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Inhibition by acidic phospholipids of protein degradation by ER-60 protease, a novel cysteine protease, of endoplasmic reticulum

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A protein (ER60) with sequence similarity to phosphoinositide-specific phospholipase C-α purified from rat liver endoplasmic reticulum (ER) degraded ER resident proteins and is really a protease [(1992) J. Biol. Chem. 265, 15152–15159]. Therefore, ER60 is called ER-60 protease. We now show that negatively charged phospholipids, phosphatidylinositol, phosphatidylinositol 4,5-bisphosphate and phosphatidylserine inhibit ER protein degradation by ER-60 protease. Phosphatidylcholine and phosphatidylethanolamine show no effect on the activity of ER-60 protease. With the use of protease inhibitors, ER-60 protease is shown to be a novel cysteine protease distinct from those of the cytosol and lysosomes.

Endoplasmic reticulum; Cysteine protease; Phospholipid; ER-60 protease; Rat

1. INTRODUCTION

A number of proteins synthesized on the rough endoplasmic reticulum (ER) is properly folded and assembled into oligomers in the lumen of ER, and then sorted to organelles. However, proteins with improper structures are known to be degraded in a pre-Golgi compartment [1]. Unassembled or partially assembled complexes of the T cell antigen receptor (TCR), which is composed of at least seven subunits, are selectively degraded in ER after their de novo synthesis [2–4]. Other unassembled subunits of oligomeric proteins [5,6] and abnormal proteins [7,8] were also observed to be degraded. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase which is an ER resident protein is also degraded in a pre-Golgi compartment through a metabolically controlled pathway [9].

Such ER protein degradation has been thought to be catalyzed by cysteine protease(s), since it is inhibited by cysteine protease inhibitors and sulfhydryl reagents [10–12]. Recently, we have purified phosphoinositide-specific phospholipase C-α family (ER60), one of the ER resident proteins [13], which degrades other ER resident proteins such as protein disulfide-isomerase and calreticulin. Therefore, we call ER60 ER-60 protease. In this paper, we report that ER protein degradation by ER-60 protease may be controlled by acidic phospholipids including phosphoinositides.

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Abbreviations: ER, endoplasmic reticulum; TCR, T cell antigen receptor; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; ALLN, *N*-acetyl-leucyl-leucyl-norleucinal; ALLM, *N*-acetyl-leucyl-leucyl-methioninal; TLCK, *N*-tosyl-L-lysyl chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanyl chloromethyl ketone; bis-Tris, bis(2-hydroxyethyl)iminotris (hydroxymethyl) methane; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGTA, ethyleneglycol bis(2-aminoethylether)tetraacetic acid.

2. EXPERIMENTAL

2.1. *Materials*

Dilinoleoyl phosphatidylcholine (PC), dilinoleoyl phosphatidylethanolamine (PE) and bovine liver phosphatidylinositol (PI) were purchased from Avanti Polar-Lipids Inc., Alabama. Phosphatidylinositol 4,5-bisphosphate (PIP_2) and bovine brain phosphatidylserine (PS) were purchased from Sigma. *N*-Acetyl-leucyl-leucyl-norleucinal (ALLN) and *N*-acetyl-leucyl-leucyl-methioninal (ALLM) were from Boehringer Mannheim. *N*-Tosyl-L-lysyl chloromethyl ketone (TLCK) was from Aldrich Chem. Co. *N*-Tosyl-L-phenylalanyl chloromethyl ketone (TPCK) was obtained from Nacalai Tesque Inc., Kyoto, Japan. Leupeptin and E-64 were purchased from Peptide Institute, Inc., Osaka, Japan. Bovine liver protein disulfide-isomerase was obtained from Takara Shuzo Co. Ltd., Kyoto, Japan.

2.2. *Protein preparations*

Male Sprague–Dawley rats (8 weeks old) were used to prepare the rough ER fraction by differential centrifugation, as described previously [13]. ER-60 protease and calreticulin were purified from the rough ER fraction through three sequential chromatographies, on DEAE Toyopearl 650, AF-heparin Toyopearl 650M and TSK gel G3000SW, as described previously [13].

2.3. *Assaying of proteolytic degradation*

The purified ER-60 protease which is composed of eight sub-species [13] was dialyzed overnight at 4°C against 10 mM bis-Tris/HCl, pH 7.0. Bovine protein disulfide-isomerase (2 μg of protein) or rat calreticulin (2 μg of protein) and ER-60 protease (1 μg of protein) were incubated for the indicated times at 37°C in 40 mM bis-Tris/HCl, pH

6.3, supplemented with 0.1 M β-mercaptoethanol in the presence or absence of an inhibitor or phospholipids, in a final volume of 9 μl. Phospholipids were sonicated for 3 min in 100 mM bis-Tris/HCl, pH 6.3, and then added to the reaction mixture. The reaction products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. *Miscellaneous*

Protein concentrations were determined using a protein assay kit from Bio-Rad, with γ-globulin as a standard.

SDS-PAGE was performed according to the method of Laemmli [14] using 12.5% acrylamide under reducing conditions. The proteins were stained with Coomassie brilliant blue R-250.

3. RESULTS AND DISCUSSION

ER-60 protease has properties like those of other neutral cysteine protease [13] and is composed of an amino acid sequence similar to that of phosphoinositide-specific phospholipase C-α of rat basophilic leukaemia cells [15]. In addition, we observed that the amino acid sequence from Asn^{438} to Pro^{487} near the carboxy- terminus exhibits 56% similarity to the amino acid sequence from Glu^{27} to Ala^{75} of human lipoprotein A-2 [16] (Fig. 1). Lipoprotein A-2, one of the high density lipoproteins, forms an amphipathic α-helical structure and associates with phospholipids [17]. With this similarity we considered that, phospholipids might be expected to interact with ER-60 protease and influence the proteolytic activity. Among the phospholipids examined, PI, PIP_2 and PS inhibited the degradation of protein disulfide-isomerase and calreticulin by ER-60 protease partially at 50 μM (Fig. 2A and B, lanes 9, 12 and 15) and completely above 200 μM (lanes 10, 11, 13, 14, 16 and 17), under the conditions that protein disulfide-isomerase and calreticulin were cleaved to produce new peptide fragments after 3-h and 10-min incubation (Fig. 2A and B, lanes 3 and 4). The inhibition by acidic phospholipids appeared not to be caused by trapping Ca^{2+} by these phospholipids, since ER60 protease was not inhibited by EGTA (Fig. 4A and B, lane 11). Neither PC nor PE affected the degradation (Fig. 2A and B, lanes 5, 6, 7 and 8). However, ER-60 protease, protein disulfide isomerase or calreticulin itself was not autocatalytically degraded even after 3 h (Fig. 2A and B, lanes 1 and 2). After incubation of ER-60 protease with PE, the band of ER-60 protease on the SDS-PAGE plate looked smeared. This suggests appreciable interaction between PE and ER-60 protease. PI in liposomes comprising PC and PE (2:1) inhibited the degradation of protein disulfide-isomerase by ER-60 protease in a concentration- dependent fashion (Fig. 3). PI and PS comprise about 13% and 5% of the total phospholipids of ER [18]. PI is distributed symmetrically between the two halves of ER membranes [19]. Possibly, ER-60 protease binds to the cisternal side of the ER through its interaction with phospholipids, similar to lipoprotein A-2. Hence, it may be that most of its proteolytic activity in situ is inhibited by acidic phospholipids and thus remains latent. There is no direct evidence of the association of ER-60 protease with the inner half layer of ER-membranes. Localization of ER-60 protease using immunogold particles indicates that almost all of the gold particles are localized in the ER lumen, but some gold particles were observed on the ER membranes by immunoelectron microscopy [13].

As to the protein degradation systems in animal cells, lysosomal and cytosolic proteases are well characterized [20], and we have characterized ER-60 protease as a cysteine protease by using protease inhibitors [13]. When the reaction mixtures were treated under the conditions (37°C, 3 h) that protein disulfide-isomerase and calreticulin were well degraded in the absence of inhibitors (Fig. 4A and B, lane 2), the degradation of both proteins by ER-60 protease was inhibited by ALLN, ALLM, leupeptin and E-64 (lanes 3, 4, 7 and 8), which are known to inhibit cathepsins B, H and L, and calpains, all cysteine proteases. Neither TLCK nor TPCK inhibited ER-60 protease (Fig. 4A and B, lanes 5 and 6) indicating that it is not a cathepsin-type protease since TPCK and TLCK inhibit cathepsins B and L [21,22]. In addition, EGTA, which inhibits the proteolytic activity of calpains [23], did not inhibit the ER-60 protease (Fig. 4A and B, lane 11). *o*-Phenanthroline, which is a metallo protease inhibitor, did not inhibit the degradation (data not shown). The degradation of protein disulfide-isomerase and calreticulin by ER-60 protease optimally occurred at pH 6.3, and did not proceed below pH 5 or above pH 7.3 (data not shown). This pH–activity relationship is different from those of cathepsins B and L, which are more acidic [20,22–24], and that of calpains, which is more alkaline [25]. In addition, the amino acid sequence of ER-60 protease is not homologous to those of any known proteases, including cellular cysteine proteases. These results may indicate that ER-60 protease is a new type of cysteine protease which is localized in ER and may be regulated by acidic phospholipids including phosphoinositides.

It has been reported that ER protein degradation is catalyzed by cysteine protease(s) [10–12]. The degradation of TCR-α and HMG-CoA reductase is inhibited by ALLN. However, TPCK inhibited the degradation of

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PIPLC-α     438-487  NDVPSPYIVKGFPTIYFSPANKKLTPKKYEGGRELNDFISYLQREATNPP
                     . : :: :. . .  ::  .  :::   .: :: .:.::   .:.:.
apo A-2      27-75   EKVKSP-ELQAQAKSYFEKSKEQLTPLIKKAGTELVNFLSYFVELGTQPA
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Fig. 1. Amino acid sequence alignment of phosphoinositide-specific phospholipase C-α (PIPLC-α) with human lipoprotein A-2 (apo A-2). The region of similarity between residues 438–487 of PIPLC-α [15] and residues 27–75 of apo A-2 [16] is shown. Double dots indicate identical amino acids. Single dots indicate amino acids with similar physicochemical properties.

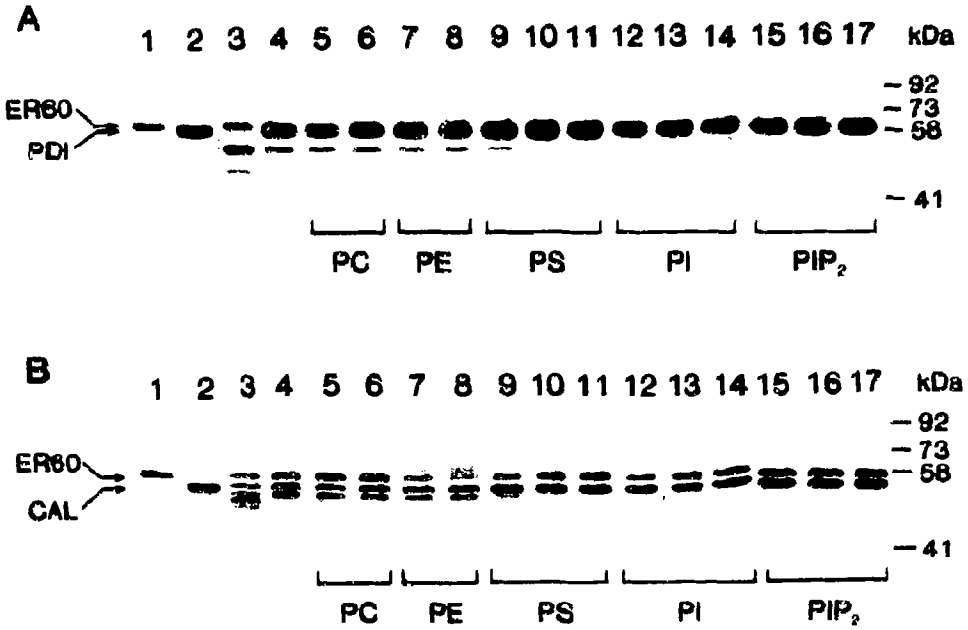

Fig. 2. Effects of phospholipids on the proteolytic activity of ER-60 protease. Protein disulfide-isomerase (PDI) (A) and calreticulin (CAL) (B) were incubated without (lane 2) or with ER-60 protease (lanes 3–17) in the absence (lanes 3 and 4) or presence of 200 μM PC (lane 5), 500 μM PC (lane 6), 200 μM PE (lane 7), 500 μM PE (lane 8), 50 μM PS (lane 9), 200 μM PS (lane 10), 500 μM PS (lane 11), 50 μM PI (lane 12), 200 μM PI (lane 13), 500 μM PI (lane 14), 50 μM PIP_2 (lane 15), 200 μM PIP_2 (lane 16) or 500 μM PIP_2 (lane 17) at 37°C for 10 min (A) or 20 min (B) except that the reactions proceeded for 3 h (lanes 2 and 3), as described under Experimental. Then the samples were subjected to SDS-PAGE. Lane 1 contained ER-60 protease (1 μg of protein) incubated at 37°C for 3 h.

TCR-α but not that of HMG-CoA reductase [26]. This suggests that more than one type of cysteine protease may participate in ER protein degradation. It has been shown that incorporation of basic or acidic amino acid residues into the central positions of transmembrane segments of proteins causes ER proteins to be degraded more rapidly [27,28]. It is interesting that the cleavage sites of the proteins attacked by ER-60 protease include a basic amino acid [13].

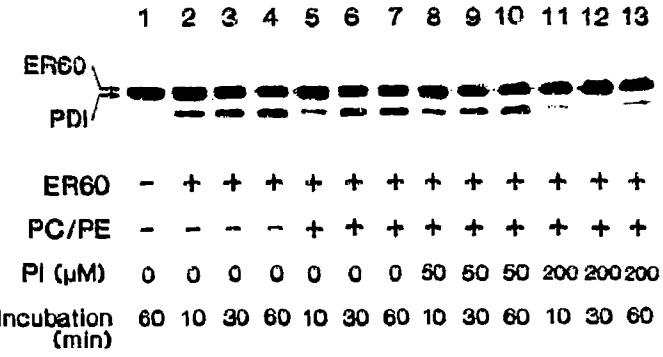

Fig. 3. Inhibition of the proteolytic activity of ER-60 protease by PI in PC/PE liposomes. Protein disulfide-isomerase (PDI) was incubated without (lane 1) or with ER-60 protease (lanes 2–13) in the absence (lanes 2–4) or presence of 300 μM PC and 150 μM PE (lanes 5–7), 300 μM PC, 150 μM PE and 50 μM PI (lanes 8–10), or 300 μM PC, 150 μM PE and 200 μM PI (lanes 11–13) at 37°C for 10 min (lanes 2, 5, 8 and 11), 30 min (lanes 3, 6, 9 and 12) or 60 min (lanes 1, 4, 7, 10 and 13), as described under Experimental. Then the samples were subjected to SDS-PAGE.

At present, there is little evidence to support the connection between PI metabolism and proteolysis in ER. It is necessary to study phospholipid metabolism link-

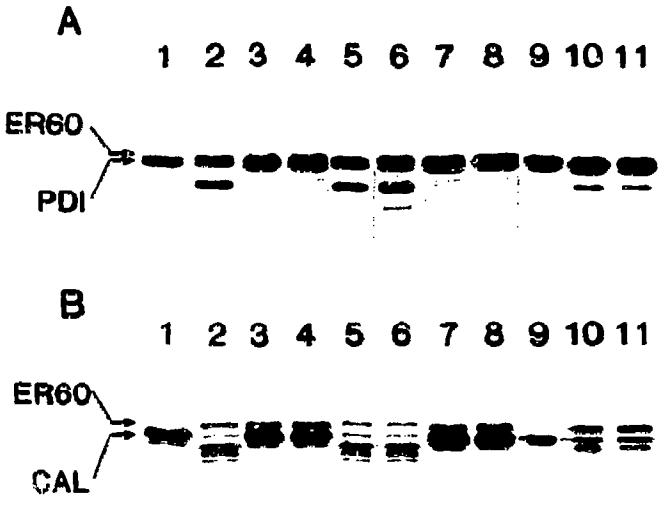

Fig. 4. Effects of protease inhibitors on the proteolytic activity of ER-60 protease. Protein disulfide-isomerase (PDI) (A) or calreticulin (CAL) (B) was incubated without (lanes 1 and 9) or with ER-60 protease (lanes 2–8, 10 and 11) in the absence (lanes 2 and 10) or presence of 10 μM ALLN (lane 3), 10 μM ALLM (lane 4), 10 μM TLCK (lane 5), 10 μM TPCK (lane 6), 10 μM leupeptin (lane 7), 10 μM E-64 (lane 8) or 1 mM EGTA (lane 11) at 37°C for 3 h (lanes 1–8), 10 min (A, lanes 9–11), or 20 min (B, lanes 9–11) as described under Experimental. Then the samples were subjected to SDS-PAGE.

ing to proteolysis in ER to understand the inhibition of ER-60 protease by acidic phospholipids.

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REFERENCES

[1] Klausner, R.D. and Sitia, R. (1990) Cell 62, 611–614.
[2] Lippincott-Schwartz, J., Bonifacino, J.S., Yuan, L.C. and Klausner, R.D. (1988) Cell 54, 209–220.
[3] Chen, C., Bonifacino, J.S., Yuan, L.C. and Klausner, R.D. (1988) J. Cell Biol. 107, 2149–2161.
[4] Bonifacino, J.S., Suzuki, C.K., Lippincott-Schwartz, J., Weissman, A.M. and Klausner, R.D. (1989) J. Cell Biol. 109, 73–83.
[5] Amara, J.F., Lederkremer, G. and Lodish, H.F. (1989) J. Cell Biol. 109, 3315–3324.
[6] Lederkremer, G.Z. and Lodish, H.F. (1991) J. Biol. Chem. 266, 1237–1244.
[7] Stoller, T.J. and Shields, D. (1989) J. Cell Biol. 108, 1647–1655.
[8] Le, A., Ferrell, G.A., Dishon, D.S., Le, Q.-Q.A. and Sifers, R.N. (1992) J. Biol. Chem. 267, 1072–1080.
[9] Goldstein, J.L. and Brown, M.S. (1990) Nature 343, 425–430.
[10] Inoue, S., Bar-Nun, S., Roitelman, J. and Simoni, R.D. (1991) J. Biol. Chem. 266, 13311–13317.
[11] Wileman, T., Kane, L.P. and Terhorst, C. (1991) Cell Regul. 2, 753–765.
[12] Stafford, F.J. and Bonifacino, J.S. (1991) J. Cell Biol. 115, 1225–123
[13] Urade, R., Nasu, M., Moriyama, T., Wada, K. and Kito, M. (1992) J. Biol. Chem. 267, 15152–15159.
[14] Laemmli, U.K. (1970) Nature 227, 680–685.
[15] Bennett, C.F., Balcarek, J.M., Varrichio, A. and Crooke, S.T. (1988) Nature 334, 268–270.
[16] Lux, S.E., John, K.M., Ronan, R. and Brewer Jr., H.B. (1972) J. Biol. Chem. 247, 7519–7527.
[17] Stoffel, W. (1983) in: Phospholipids and Atherosclerosis (Avogaro, P., Mancini, M., Ricci, G. and Paoletti, R. eds.) pp. 115–130, Raven Press, New York.
[18] Higgins, J.A. and Dawson, R.M.C. (1977) Biochim. Biophys. Acta 470, 342–356.
[19] Sundler, R., Sarcione, S.R., Alberts, W. and Vagelos, P.R. (1977) Proc. Natl. Acad. Sci. USA 74, 3350–3354.
[20] Bond, J.S. and Butler, P.E. (1987) Annu. Rev. Biochem. 56, 333–364.
[21] Towatari, T., Kawabata, Y. and Katunuma, N. (1979) Eur. J. Biochem. 102, 279–289.
[22] Towatari, T., Tanaka, K., Yoshikawa, D. and Katunuma, N. (1978) J. Biochem. (Tokyo) 84, 659–671.
[23] Pontremoli, S. and Melloni, E. (1986) Annu. Rev. Biochem. 55, 455–481.
[24] Barrett, A.J. and Kirschke, H. (1981) Methods Enzymol. 80, 535–561.
[25] Waxman, L. (1981) Methods Enzymol. 80, 664–680.
[26] Inoue, S. and Simoni, R.D. (1992) J. Biol. Chem. 267, 9080–9086.
[27] Bonifacino, J.S., Cosson, P. and Klausner, R.D. (1990) Cell 63, 503–513.
[28] Bonifacino, J.S., Cosson, P., Shah, N. and Klausner, R.D. (1991) EMBO J. 10, 2783–2793.