Volume 214, number 1, 135–138 FEB 04577 April 1987

Brefeldin A arrests the intracellular transport of a precursor of complement C3 before its conversion site in rat hepatocytes

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Received 5 January 1987

The effects of brefeldin A on intracellular transport and posttranslational modification of complement C3 (C3) were studied in primary culture of rat hepatocytes. In the control culture C3 was synthesized as a precursor (pro-C3), which was processed to the mature form with α- and β-subunits before its discharge into the medium. In the presence of brefeldin A the secretion of C3 was strongly blocked, resulting in accumulation of pro-C3. However, after a prolonged interval the mature form of C3 was finally secreted. The results indicate that brefeldin A impedes translocation of pro-C3 to the Golgi complex where pro-C3 is converted to the mature form, but not its proteolytic processing, in contrast to the effects of monensin and weakly basic amines.

Brefeldin A; Secretion-blocking agent; Complement C3; Complement precursor accumulation; (Rat hepatocyte)

1. INTRODUCTION

Brefeldin A is a macrolide antibiotic having a potent inhibitory activity against virus multiplication [1]. Takatsuki and Tamura [2] have recently found that brefeldin A inhibits the cell-surface expression of G-protein in vesicular stomatitis virus-infected baby hamster kidney cells, resulting in the accumulation of G-protein with high-mannose type oligosaccharides. The results indicate that inhibition of virion assembly on the cell surface is a cause of the antiviral effect of brefeldin A. These findings prompted us to elucidate the effects of brefeldin A on intracellular transport of secretory proteins as a new perturbant of the secretory pathway. In [3], we reported the effects of brefeldin A on intracellular transport and concomitant processing of albumin, α_1-protease inhibitor and haptoglobin. The results clearly demonstrate that the mode of action of brefeldin A is different from that of other perturbants such as monensin [4–6], antimicrotubular agents [5,7] and weakly basic amines [8,9], suggesting that brefeldin A is a potent agent for dissecting the intracellular transport pathway of secretory proteins [3] as well as membrane proteins [2].

The third component of complement (C3) is synthesized as a proform (pro-C3, M_r 185000) and intracellularly converted to the α- (M_r 115000) and β- (M_r 65000) subunits before its secretion [10]. We have recently demonstrated that the proteolytic conversion of pro-C3 occurs in the Golgi complex using an immunoblotting method [11]. Here, we examined the effects of brefeldin A on the proteolytic processing of pro-C3 in cultured rat

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Abbreviations: C3, the third component of complement; MEM, minimum essential medium

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

hepatocytes to characterize further the effects of brefeldin A on the secretory pathway.

2. MATERIALS AND METHODS

2.1. *Materials*

L-[^{35}S]Methionine (1200 Ci/mmol) was purchased from New England Nuclear (Boston, MA), cycloheximide from Sigma (St. Louis, MO), monensin from Calbiochem-Behring (La Jolla, CA), nigericin from Lilly (Indianapolis, IN), MEM from Nissui Seiyaku (Tokyo) and goat anti-rat C3 antiserum from Cappel (West Chester, PA). Brefeldin A was isolated in crystalline form from FL-24 fungi, as described [2]. A stock solution was made in methanol (5 mg/ml) and stored at −20°C until use. Rat serum C3 was purified from freshly prepared rat serum by affinity chromatography through an anti-(rat C3)-IgG-Sepharose column [12].

2.2. *Hepatocyte culture*

Hepatocytes were isolated from adult male Wistar rats, weighing 200–250 g, by the collagenase perfusion method [13]. Isolated hepatocytes were cultured at 37°C for 2 days before experiments as in [4,5].

2.3. *Labeling and immunoprecipitation of C3*

Cells were preincubated for 1 h in methionine-free MEM in the absence or presence of brefeldin A (5 μg/ml). The cells were then pulse-labeled with [^{35}S]methionine (100 μCi/1.5 ml per dish) for 15 min and chased in the presence of 100 μM cycloheximide. Brefeldin A was present throughout experiments. At the indicated times, cell lysates and media were prepared and used for immunoprecipitation of C3 as in [6,12]. For steady labeling, cells were preincubated for 30 min and labeled with 25 μCi [^{35}S]methionine for 2 h in the absence or presence of either monensin (5 μM), nigericin (5 μM) or methylamine (10 mM), followed by immunoprecipitation of C3 as above.

2.4. *Polyacrylamide gel electrophoresis*

Immunoprecipitates were analyzed by electrophoresis on 7.5% polyacrylamide gels in the presence of SDS, followed by fluorography as described in [9,10].

3. RESULTS AND DISCUSSION

In a previous paper [10] we have shown that pro-C3 is converted to mature C3 with α- and β-subunits in the Golgi complex before being released into the medium. This is based on the findings that pro-C3 is not detected in the medium and that the proteolytic conversion of pro-C3 is inhibited by monensin, a perturbant of the Golgi complex [10]. However, we failed to demonstrate the presence of the α- and β-subunits within the cells, suggesting the possibility that pulse-chase experiments did not proceed properly and/or that the mature C3, once formed, is quickly exported out of the cells. In the present study we carried out the chase experiments in the presence of cycloheximide so that complete cessation of labeled amino acid incorporation into pro-C3 after a pulse period could increase the ratio of the newly converted mature C3 to the precursor.

Fig.1 illustrates the effect of brefeldin A on secretion of C3. Cells were pulse labeled with [^{35}S]methionine for 15 min and chased for various times in the absence or presence of brefeldin A (5 μg/ml). In the control culture C3 appeared in the medium within 30 min after start of the chase. This is consistent with previous experiments without cycloheximide [10], indicating that continuous protein synthesis is not required for the in-

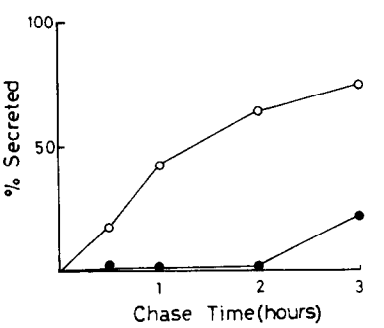

Fig.1. Time course of secretion of complement C3 in a primary culture of rat hepatocytes. Hepatocytes were pulse-labeled for 15 min with [^{35}S]methionine and chased in the absence (○) or presence (●) of brefeldin A (5 μg/ml) as described in section 2. At the indicated times, immunoprecipitates of C3 were prepared from cell lysates and media, and the radioactivity determined. Values are expressed as percentages of the radioactivity secreted of the total radioactivity incorporated into C3 during 15 min pulse.

tracellular transport of newly synthesized C3. However, in the presence of brefeldin A the secretion of C3 was delayed more than 2 h as compared with that in the control culture.

The proteolytic processing of pro-C3 was analyzed by pulse-chase experiments, followed by SDS-polyacrylamide gel electrophoresis (fig.2). In the control culture (fig.2A), the proform which appeared as a single form just after the pulse labeling (lane 1) started to be processed to the mature form with α- and β-subunits within 30 min of chase (lane 2), indicating that pro-C3 reached its conversion site by that time. C3 was finally released into the medium after being completely converted to the mature form, since no proform was detected in the medium (lane 6). Most recently we have demonstrated the presence of the α- and β-subunits in Golgi fractions but not in rough and smooth microsomes isolated from rat liver by means of immunoblotting [11]. Thus, the appearance of the subunits of C3 can be used as a good marker to monitor the intracellular transport of C3 from the endoplasmic reticulum to the Golgi complex.

In the presence of brefeldin A, however, the appearance of the subunits of C3 was markedly delayed (fig.2B). Only the proform was present in cells up to 2 h of chase (lanes 1–4), after which the

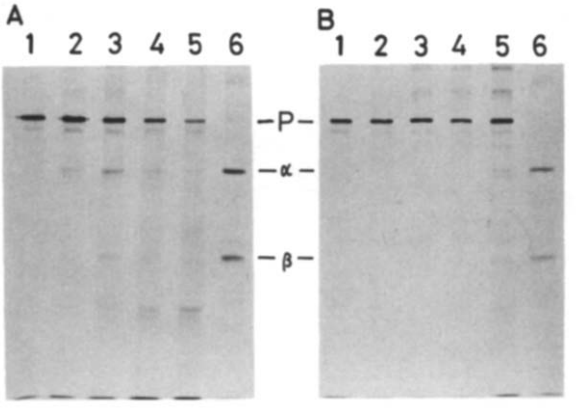

Fig.2. Effect of brefeldin A on intracellular processing of complement C3. Cells were pulse-labeled and chased in the absence (A) or presence (B) of brefeldin A (5 μg/ml) as shown in fig.1. Immunoprecipitates of C3 were prepared from cell lysates (lanes 1–5) and media (lane 6), and analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. Lanes: 1, no chase; 2, chase 30 min; 3, 1 h; 4, 2 h; 5,6, 3 h. P, α and β denote the proform, α- and β-subunits, respectively, of C3.

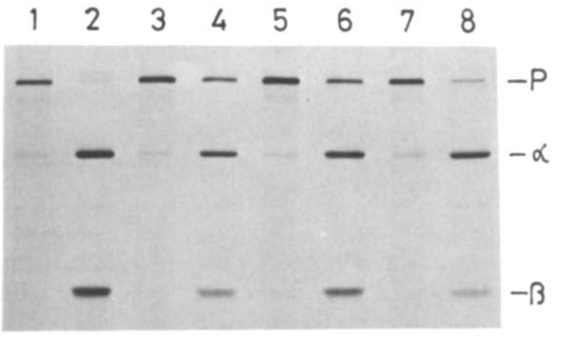

Fig.3. Effects of perturbants of the Golgi complex on the proteolytic processing of complement C3. Cells were labeled for 2 h with [^{35}S]methionine in the absence or presence of the following drugs as described in section 2. Immunoprecipitates prepared from cell lysates (lanes 1,3,5,7) and media (lanes 2,4,6,8) were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. Lanes: 1,2, no drug; 3,4, monensin (5 μM); 5,6, nigericin (5 μM); 7,8, methylamine (10 mM). P, α and β denote the proform, α- and β-subunits, respectively, of C3.

processed form was detectable in the cells (lane 5) and secreted into the medium (lane 6), coincident with the time course of secretion (fig.1). The delayed secretion was accompanied by a morphological alteration with marked dilation of the endoplasmic reticulum as shown in [3]. The finding that no proform was detectable in the medium as in the control culture (lane 6 in fig.2A,B) suggests that the drug causes no significant inhibition of proteolytic cleavage of pro-C3. Taken together, these results indicate that brefeldin A primarily impedes intracellular transport of the proform to its conversion site, the Golgi complex, resulting in delayed secretion of the mature form after prolonged accumulation of the proform in cells. This effect is different from those of other secretion-blocking agents such as carboxylic ionophores and weakly basic amines, which commonly cause not only swelling of the Golgi elements, but also dysfunctions of the Golgi complex [4,5,8–10]. In fact, when the cells were treated with these drugs, a considerable amount of pro-C3 was released directly into the medium, as typified in fig.3 for monensin (lane 4), nigericin (lane 6) and methylamine (lane 8).

The present study and the previous one [3] demonstrate that brefeldin A markedly retards the

translocation of pro-C3 and proalbumin to the site where proteolytic cleavage of both precursors takes place. The most proximal site for proteolytic conversion of both precursors is supposed to be an acidic *trans* region of the Golgi complex, since acidotropic agents including carboxylic ionophores and weakly basic amines disturb this particular processing occurring in the Golgi complex [8,9,14]. Thus, we cannot exclude the possibility that brefeldin A affects the intra-Golgi transport from *cis* to *trans* Golgi subcompartments other than translocation from the endoplasmic reticulum to the Golgi complex. Analysis of processing of asparagine-linked oligosaccharides in the presence of brefeldin A should provide a clue for specifying the target site of this novel perturbant of the exocytotic pathway.

ACKNOWLEDGEMENT

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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