat et al., 1997). On the other hand, the observed molecular mass of the protein was 46 kDa. The sequence determined by Edman degradation starts from residue 195, indicating that the CLN2 precursor contains a long pro-region (Sleat et al., 1997).

However, post-translational modification of the precursor to the mature enzyme is not known. The PPICP that was the first example among the PICP characterized contained 370 amino acid residues (40 kDa) with one methionine and one disulfide bond when the amino acid sequence was determined by chemical procedures (Hayashi et al., 1995). *E. coli* carrying a plasmid containing the cloned PPICP gene produced a 62-kDa protein. The 62-kDa protein was then processed and secreted into the periplasm of *E. coli* as a 43-kDa protein, which was converted to a 40-kDa PPICP autocatalytically under acidic conditions (Oda et al., 1994). The PICP homologue of *Xanthomonas* was also processed from a precursor (84 kDa) to an active form (42-kDa) under an acidic condition (Oda et al., 1996).

E. coli transformed with pBSK-lys45 produced two differentially stained immunopositive proteins. Between the two products a 45-kDa protein had a molecular mass equivalent to that of the protein encoded by the gene. Thus the less stained 43-kDa band appeared to be the partially processed product from the 45-kDa protein. We also detected multiple immunopositive proteins produced in *E. coli* transformed with pBSK-lys60. Among them a 64-kDa protein was close in molecular mass to the one expected from the sequence analysis. The other protein bands reacting with the mAb appeared to be partially processed products of the 64-kDa protein in *E. coli*. Considering the molecular masses and processing of homologous proteins, the 64-kDa protein was thought to be the primary product from the cDNA in *E. coli*. The processing of human PICP was assumed in lysosomes (Sleat et al., 1997), but has not been tested in *E. coli* transformed with the gene. However, it was surprising that eukaryotic PICP homologues of amoebae were processed to low molecular forms in *E. coli*.

If Lys60 is processed as its human homologue (Fig. 4), it may have a 177-amino acid pro-region. A mature enzyme may contain 353 amino acids (40 kDa) and one potential site for an asparagine-linked core oligosaccharide. Since the observed masses of the protein in amoebae were 60 kDa and 45 kDa, carbohydrates may account for 4-5 kDa of this protein. The immunostained 45-kDa major protein band of lysosome fractions and total proteins of amoebae (Fig. 1) apparently contain both the processed end products of Lys60 and Lys45 proteins.

PICP is an unusual carboxy proteinase that is not inhibited by pepstatin, the classical inhibitor of pepsin, cathepsin D, and other aspartyl proteases (Oda and Murao, 1991). In pepstatin-sensitive carboxyl proteinases, sequences around two active sites with aspartyl residues are well conserved as D*TG, E*TG and D*TSG (Oda and Murao, 1991). The PICP enzyme was found as a unique carboxyl proteinase as these catalytic sequences are not conserved (Hayashi et al., 1995). In the study of substrate specificities, PPICP had preference for cleavage between hydrophobic and bulky amino acid residues and had a narrow substrate specificity compared with those of other carboxyl proteinase enzymes (Oda and Murao, 1991). While PICPs have been characterized in a few organisms, none of the consensus motifs are known. In the alignment of amino acid sequences of PICPs (Fig. 4), we found that all PICPs, including Lys60 and Lys45 of amoebae, had a putative serine active center, GTS*XXXTXFXG, for serine protease in common. Glycine was the most conserved amino acid as in subtilisin-like serine proteases (SLSP) (Siezen et al., 1991). SLSPs have three amino acid residues, ^{32}DTG, ^{64}HGT and GTS^{221}, which are noted as the essential catalytic residues and membrane anchor segments. However, ^{32}DTG and ^{64}HGT analogues were not found in PICPs.

Lysosomal proteins contain N-glycosylation sites (NX[ST]) and many O-glycosylation sites [ST]. The extracellular domain has numerous contiguous runs of serine, threonine, and proline, which could act as attachment sites for O-linked carbohydrates (Wilson et al., 1991). When we compared amino acid compositions of Lys60 and Lys45 with those of well characterized lysosomal enzymes and membrane proteins (lamp), Lys60 and Lys45 proteins had as many serine, threonine, and proline as did lamp (data not shown). The extracellular domain of the lamp family has a bipartite organization divided by an extended proline hinge (Viitala et al., 1988; Holness and Simmons, 1993). However, prolines of Lys45 and Lys60 were not clustered. Thus, Lys45 and Lys60 do not seem to belong to lamp.

This study was initiated to find a biochemical marker for a late lysosomal subpopulation in amoebae. Amoebae apparently have two different PICP enzymes, which could be unambiguous enzyme markers for late lysosomes. Further work is in progress to discover additional lysosomal markers thought to exist by other immunofluorescence microscopic studies of amoebae (Choi et al., 1992; Kim and Jeon, 1993), and such markers would help us characterize subpopulations of lysosomes.

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