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Brefeldin A Inhibits the Targeting of Cathepsin D and Cathepsin H to Lysosomes in Rat Hepatocytes

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Effect of brefeldin A on the transport of lysosomal acid hydrolases (cathepsins D and H) was investigated in primary cultured rat hepatocytes. Both cathepsins were synthesized as proenzymes and progressively converted to mature enzymes in the control cells. However, BFA strongly inhibited the appearance of the mature enzymes in the cells in a dose dependent manner, suggesting that transport of newly synthesized lysosomal enzymes from the endoplasmic reticulum to lysosomes is blocked by the drug. The inhibitory effect by brefeldin A was reversible. Upon recovery from brefeldin A-intoxication, procathepsin D was effectively targeted into lysosomes, whereas a substantial amount of procathepsin H was found to be missorted, resulting in its secretion into the culture medium.

Brefeldin A (BFA), a macrolide antibiotic, has a potent inhibitory effect on the intracellular transport of G protein in vesicular stomatitis virus-infected baby hamster kidney cells (1). In cultured rat hepatocytes BFA strongly blocks secretion of plasma proteins such as albumin and α_1-protease inhibitor (α_1-PI) (2,3). Using an immunocytochemical technique we have recently demonstrated that BFA evokes the disassembly of the Golgi stacks and concomitantly causes the

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ABBREVIATIONS: BFA, brefeldin A; α_1-PI, α_1-protease inhibitor; ER, endoplasmic reticulum; SDS, sodium dodecyl sulfate.

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accumulation of albumin in the endoplasmic reticulum (ER) and the nuclear envelope, indicating that BFA blocks the exocytotic pathway at the exit from the ER (4). Interestingly, effects by BFA are reversible in rat hepatocytes. Reassembly of the Golgi stacks occurs in the BFA-treated cells after a long incubation time, when the secretion of plasma proteins resumes (4).

In the present study we investigated the effect of BFA on the targeting of newly synthesized cathepsins (D and H) to lysosomes in primary cultured rat hepatocytes.

MATERIALS AND METHODS

Materials: L-[^{35}S]Methionine (>800 Ci/mmol) and En^{3}Hance were obtained from Du Pont-New England Nuclear. Pansorbin (fixed Staphylococcus aureus cells) from Behring Diagnotics. Antibodies against rat α_1-PI (5), rat cathepsin D (6) and rat cathepsin H (7) were raised in rabbits. BFA was a gift from Dr. A Takatsuki (University of Tokyo). BFA was dissolved in methanol and stored at -20°C.

Hepatocyte culture and Labelling cells: Hepatocytes were prepared from Wistar rats (200-250 g) and cultured for 24 h before use as described previously (8). Pulse-chase experiments were performed as described previously (8). Unless otherwise indicated BFA was included throughout the experiments. At the indicated times cells were separated from media, lysed in 0.5 ml of phosphate buffered saline containing 1 % Triton X-100, 0.5 % sodium deoxycholate and 0.1 % sodium dodecyl sulfate (SDS) and used for immunoprecipitation.

Immunoprecipitation: From cell lysates and media, α_1-PI was purified using anti-α_1-PI IgG conjugated Sepharose 4B as described previously (8). For precipitation of cathepsins cell lysates and media were incubated with 80-100 µg each of monospecific IgG against cathepsin D or cathepsin H for 12 h at 4°C and further incubated with 100 µl of a 10 % suspension of Pansorbin for 12 h at 4°C. Pansorbin-immunocomplex was extensively washed (9). Samples were analyzed on 12.5 % SDS-polyacrylamide gel according to Laemmli (10), followed by fluorography.

RESULTS AND DISCUSSION

Both cathepsins (D and H) are known to be synthesized as preproenzymes (11-15). Since prepieces were cleaved cotranslationally, procathepsin D and procathepsin H with apparent molecular masses of 45 KDa or 41 KDa, respectively, appeared in

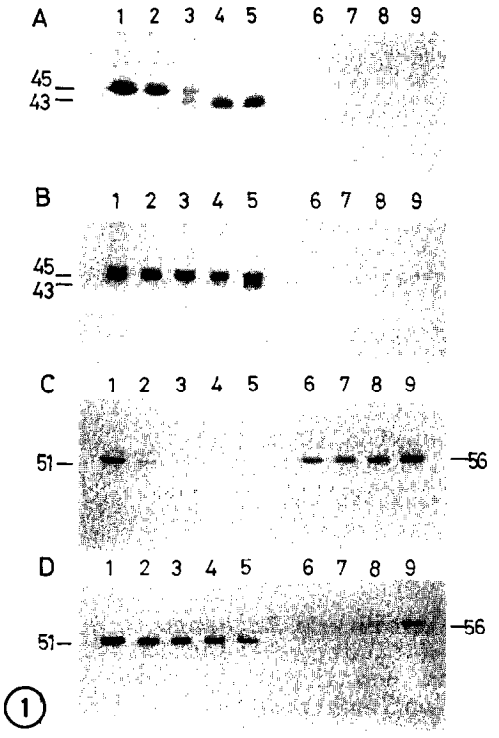

Figure 1. Effects of BFA on the synthesis, transport and processing of cathepsin D. Hepatocytes were incubated with or without 2.5 µg/ml of BFA prior to pulse-labeling with 100 µCi of [^{35}S]methionine for 30 min and chased with (B and D) or without (A and C) 7.5 µg/ml of BFA. At the indicated times cell lysates (lanes 1-5) and media (lanes 6-9) were prepared and subjected to immunoprecipitation of cathepsin D (A and B) and subsequently of α_1-PI (C and D). Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoreis, followed by fluorography. Lane 1, no chase; lanes 2 and 6, 1 h-chase; lanes 3 and 7, 2 h-chase; lanes 4 and 8, 3 h-chase; lanes 5 and 9, 5 h-chase. The positions of the 45 KDa procathepsin D and the 43 KDa cathepsin D (A and B) and the 51 KDa form and the 56 KDa form of α_1-PI are marked.

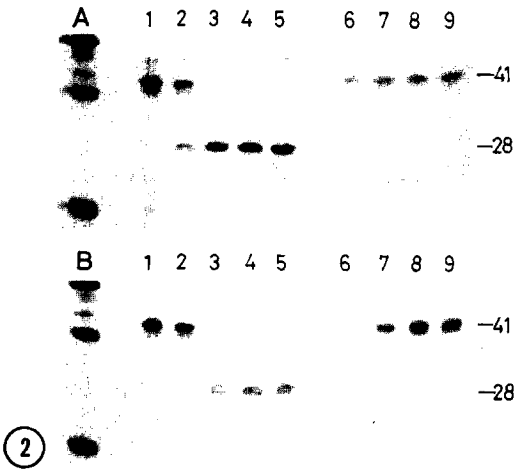

Figure 2. Effects of BFA on the synthesis, transport and processing of cathepsin H. Hepatocytes were labeled with [^{35}S]methionine as described in the legend to figure 1 with (B) or without (A) 2.5 µg/ml BFA throughout the experiment. Cell lysates (lanes 1-5) and media (lanes 6-9) were subjected to immunoprecipitation of cathepsin H. Samples were analyzed by SDS-polyacrylamide gel electrophoresis. Lane 1, no chase; lanes 2 and 6, 1 h-chase; lanes 3 and 7, 3 h-chase; lanes 4 and 8, 5 h-chase; lanes 5 and 9, 8 h-chase. At the left of the gel, [^{35}S]-methionine labeled markerks were run: α_1-PI (56 KDa), haptoglobin β subunit (36 KDa) and $\alpha_{2\mu}$-globulin (20 KDa) from the top (8). The positions of the 41 KDa procathepsin H and the 28 KDa cathepsin H are marked.

the cells after the pulse-label (Figs. 1A and 2A, lane 1). With continued incubations, the proenzymes disappeared and concomitantly mature enzymes with apparent molecular masses of 43 KDa

(cathepsin D) and 28 KDa (cathepsin H), respectively, accumulated in the cells (Fig. 1A and Fig. 2A, lanes 3-5). The mobility shift on SDS-polyacrylamide gel corresponds to the cleavage of propeptides from the proenzymes (13-15), which is believed to occur in the late stage of transport (prelysosomal compartments and/or lysosomes) (16,17). Thus newly synthesized cathepsin D and cathepsin H migrate to their destination with approximate half-times of transport of ~3 h and ~1 h, respectively. BFA blocked the appearance of the mature enzymes (Figs. 1B and 2B, lanes 1-4), demonstrating that BFA blocks the intracellular transport of lysosomal enzymes. Preliminary immunocytochemical observations showed the accumulation of cathepsin D in the ER and the nuclear envelope (S. Fujiwara et. al. unpublished results). It is therefore most likely that BFA inhibits the transport of cathepsin D at the exit from the ER, as demonstrated in the cases of albumin (4) and a T cell receptor (18).

Interestingly, in the control incubation a significant amount of procathepsin H but not cathepsin D was found to be rapidly secreted (Fig. 2A, lanes 6-9), suggesting that part of procathepsin H failed to be segregated for delivery to lysosomes, resulting in the release into the culture medium. This finding, though different from the results obtained in other studies (15), allowed us to analyze the transport of both lysosomal and secretory proteins simultaneously. As pointed out previously (2-4), the effect of BFA is reversible in rat hepatocytes. After a prolonged incubation both α_1-PI and most procathepsin H were finally released into the culture medium (Fig. 1D, lanes 8 and 9, and Fig. 2B, lanes 7-9), indicative of reassembly of the Golgi stack (4). At the same time the mature enzymes were found in the BFA-treated cells

(Figs. 1B, lane 5 and Fig. 2B, lanes 3-5), suggesting that when the blockade by BFA is released, the proenzymes once accumulated in response to BFA are delivered to lysosomes and converted to the mature enzymes. However, it is evident that more procathepsin H was released into the medium from the BFA-treated cells than from the control cells (Figs. 2 A vs. B, lanes 6-9), indicating that missorting of cathepsin H did occur during the process of the Golgi reassembly in contrast to cathepsin D (Fig. 1B). If the difference in the transport rate of the two enzymes is mostly accounted for by different transit times within the ER as reported in the case of plasma proteins (19), it is conceivable that when the block by BFA was released procathepsin H moved relatively rapidly to the Golgi, which was still in the process of reassembly and functionally incomplete, whereas by the time when procathepsin D migrated to the Golgi reassembly of the Golgi had been completed and cathepsin D was sorted properly. In fact we have noted that reassembly of the Golgi stack is a time-dependent process and Golgi stacks with aberrant structure appeared in the BFA-treated cells (4). To our knowledge this is the first report describing the effect of BFA on the targeting of lysosomal enzymes.

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