Apolipoprotein B Is Intracellularly Associated with an ER-60 Protease Homologue in HepG2 Cells

Khosrow Adeli‡§, Joseph Macri‡, Abbas Mohammadi‡, Makato Kito‡, Reiko Urade†, and Dora Cavollo‡

From the ‡Department of Chemistry and Biochemistry, University of Windsor, Windsor, Ontario, N9B 3P4 Canada and the §Research Institute for Food Science, Kyoto University, Uji, Kyoto 611, Japan

Two ALLN (N-acetyl-leucyl-leucyl-norleucinal)-sensitive endoplasmic reticulum (ER)-localized proteases (ER-60 and ER-72) were recently purified from rat liver. We used an antibody to rat ER-60 to investigate the possible role of this protease in apolipoprotein B (apoB) degradation. First, immunoprecipitation and immunoblotting experiments with the anti-rat ER-60 antibody suggested that HepG2 cells contain a homologue of ER-60 with an approximate molecular mass of 58–60 kDa. The ER-60 homologue was mostly associated with the luminal contents of HepG2 microsomes. Evidence from co-immunoprecipitation and cross-linking experiments appear to suggest that the ER-60 homologue in HepG2 cells is associated with apoB intracellularly. A small pool of apoB was recovered when HepG2 lysates were subjected to immunoprecipitation with anti-rat ER-60 antibody followed by a second immunoprecipitation with anti-apoB antibody. Furthermore, cross-linking of permeabilized cells with dithiobis(succinimidyl-propionate) further demonstrated association of apoB with the ER-60 homologue in HepG2 cells. Three polypeptides with molecular masses of 78, 66, and 50 kDa were consistently found to be associated with apoB as well as the 58-kDa ER-60 homologue. The 78-kDa protein associated with both apoB and ER-60 appeared to represent immunoglobulin heavy chain-binding protein (BiP) based on immunoprecipitation with a monoclonal antibody. Cross-linking and immunoblotting experiments suggested the association of the 78-kDa BiP with both the 58-kDa ER-60 homologue as well as the 550-kDa apoB.

In summary, the data suggests that HepG2 cells contain a 58-kDa protein which is homologous to the rat liver ER-60 in size, antigenecity, and intracellular localization. The ER-60 homologue in HepG2 cells appears to be closely associated with apoB, as well as other ER-associated proteases possibly representing ER chaperones such as BiP. We hypothesize that the ER-60 homologue may be involved in the degradation of apoB in the ER lumen of HepG2 cells.

Post-translational degradation of apoB has been shown to modulate the intracellular levels of newly-synthesized apoB molecules (1–19). Recent evidence suggest that apoB degradation may occur in the cytosol by the proteasome (19) as well as in the ER lumen by an unidentified ER protease(s) (17, 18). The identity of the ER-associated protease involved in apoB degradation has remained elusive, however, some characteristic features of this degradative system have recently been documented. The ER-associated protease appears to be responsible for fragmentation of apoB into a number of distinct degradation intermediates including an abundant 70-kDa fragment (12, 16). The activity of the ER-associated protease is also inhibitable by ALLN in a dose-dependent manner (12, 16). Intraluminal degradation of secretion-competent apoB associated with nascent HDL-like and LDL-like lipoprotein particles in the secretory pathway is also ALLN-sensitive and may be mediated by a putative ER-localized protease (17). Work by Ginsberg and co-workers (18) has recently shown that the luminal degradative process is also DTT sensitive.

The ER lumen contains a number of ER-resident proteins including molecular chaperones and proteases. Recently, two ER-associated proteases were purified to homogeneity and characterized (20, 21). One of these proteases, the ER-60 protease was first purified from the ER of rat liver, and was shown to be a cysteine protease (20, 22). ER-60 has 98% homology in amino acid sequence to rat phosphoinositide-specific phospholipase C (20). This protease has been implicated in degradation of human lysozyme (23). ER-60 was shown to be chemically cross-linked to misfolded mutant lysozyme. It was also found to degrade the reduced and denatured form of lysozyme, but not the native form in vitro (23).

In the present report, we demonstrate that HepG2 cells contain a homologue of rat liver ER-60, an ER-localized cysteine protease. Evidence from co-immunoprecipitation, chemical cross-linking, and a combination of immunoprecipitation and immunoblotting experiments suggest that apoB-100 is associated intracellularly with the ER-60 homologue in HepG2 cells. It is thus postulated that the ER-60 homologue may be involved in a degradative system affecting apoB in the ER. Such a protease may be responsible for the ER luminal protease activity fragmenting apoB into distinct degradation intermediates.

EXPERIMENTAL PROCEDURES

Materials—HepG2 cells (ATCC HB 8065) were obtained from American Type Culture Collection. Fetal bovine serum (certified grade) and cell culture media were obtained from Life Technologies (Toronto, Ontario, Canada). Monoclonal anti-BiP was obtained from StressGen (Victoria, Canada). Two ALLN (N-acetyl-leucyl-leucyl-norleucinal)-sensitive endoplasmic reticulum (ER)-localized proteases (ER-60 and ER-72) were recently purified from rat liver.

© 1997 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

This paper is available on line at http://www.jbc.org

22489
Permeabilized cells were incubated with DSP (1 mM) for 30 min on ice, were normally first permeabilized with digitonin as described above. They were then subjected to immunoprecipitation as described below.

Subcellular Fractionation and Isolation of Total Microsomes—Total microsomes were isolated from intact cells (4 × 10⁶-mm dishes) essentially as described (3, 5, 17, 25). In some experiments, total microsomes were extracted with sodium carbonate and fractionated by ultracentrifugation as described (3, 5, 17, 25) to isolate a luminal fraction and a membrane-enriched fraction. The membrane and luminal fractions were then subjected to immunoprecipitation as described below.

Cross-Linking of Cells with DSP—To cross-link HepG2 cells, the cells were normally first permeabilized with digitonin as described above. Permeabilized cells were incubated with DSP (1 mM) for 30 min on ice, and the cross-linking reaction was stopped by the addition of 2 mM glycine and incubation for 15 min on ice. Cross-linked cells were washed with phosphate-buffered saline, solubilized in solubilization buffer as above, and subjected to immunoprecipitation.

Immunoprecipitation and Immunoblotting—Immunoprecipitation was performed using Immunoprecipitin (Life Technologies) as described previously (12, 26). In procedures where monoclonal antibodies were employed, the first antibody was bound to Affi-Gel beads (Bio-Rad) then washed and used to immunoprecipitate the second antibody conjugated to peroxidase. Detection of peroxidase activity was carried out using an AEC Turbo substrate (Dimension Lab Inc., Mississauga, Canada).

SDS-PAGE and Fluorography—SDS-PAGE was performed essentially as described (27). Gels (16 × 12 cm) were used or 6% resolving, 10% resolving, or gradient gels (3–15%). The gels were fixed, stained, and were fluorographed by incubating in Amplify (Amersham). The gels were dried, and exposed to DuPont autoradiographic film at −80 °C for 1–4 days.

RESULTS

Detection of an ER-60 Protease Homologue in HepG2 Cells—The ER-60 protease has been previously detected in rat liver (20). We first attempted to detect the presence of an ER-60 homologue in HepG2 cells. Two approaches were used. First, intact HepG2 cells were pulsed for 1 h, chased briefly to label intracellular proteins, and then immunoprecipitated with a polyclonal antibody against purified rat ER-60. As shown in Fig. 1A (lanes 2 and 3), we consistently detected a protein with an approximate size of 58 kDa following immunoprecipitation. The 58-kDa band immunoprecipitated from HepG2 cells thus resembles rat ER-60 in both size and antigenecity. In a second approach, we solubilized HepG2 cells and separated the total cell extract by SDS-PAGE and immunoblotted with either non-immune rabbit serum or with rabbit anti-rat ER-60 antibody (Ab). Immunoprecipitates were analyzed by SDS-PAGE and fluorography. Lanes 3 and 4, immunoprecipitated with non-immune rabbit serum. Lanes 5 and 6, immunoprecipitation with rabbit anti-ER-60 antibody. In contrast, non-immune rabbit serum (lanes 1 and 2) did not immunoprecipitate a similarly sized band from HepG2 cells, suggesting the specificity of the immunoprecipitation with the anti-rat ER-60 antibody. The 58-kDa band immunoprecipitated from HepG2 cells thus resembles rat ER-60 in both size and antigenecity.

ApoB Is Associated Intracellularly with the ER-60 Protease
with non-immune rabbit serum (lane 2) or with goat anti-human apoB antibody (lane 3) in the ER-60 immunoprecipitate. A small amount of apoB was detected (Fig. 2A, lane 3) in the ER-60 immunoprecipitate. This further suggests that the ER-60 homologue in HepG2 cells is associated with a small pool of apoB.

Experiments were also performed to demonstrate the association of apoB with the ER-60 homologue in CHAPS-solubilized HepG2 cells. Solubilization in CHAPS appears to provide a less stringent condition allowing for a better detection of intracellular protein-protein interactions (28), and has been previously employed to study intracellular association of ER chaperones with secretory and membrane proteins. As shown in Fig. 2B, immunoprecipitation of apoB from CHAPS-solubilized HepG2 cells resulted in the detection of a number of additional proteins, which may represent proteins genuinely associated with apoB or nonspecifically immunoprecipitated. Probing CHAPS-solubilized HepG2 cells with the anti-rat ER-60 antibody again detected a 58-kDa protein, however, other bands were also detected in the ER-60 immunoprecipitate including a protein migrating at around 550 kDa. Thus, the data from CHAPS-solubilized HepG2 cells appears to support the notion that apoB and the ER-60 homologue may be intracellularly associated.

To confirm the observations in Fig. 2, A and B, we performed experiments in which 35S-labeled HepG2 lysates were first immunoprecipitated with either non-immune rabbit serum or non-immune goat serum. The immunoprecipitates were then dissociated and re-immunoprecipitated with either rabbit anti-rat ER-60 antibody or with goat anti-human apoB, respectively. As shown in Fig. 2C, no detectable bands were observed in either co-immunoprecipitation experiments, suggesting that the small amount of apoB detected in Fig. 2A, lane C, is not due to nonspecific co-immunoprecipitation. Further confirmation for the specific association of the ER-60 protease homologue and apoB came from the experiment in Fig. 2D. In this experiment, HepG2 lysates were first immunoprecipitated with either non-immune rabbit serum or with rabbit anti-rat ER-60 antibody. Both immunoprecipitates were then dissociated and re-immunoprecipitated with goat anti-human apoB antibody. The immunoprecipitates were then fractionated by SDS-PAGE and immunoblotted with goat anti-human apoB antibody. As shown in Fig. 2D, no detectable band was observed when the HepG2 lysates were first immunoprecipitated with non-immune rabbit serum (lane 1). However, immunoprecipitation with rabbit anti-rat ER-60 antibody (lane 2) clearly resulted in the detection of a 550-kDa protein when immunoblotted with goat anti-human apoB antibody. These observations thus elaborate our earlier observations suggesting the intracellular association of apoB and ER-60 protease homologue in HepG2 cells.

ApoB Can Be Cross-linked to the ER-60 Protease Homologue in Permeabilized HepG2 Cells—We also attempted to cross-link apoB and the ER-60 protease homologue using a commonly used chemical cross-linking agent, DSP. Preliminary experiments with DSP showed that prior permeabilization of HepG2 cells increased the efficiency of DSP-mediated cross-linking of proteins and enhanced the sensitivity of detection of apoB cross-linking to ER-60. We thus performed all our subsequent cross-linking experiments in permeabilized cells. Intact HepG2 cells were pulsed, briefly chased, and then permeabilized with digitonin. Permeabilized cells were then cross-linked with DSP, solubilized, and immunoprecipitated with antibodies.
against apoB and ER-60. The immunoprecipitates were treated with a high concentration of DTT to dissociate cross-linked proteins before analysis by SDS-PAGE and fluorography. Fig. 3A shows the apoB immunoprecipitates from control and DSP-cross-linked permeabilized HepG2 cells. As shown in Fig. 3A (lane 1) goat anti-human apoB antibody consistently recovered from non-cross-linked cells with the full-length apoB, as well as a number of nascent chains. Cross-linking of HepG2 cells resulted in the recovery of apoB, its nascent chains, as well as a few other proteins (Fig. 3A, lane 2). Two bands with approximate sizes of 58 and 78 kDa became more visible in the apoB immunoprecipitates when cells were cross-linked with DSP (observed in several independent experiments).

When probed with the anti-rat ER-60 antibody (Fig. 3A, lane 3), a single 58-kDa protein band was detected in control cells (as expected). A strong band was also visible with an approximate size of 300–400 kDa. This band has been previously observed when immunoprecipitation is performed with Immunoprecipitin. It is detected in some immunoprecipitation experiments (when a preclumping step with Immunoprecipitin is not performed) and is the result of nonspecific binding of the unknown protein with the Immunoprecipitin (the protein can be recovered by incubating the HepG2 lysate with Immunoprecipitin without any added antibody) (data not shown).

Four bands were recovered from cross-linked cells when immunoprecipitated with rabbit anti-rat ER-60 antibody (50, 58, 66, and 78 kDa) (Fig. 3A, lane 4). Among these four bands, the 58-kDa band appeared to represent the ER-60 homologue itself. The ER-60 homologue was consistently found to cross-link to the protein species at 50, 66, and 78 kDa in several experiments, two of which are shown in Fig. 3B. In some immunoprecipitates, a small amount of the 550-kDa apoB was also detected after cross-linking of permeabilized HepG2 cells and immunoprecipitation with the anti-ER-60 antibody. However, long exposures of the fluorograph were needed to visualize the apoB bands, suggesting the cross-linking of only a very small amount of apoB with ER-60 homologue.

To further confirm the association of apoB and ER-60, permeabilized and cross-linked HepG2 cells were solubilized and immunoprecipitated with the goat anti-human apoB antibody. The apoB immunoprecipitates were then analyzed by SDS-PAGE and fluorography. The apoB immunoprecipitates were treated with sample buffer containing 400 mM DTT to reduce the cross-links and then analyzed by SDS-PAGE and fluorography. B, HepG2 cells were pulsed, permeabilized, and cross-linked as in A, and immunoprecipitated with rabbit anti-rat ER-60 antibody. The immunoprecipitates were treated as in A and then analyzed by SDS-PAGE and fluorography. The figure shows the results using duplicate dishes with (lanes 2 and 4) or without (lanes 1 and 3) cross-linking with DSP. C, HepG2 cells were permeabilized and cross-linked as in A. Control and cross-linked cells were immunoprecipitated with goat anti-apoB antibody bound to Affi-Gel. The immunoprecipitates were treated as in A and then analyzed by SDS-PAGE and fluorography. The figure shows the results using duplicate dishes with (lanes 2 and 4) or without (lanes 1 and 3) cross-linking with DSP. D, HepG2 cells were permeabilized and cross-linked as in A. Control and cross-linked cells were immunoprecipitated with non-immune goat serum. The immunoprecipitates were treated with sample buffer to reduce the cross-links, and then analyzed by SDS-PAGE, followed by immunoblotting with rabbit anti-rat ER-60 antibody. Lane 1, immunoprecipitation of non-cross-linked cells. Lane 2, immunoprecipitation of cross-linked cells. D, HepG2 cells were permeabilized and cross-linked as in A. Control and cross-linked cells were immunoprecipitated with non-immune goat serum. The immunoprecipitates were treated with sample buffer to reduce the cross-links, and then analyzed by SDS-PAGE, followed by immunoblotting with rabbit anti-rat ER-60 antibody. Lane 1, immunoprecipitation of non-cross-linked cells. Lane 2, cross-linked cells.
Intracellular Association of ApoB with ER-60 Protease

The fraction of newly-synthesized apoB associated with the ER-60 homologue appears to be quantitatively very low. In addition, cross-linking experiments in permeabilized HepG2 cells showed that the ER-60 protease homologue is intracellularly associated with other proteins with molecular masses of 50, 66, and 78 kDa. The 78-kDa protein which consistently cross-linked to ER-60 appeared to represent BiP. In addition, immunoprecipitation of BiP from CHAPS-solubilized HepG2 cells as well as cross-linking experiments showed that BiP may also be associated with apoB. These findings raise the intriguing possibility of a three way association between apoB, ER-60, and BiP in the ER lumen. Whether association of ER-60 and apoB with BiP is an indication of a regulatory process for targeting of apoB for degradation remains to be demonstrated and requires further investigation. It would not be surprising if apoB associates with BiP in the lumen of the ER since the folding and biogenesis of the extremely large apoB polypeptide would be expected to involve the action of intraluminal molecular chaperones. ApoB is already known to associate with microsomal triglyceride transfer protein, protein disulfide isomerase, and possibly calnexin (29, 30) during its biogenesis in the ER. Patel and Grundy (30) also showed association of apoB41 with KDEL-bearing proteins including a 78-kDa protein resembling BiP in COS cells.

The fraction of newly-synthesized apoB associated with the ER-60 homologue appears to be quantitatively very low. In several experiments performed it appears that only a small fraction of newly-synthesized apoB pool could be recovered in association with the 58-kDa ER-60 homologue. This finding is not surprising and should be expected since it is unlikely that a large portion of the apoB pool would be associated with the ER-60 homologue at any given time. In addition, a major fraction of the newly-synthesized apoB pool is normally found on the cytosolic face of the ER membrane and is unlikely to associate with the ER-60 homologue which appears to be luminal in nature. Presumably only the apoB pool that is fully-translocated and is present in the ER lumen would be targeted by a luminal protease such as the ER-60 protease homologue.

Although we do not have direct evidence implicating the
ER-60 protease homologue in apoB degradation, we hypothesize that the ER-60 protease is involved in intraluminal degradation of apoB in the ER, based on the evidence showing apoB-ER-60 association and the cysteine protease activity of ER-60 (20, 21). Recent evidence from our laboratory and others (18) suggest the existence of an ER-localized proteolytic system distinct from the ubiquitin-proteasome system. Ginsberg and co-workers (18) reported that even after apoB is translocated into the ER lumen, it can potentially be degraded by a luminal DTT-sensitive degradative system that may be responsible for degrading the N-terminal 70-kDa apoB fragment. We have previously reported that degradation of apoB in permeabilized HepG2 cells can generate specific apoB fragments in the ER including an abundant 70-kDa intermediate (12, 16, 17, 26). More recent experiments in our laboratory support the hypothesis that apoB degradation may occur in two steps. Our data in the present report demonstrating the association of apoB with an ER-resident protease, ER-60, supports the above notion that proteases other than the proteasome may be involved in the apoB degradation process. It is thus reasonable to hypothesize that the ER-60 protease homologue detected in HepG2 cells may be involved in a luminal proteolytic system that degrades fully-translocated apoB. The luminal process appears to be distinct from the proteasome-mediated degradative process (19) operating on the cytosolic side of the ER membrane, which degrades the bulk of apoB co-translationally. Efforts are underway to further characterize the ER-associated degradation pathway and confirm its subcellular localization and the involvement of the ER-60 protease in this pathway. It would also be interesting to investigate the role of the ER-associated protease in degradation of nascent apoB-containing lipoprotein particles in the lumen of the ER, and the possibility of association of the ER-60 protease homologue with these lipoprotein particles.

REFERENCES

1. Davis, R., Prewett, A., Thompsett, J., Chan, P., Borchardt, R., and Gallacher, W. (1991) J. Lipid Res. 32, 941–950
2. Davis, R. A., Thrift, R. N., Wu, C. C., and Howell, K. E. (1990) J. Biol. Chem. 265, 10055–10061
3. Boren, J., Wettesten, M., Sjøberg, A., Thorlin, T., Bondjers, G., Wicklund, O., and Olofsson, S.-O. (1990) J. Biol. Chem. 265, 10556–10564
4. Thrift, R. N., Drisko, J., Dueland, S., Trawick, J. D., and Davis, R. A. (1992) J. Biol. Chem. 267, 20937–20942
5. Boren, J., Graham, L., Wettesten, M., Scott, J., White, A., and Olofsson, S.-O. (1992) J. Biol. Chem. 267, 9858–9867
6. Furukawa, S., Sakata, N., Ginsberg, H. N., and Dixon, J. L. (1992) J. Biol. Chem. 267, 22630–22635
7. Sakata, N., Wu, X., Dixon, J. L., and Ginsberg, H. N. (1993) J. Biol. Chem. 268, 22967–22970
8. Sato, R., Imanaka, T., Takatsuki, A., and Takano, A. (1996) J. Biol. Chem. 265, 11880–11884
9. Cartwright, I. J., Hebbachi, A.-M., and Higgins, J. A. (1993) J. Biol. Chem. 268, 20937–20952
10. Verkade, H. J., Fast, D. G., Rusinol, A. E., Scraba, D. G., and Vance, D. E. (1993) J. Biol. Chem. 268, 24990–24996
11. Sparks, J. D., and Sparks, C. E. (1993) Curr. Opin. Lipidol. 4, 177–186
12. Adeli, K. (1994) J. Biol. Chem. 269, 9166–9175
13. Du, E. Z., Kurth, J., Wang, S.-L., Hamiston, P., and Davis, R. A. (1994) J. Biol. Chem. 269, 24169–24176
14. Bonnardel, J. A., and Davis, R. A. (1995) J. Biol. Chem. 270, 28892–28896
15. Wang, C.-N., Holman, T. C., and Brindley, D. N. (1995) J. Biol. Chem. 270, 24924–24931
16. Sallach, S., and Adeli, K. (1995) Biochim. Biophys. Acta. 1265, 29–32
17. Adeli, K., Wettesten, M., Arai, A., Mohammadi, M., Kito, R., Urade, R., and Olofsson, S.-O. (1997) J. Biol. Chem. 272, 5031–5039
18. Wu, X., Sakata, N., Lele, K. M., Zhou, M., Jiang, H., and Ginsberg, H. N. (1997) J. Biol. Chem. 272, 11575–11580
19. Yeung, S. J., Chen, S. H., and Chan, L. (1996) Biochemistry 35, 13843–13848
20. Urade, R., Nasu, M., Moriyama, T., Wada, K., and Kito, M. (1992) J. Biol. Chem. 267, 15152–15159
21. Urade, R., Takenaka, Y., and Kito, M. (1993) J. Biol. Chem. 268, 22004–22009
22. Urade, R., and Kito, M. (1992) FEBS Lett. 312, 83–86
23. Otsu, M., Urade, R., Kito, M., Omura, F., and Kituchi, M. (1995) J. Biol. Chem. 270, 14558–14561
24. Adeli, K., and Sinkovitch, C. (1990) FEBS Lett. 2, 345–348
25. Bostrom, K., Wettesten, M., Boren, J., Bondjers, G., Wicklund, O., and Olofsson, S.-O. (1986) J. Biol. Chem. 261, 13800–13806
26. Macri, J., and Adeli, K. (1997) J. Biol. Chem. 272, 7282–7337
27. Laemmli, U. K. (1970) Nature 227, 680–685
28. Hjelmeland, I. H. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 6368–6372
29. Ginsberg, H. N. (1995) Biochim. Biophys. Acta. 1265, 29–32