FEBS Letters 349 (1994) 265–269

FEBS 14271

Immunological significances of invariant chain from the aspect of its structural homology with the cystatin family

Nobuhiko Katunuma[a,*], Hisao Kakegawa[a], Youichi Matsunaga[b], Toshiji Saibara[c]

[a]*Institute for Health Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770, Japan*
[b]*Third Department of Internal Medicine, School of Medicine, The University of Tokushima, Tokushima 770, Japan*
[c]*Department of First Internal Medicine, The Kochi Medical School, Kochi 783, Japan*

Received 2 June 1994

Abstract

The primary structure of p31 of invariant chain (Ii-chain) shows about 50% homology with those of the cystatin family which are endogenous cysteine protease inhibitors. The binding domains between Ii-chain and HLA-DR-7 were estimated from the structural homology between cystatin and Ii-chain and also between cathepsins and DR-7, respectively. The QL_{64-71} and GS_{76-88} of Ii-chain were estimated to be the binding domains with GG_{45-51} and VS_{57-63} of HLA-DR7, respectively. The purified human Ii-chain from spleen is capable of forming four molecular forms from monomer to tetramer by redox-potential dependent disulfide bond formation. The Ii-chain inhibits cathepsin L and H competitively as a dimer and the K_i value for cathepsin L was 4.1×10^{-8} M, but cathepsin B was not inhibited at all. The Ii-chain showed mainly a dimer (60 kDa) under the assay condition of cathepsins with cysteine and was not degraded by these cathepsins. The Ii-chain may play an important role in the regulation of antigenic peptide presentation to MHC class II.

Key words: Invariant chain; Cathepsin B; Antigen processing

1. Introduction

We have reported previously that lysosomal cathepsin B of macrophages plays an essential role in the processing of some exogenous antigens to present with MHC class II [1,2]. The immune responses to virus vaccines of hepatitis B and rabies as antigens were suppressed by specific inhibitors of cathepsin B (E-64 [3] and CA-074 [4]), anti-cathepsin B antibody [21] and specific substrate of cathepsin B [30]. Furthermore, one of the active sites of cathepsin B [5,6], $VN_{217-222}$ shares high homology with a part of the desetope, VN_{57-62} of the HLA-DR7 [7,8]. This evidence suggests that the fragments formed from antigens by selective proteolysis of cathepsin B are capable of binding commonly with the desetope of MHC class II, β-chain. We found that the amino acid sequence of the Ii-chain shares high homology with that of the cystatin family which are the endogenous inhibitors of cathepsins and the Ii-chain strongly inhibits the activities of cathepsins. We first purified the Ii-chain from human spleen and the properties and the molecular forms were studied. Herein we discuss a new aspect of immunological significance of Ii-chain in the regulation of antigenic peptide presentation to MHC class II.

2. Materials and methods

2.1. Materials

The substrates for cathepsins, Z-Arg-Arg-MCA, Z-Phe-Arg-MCA and Arg-MCA were purchased from Institute for Protein Research, Peptide Inst. Inc., Osaka. Cathepsin B and L were purified from rat liver by Katunuma's method [24,25]. Silver staining kit was purchased from Wako Pure Chemical Co., Japan. The mouse antibody against CD74 as a first antibody was purchased from The Binding Site Ltd., USA and anti-human CD74 (LN-2) as a first antibody was purchased from Seikagaku Co., Japan. Goat anti-mouse IgG Fab'-conjugated alkaline phosphatase was purchased from EY Laboratories Inc. and picoBlue Immunoscreening Kits from Stratagene, CA. Sephadex G-75, DEAE-Sepharose FF and Superdex 200HR 10/30 were purchased from Pharmacia and YM-10 membrane from Amicon, Japan and Jasco-HPLC system from Jasco Co., Japan were used.

2.2. Enzyme assay

Various cysteine protease activities were measxured with Z-Arg-Arg-MCA for cathepsin B, Z-Phe-Arg-MCA for cathepsin L plus B and Arg-MCA for cathepsin H at pH 5.5 by the method of Barrett and Kirschke [30]. Purified Ii-chains were briefly preincubated with cathepsin and the reactions were started by the addition of the relevant substrates. The fluorescence of 7-amino-4-methylcoumrin (MCA) liberated from substrates were monitored by a fluorescence spectrometer (Hitachi F-2000). Protein concentration in the reaction mixtures were determined by the BCA method (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as a standard.

2.3. Gel electrophoresis and western blotting analysis of Ii-chain

SDS-PAGE was carried out by the method of Laemmli [19] in a 15–25% gradient gel containing 0.1% SDS at room temperature. The Ii-chain (400 ng) was incubated with and without 500 μg of 2-mercaptoethanol for 10 min at room temperature or 100°C in the same buffer. The gel was stained with silver stain kit and the immunoblotting was developed using a mouse antibody against human CD74 as a first antibody and the anti-human CD74 (LN-2) was also used as a first antibody. The bound antibodies were detected by goat anti-mouse IgG Fab'-conjugated alkaline phosphatase and the alkaline phosphatase reaction was performed using picoBlue Immunoscreening Kits. The SDS-PAGE molecular weight markers (Bio-Rad, Richmond) were bovine serum albumin (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa) and soybean trypsin inhibitor (27.5 kDa).

2.4. Search for sequence homology between Ii-chain and cystatin family

Optimal pairwise alignments were calculated by use of computer implementation using GENETYX ver.7.0 (Software Development, Tokyo, Japan). The homological part of amino acid sequences between

*Corresponding author. Fax: (81) (886) 22-3217.

SSDI 0014-5793(94)00657-H

total sequences of cystatins and p31 of human Ii-chain were compared and the homology matching was calculated under the consideration of X-ray co-crystal structure of cystatin B and cathepsin B [27].

3. Results and discussion

3.1. Homology of amino acid sequences between Ii-chain and cystatin family and possible binding domains between Ii-chain and HLA-DR7

The amino acid sequence of human Ii-chain has been reported as 232 amino acid residues by gene cloning [9]. The human Ii-chain contains 2 mol of cysteines located near N-terminus, whereas the mouse and rat Ii-chains contain only 1 mol of cysteine and the sorting signal motif of EP_{28-31} for introduction to the endosome is also located near N-terminus [10]. The N-terminus domain of p31 in Ii-chain [16,17] shows more than 50% homology with that of the cystatin family [11–15] as shown in Fig. 1. The homology search of their sequence matching between cystatin family and Ii-chains was calculated by computer implementation under consideration of X-ray co-crystal structure of cystatin B with cathepsin B [27]. Although the Ii-chain contains sugar unlike the cystatin family, it may be considered to belong to the cystatin super family. The sequences of cystatin B, QL_{46-55} and GS_{76-83} which bind with the two active sites of cathepsin B [6] show the highest homology with two domains of Ii-chain, QL_{64-71} and GS_{76-83}, respectively. As mentioned previously, the two domains of active sites of cathepsin B, $GG_{193-215}$ and $VT_{217-223}$ show significant homology with those of the desetope of HLA-DR7 [16], GG_{45-51} and VS_{57-63}, respectively. Therefore, these two domains of Ii-chain, QL_{64-71} and GS_{76-88} are estimated to be the binding domains with those of the desetope of HLA-DR7, GG_{45-51} and VS_{57-63}, respectively. The stereostructures of cathepsin B-cystatin β-co-crystal [6,27] and crystal of MHC class II [18] by X-ray crystallography also support our hypothesis proposed on the binding domains between Ii-chain and HLA-DR7, as illustrated in Fig. 2. Furthermore, it was reported recently that the two complexes between MHC class II β-chain and a part of Ii-chain, that is, 96–122 domain of Ii-chain plus HLA-DR1 and 96–112 domain plus HLA-DR3, were isolated [28,29]. These domains of Ii-chain correspond to the binding domain with HLA-DR7, β-chain estimated.

3.2. Purification of human Ii-chain from spleen

We first purified the Ii-chain from human spleen, and the properties and the interconvertible molecular forms were studied. Homogenized human spleen in 20 mM Tris-HCl buffer, pH 7.5, was sonicated. The supernatant after centrifugation was treated with 0–70% of $(NH_4)_2SO_4$. The precipitate was applied to a column of Sephadex G-75. The fractions containing Ii-chain detected using immunoblotting analysis were concentrated

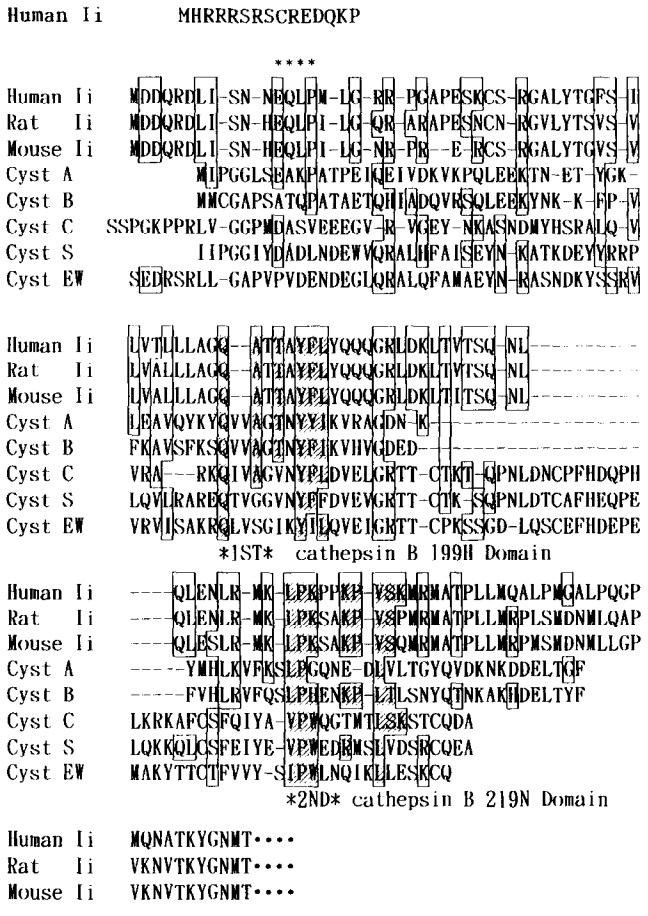

Fig. 1. Sequence homology between Ii-chain and the cystatin family. Sequence homology between total sequences of cystatins and p31 of human Ii-chain were compared. The entrenched symbols indicate homologous (or same) sequences between Ii-chain and the cystatin family. Two domains of cystatin B, QL_{45-55} and GS_{60-73}, to bind with active sites of histidine-199 area and asparagine-217 area which are substrate binding sites of cathepsin B show the highest homology with the corresponding two domains of Ii-chain, QL_{64-71} and GS_{76-83}, respectively, and these domains, illustrated by shadow lines, seem to be the binding domains with the HLA-DR7. ****EQLP shows the sorting signal sequence of Ii-chain into lysosome.

by YM-10 membrane. The Ii-chain fraction was then applied to a DEAE-Sepharose FF column and the non-absorbed eluents with the same buffer were collected. The eluent was then applied to a solumn of Superdex 200 HR 10/30 connected in series with a Jasco HPLC system equilibrated with the same buffer containing 0.2 M Na_2SO_4. Two protein peaks were eluted from the column and Ii-chain was eluted in the first peak corresponding to 120 kDa. Finally, the Ii-chain was purified by the repeated HPLC as a single peak. Then, the purified Ii-chain were subjected to SDS-PAGE (10–20% gradient gel containing 0.1% SDS) by the modified method of Laemmli [19]. The gels were stained with silver stain kit and the immunoblotting was developed using the antibody against Ii-chain mentioned in section 2. All positive bands with silver staining proteins also showed positive

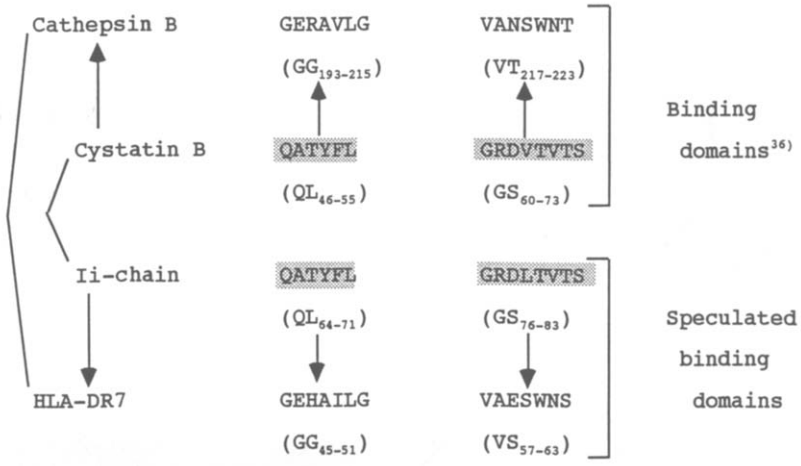

Fig. 2. Possible binding domains between Ii-chain and HLA-DR7. The two domains of $GG_{193-215}$ and $VT_{217-223}$ of cathepsin B that correspond to the second and the third active site of cathepsin B are exposed on the surface of the substrate binding crack by X-ray crystallographical data. It is well known that the two domains of cystatin B, QL_{46-55} and GS_{60-73}, which show the highest homology with the corresponding two domains of the Ii-chain, are binding domains with two active sites of cathepsin B. The domain of VS_{57-63} of HLA-DR7 is generally admitted to be desetope to bind with the antigenic peptide.

reactions with the immune staining of the Ii-chain antibody on the SDS-PAGE electrophoresis by Western blotting as shown in Fig. 3. The purified Ii-chain gave a single component of about 30 kDa mobility on the gel under strong reduced condition, while the subunit molecular weight of the Ii-chain was calculated to be 30 kDa from the amino acid sequences by molecular cloning.

3.3. *Interconvertibility of molecular forms of human Ii-chain by disulfide bond formation*

When the purified Ii-chain was placed on suitable oxide-redox conditions, the Ii-chain converted into four different molecular forms of 30, 60, 90 and 120 kDa, while the oxidized form showed only a 120 kDa tetramer, as shown in Fig. 3. Since the subunit molecular weight of the Ii-chain calculated from the primary structure is about 30 kDa, including carbohydrates, these bands correspond to the monomer, dimer, trimer and tetramer of the 30 kDa subunit, respectively. Since the human Ii-chain contains 2 mol of cysteine residue near the N-terminus, the Ii-chain can possibly form logically four kinds of molecular types from monomer to tetramer, mediated by disulfide bridges depending upon the redox potential. However, under the assay conditions of cathepsin activities in the presence of 10^{-3}–10^{-4} M of L-cysteine, the human Ii-chain showed mainly a dimer of 60 kDa as shown in Fig. 4. Therefore, the 60 kDa dimer form may be the most stable conformation under acidic and reduced conditions in physiological lysosome. While

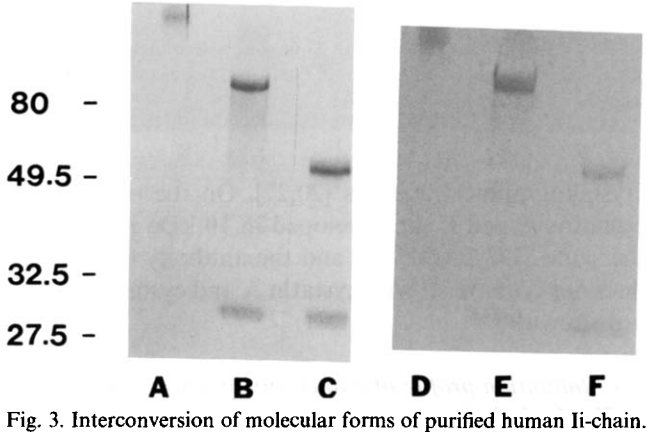

Fig. 3. Interconversion of molecular forms of purified human Ii-chain. The purified Ii-chains under various conditions were subjected to SDS-PAGE (10–20% gradient gel with 0.1% SDS). Lanes A, B and C are stained with silver; lane A = non-treatment; lane B = mercaptoethanol treatment at room temperature; lane C = mercaptoethanol treatment at 100°C. Lanes D, E and F are immunoblotting; lane D = non-treatment; lane E = mercaptoethanol treatment at room temperature; lane F = mercaptoethanol treatment at 100°C.

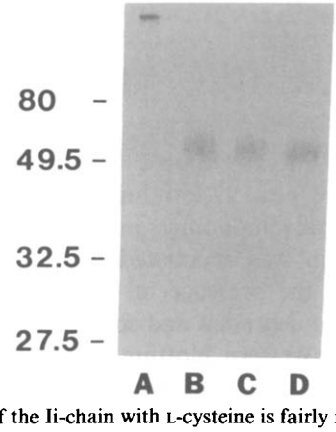

Fig. 4. Dimer of the Ii-chain with L-cysteine is fairly resistant to proteolysis by cathepsin B and cathepsin L. The Ii-chain (450 ng) was incubated with and without cathepsin B (94 ng) or L (74 ng) in the presence of 10^{-3} M L-cysteine for 30 min at 37°C in pH 5.5 acetate buffer containing 10^{-3} M EDTA·2Na. Lane A = non-treatment; lane B = L-cysteine treatment; lane C = L-cysteine and cathepsin B treatments; lane D = L-cysteine and cathepsin L treatments.

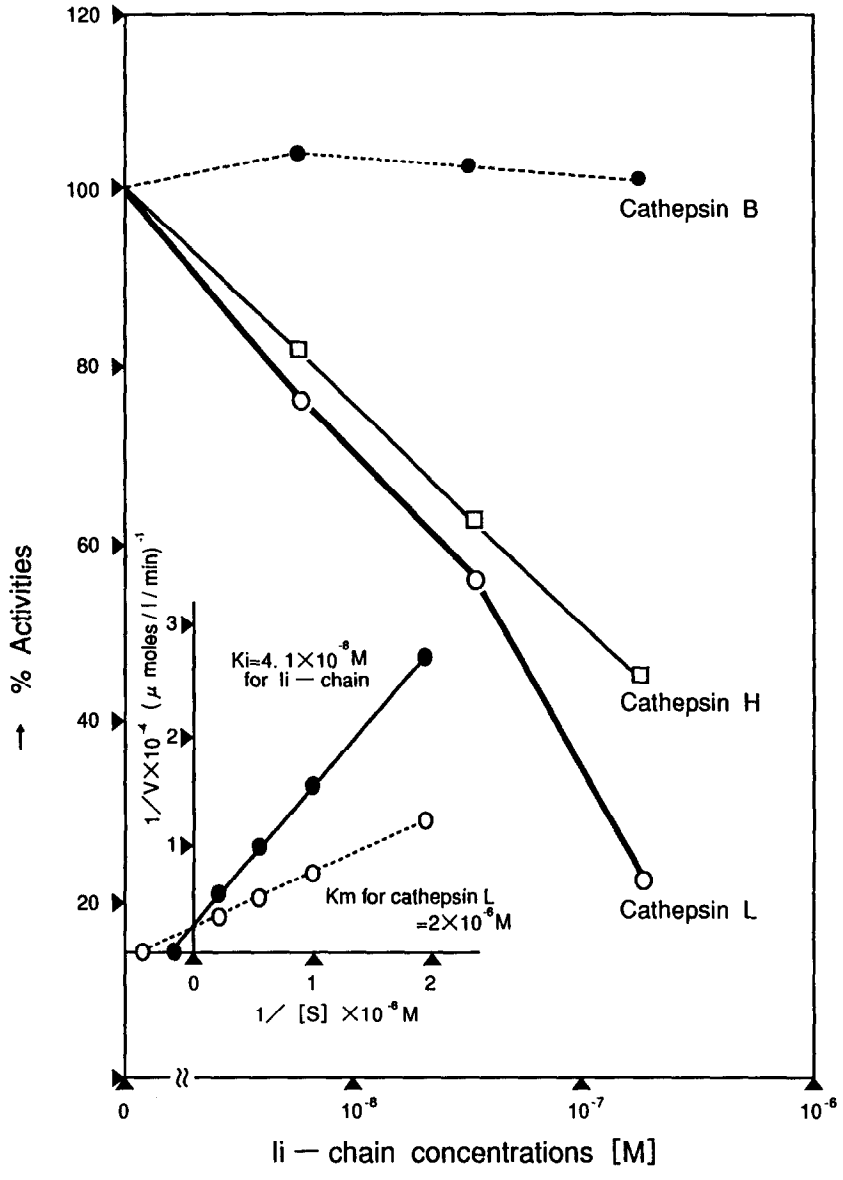

Fig. 5. Inhibition profile of cathepsins L, H and B by purified human Ii-chain. The reactions were carried out in 50 mM acetate buffer, pH 5.5, with 10^{-3} M of L-cysteine for 30 min and the released MCA was assayed by fluorometrically. The molar concentrations of the Ii-chain added were calculated as 60 kDa dimer form. The K_m for cathepsin L was 1.97×10^{-6} and the K_i value for Ii-chain was 4.1×10^{-8} M.

the Ii-chain of rat and mouse contain only 1 mol of cysteine residue near N-terminus, these Ii-chains can only form logically, monomers and/or dimers. When the purified Ii-chain was incubated with cathepsin B or cathepsin L in the presence of 10^{-3} M L-cysteine, the Ii-chain was not degraded and showed only the 60 kDa dimer form by Western blotting on the SDS-PAGE as shown in Fig. 4. It is also already well known that the cystatins are not hydrolyzed by cathepsins. Under intralysosomal conditions of acidic pH and reduced potential, the Ii-chain may choose the 60 kDa dimer, and also the native dimer form of Ii-chain is not hydrolyzed by cathepsin L and B. The binding domains between cystatin β and cathepsin B have been well clarified by X-ray crystallographical analysis [20,27]. On the other hand, cystatins A and C are developed in 10 kDa position on the same SDS-PAGE [21] and the antibody for Ii-chain does not cross-react with cystatin A and cystatin C (data not shown).

3.4. Inhibition profile of cathepsins by human Ii-chain

The Ii-chain is considered to be cystatin family from the aspect of their sequence homology, as mentioned above. The inhibition of cathepsin activities by the Ii-chain was then tested directly by purified human Ii-chain and various cathepsins in the presence of L-cysteine [25,26]. The Ii-chain of 3.8×10^{-7} M inhibited 75% of cathepsin L and 55% of H activities, while cathepsin B

activity was not inhibited at all, as shown in Fig. 5. The inhibition mode of cathepsin L by the Ii-chain shows typical competitive kinetics and the K_i value of the Ii-chain for cathepsin L was 4.1×10^{-8} M. It may considered, that the antigen processing by cathepsin B is not interfered with by released Ii-chain and also further degradation of processed antigenic peptides by cathepsin L and H may be protected by the released Ii-chain. The stereo-mechanisms of binding between cystatin and cathepsin B is well established by X-ray crystallographical analysis by our studies, and also it is well known that the cystatins are not cleaved by cathepsins [6,20–22]. These data also suggest that the Ii-chain is not cleaved by cathepsins judging from the aspect of their similar binding mechanism. Furthermore, the purified human Ii-chain was not degraded by cathepsin B and cathepsin L in the usual reaction conditions with L-cysteine as shown in Fig. 4. As we have reported previously, although the immune responses to high molecular weight antigens are inhibited by cathepsin B inhibitors, the immune responses against the processed antigenic peptide are not suppressed by cathepsin B inhibitors [1,2]. These accumulated findings suggest that the cathepsin B inhibitors do not interfere with any processes of immune-responses to the antigens by cathepsin B to expose the MHC class II surface as suggested in previous papers [23,24]. The redox potential and acidic pH in the endosome may play the most possible role for the release of the Ii-chain from MHC class II.

References

[1] Matsunaga, Y., Saibara, T., Kido, H. and Katunuma, N. (1993) FEBS Lett. 324, 325–330.

[2] Katunuma, N., Kakegawa, H., Matsunaga, Y., Nikawa, T. and Kominami, E. (1993) Agents and Actions Supplements (Chercnis, J.C. and Repine, J.E. eds.) Vol. 42, pp. 195–210, Bir-Hauser-Verlag, Basel.

[3] Hashida, S., Kominami, E. and Katunuma, N. (1982) J. Biochem. 91, 1373–1380.

[4] Towatari, T., Nikawa, T., Murata, M., Yokoo, C., Hanada, K., Tamai, M. and Katunuma, N. (1991) FEBS Lett. 280, 311–315.

[5] Takio, K., Towatari, T., Katunuma, N., Tetter, D. and Titani, K. (1993) Proc. Natl. Acad. Sci. USA 80, 3666–3670.

[6] Musil, D., Zucic, D., Turk, D., Engh, R.A., Mayr, I., Huber, R., Popovic, T., Turk, V., Towatari, T., Katunuma, N. and Bode, W. (1991) EMBO J. 10, 2321–2330.

[7] Marsh, S.G.B. and Bodmer, J.G. (1992) Immunogenetics 37, 79–94.

[8] Young, J.A.T., Wilkinson, D., Bodmer, W.F. and Trowsdale, J. (1987) Proc. Natl. Acad. Sci. USA 84, 4929–4933.

[9] Strubin, M., Mach, B. and Long, E.O. (1984) EMBO J. 3, 869–872.

[10] Bakke, O. ,and Dobberstein, B. (1990) Cell 63, 707–716.

[11] Machleidt, W., Borchart, U., Brzin, J., Ritonja, A. and Turk, V. (1983) Hoppe-Seyler's Z. Physiol. Chem. 364, 1481–1486.

[12] Ritonja, A., Machleidt, W. and Barrett, J., Biochem. Biophys. Res. Commun. 131, 1187–1192.

[13] Grubb, A. and Lofberg, H. (1982) Proc. Natl. Acad. Sci. USA 79, 3024–3027.

[14] Isemura, S., Saitoh, E., Ito, S., Isemura, M. and Sanada, K. (1984) J. Biochem. (Tokyo) 96, 1311–1314.

[15] Turk, V., Brzin, J., Longer, M., Ritonja, A. and Eropkin, M. (1983) Hoppe-Seyler's Z. Physiol. Chem. 364, 1487–1496.

[16] McKnight, A.J., Manson, D.W. and Barclay, A. (1989) Nucleic Acid Res. 17, 3983–3984.

[17] Singer, P.A., Lauer, W., Dembic, Z., Mayer, W.E., Lipp, J., Koch, N., Hammerling, G., Klein, J. and Dobberstein, B. (1984) EMBO J. 3, 873–877.

[18] Brown, J.H., Jardetzky, T.S., Gorga, J.C., Stern, L.J., Urban, R.G., Strominger, J.L. and Wiley, D.C. (1993) Nature 364, 33–39.

[19] Laemmli, U.K. (1970) Nature 227, 680.

[20] Bode, W., Musil, D., Zucic, D., Turk, D., Engh, R.A., Huber, R., Kos, J., Popovic, T., Towatari, T., Katunuma, N., and Turk, V. (1994) Biological Functions of Proteases and Inhibitors (Katunuma, N., Suzuki, K., Travis, J. and Fritz, H. Eds.) pp. 23–34, Japan Scientific Societies Press Tokyo and S. Karger, Basel.

[21] Katunuma, N. (1989) RBC Cell Biology Reviews (Knecht, E. and Grisolia, S. Eds.) vol. 20, pp. 35–61, Springer International, Berlin.

[22] Katunuma, N. and Kominami, E. (1985) Curr. Top. Cell. Regul. 27, 345–360.

[23] Teyton, L., Sullivan, D.O., Dickson, P.W., Lotteau, V., Sette, A., Fink, P. and Peterson, P.A. (1990) Nature 348, 39–44.

[24] Roche, P.A. and Cresswell, P. (1991) Proc. Natl. Acad. Sci. USA 88, 3150–3154.

[25] Towatari, T. and Katunuma, N. (1988) FEBS Lett. 236, 57–61.

[26] Towatari, T. and Katunuma, N. (1978) Biochem. Biophys. Res. Commun. 83, 513–520.

[27] Stubbs, M.T., Laber, B., Bode, W., Huber, R., Jerala, R., Lenarcic, B. and Turk, V. (1990) EMBO J. 9, 1939–1947.

[28] Roman, M.C., Robert, G.U., William, S.L., Joan, C.G., Lawrence, J.S., Dario, A.A.V. and Jack, L.S. (1992) Nature 358, 764– 768.

[29] Janice, M.R., John, R.N., Michael, J.S., James, A.B. and Peter, C. (1992) Nature 360, 474–477.

[30] Barret, A.J. and Kirschke, H. (1981) Methods Enzymol. 80, 535–561.