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BREFELDIN A AFFECTS THE CELLULAR DISTRIBUTION OF ENDOCYTIC RECEPTORS DIFFERENTIALLY

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The cell surface expression of three endocytic receptors was studied in human hepatoma Hep G2 cells treated with brefeldin A (BFA). Ligand binding and cell surface iodination revealed that BFA increased the number of mannose 6-phosphate/insulin-like growth factor II receptors twofold and decreased the amount of asialoglycoprotein and transferrin receptors by 40-60 %. The altered expression of receptors at the cell surface was paralleled by changes in the respective ligand uptake. The implications of this finding on our understanding of intracellular trafficking are discussed.

The fungal metabolite brefeldin A (BFA) has dramatic, well documented effects on the structural and functional organization of the Golgi complex. BFA blocks anterograde protein transport out of the ER and causes a rapid disassembly of the Golgi apparatus. The Golgi components redistribute through a retrograde, microtubule dependent pathway back to the ER (1-3). BFA prevents the membrane assembly of cytosolic coat proteins which regulate the formation of both coated transport vesicles and uncoated tubules (4, 5; for review see 6).

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Abbreviations: Man6P, mannose 6-phosphate; IGF, insulin-like growth factor; BFA, brefeldin A; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; PMP-BSA, pentamannose 6-phosphate coupled to bovine serum albumin; ASOR, asialoorosomucoid; ASGPR, asialoglycoprotein receptor; TfR, transferrin receptor

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Recent immunocytochemical and electronmicroscopic studies have demonstrated that BFA treatment also induces the formation of tubules from the trans Golgi network (TGN) and endosomes resulting in a fused TGN/endosomal network (7, 8). While the cycling between the plasma membrane and the TGN/endosomal network continued the number of the mannose 6-phosphate/insulin-like growth factor II (Man6P/IGF II) receptor and the 46 kD Man6P receptor (MPR 46) at the cell surface was increased (7, 9). In the present study we examined the effects of BFA on two other endocytic receptors, the receptors for transferrin and asialoglycoproteins, which are different to Man6P/IGF II receptors in regard to their cellular distribution and their cycling pathway (10, 11). The results demonstrate that in contrast to the Man6P/IGF II receptor in BFA treated human hepatoma cells (Hep G2) the number of transferrin and asialoglycoprotein receptors at the cell surface was decreased while the expression of the Man6P/IGF II receptor was increased.

MATERIALS AND METHODS

Materials: The human hepatoma cell line Hep G2 was maintained in minimal essential medium (MEM) containing 10 % fetal calf serum. Pentamannosyl 6-phosphate was coupled to bovine serum albumin (PMP-BSA) as described previously (12). Man6P and human transferrin were obtained from Sigma and Pansorbin from Calbiochem. Brefeldin A was kindly provided by Dr. Nespital, Sandoz (Nürnberg). Orosomucoid was a gift from American National Red Cross Research Laboratory (Bethesda, ML) and desialylated according to (13). Na[^{125}I] was obtained from Amersham. Antisera against the human Man6P/IGF II receptor, the human H1-ASGPR (KI-5b) and the human transferrin receptor were those as described (10, 14, 15). PMP-BSA, ASOR and diferric transferrin were iodinated (12) with the aid of iodogen (Pierce) to specific activities of 150 μCi/μg, 6 μCi/μg and 4 μCi/μg, respectively.

Binding and endocytosis of ligands: Binding and endocytosis of [^{125}I]PMP-BSA, [^{125}I]ASOR and [^{125}I]diferric transferrin were carried out as described earlier (16-18). The cellular protein content was determined (19) using BSA as standard. The number of cell surface receptors and their ligand dissociation constants (K_d) were estimated by Scatchard analysis.

Internalization of [^{125}I]transferrin: Hep G2 cells were incubated at 4°C for 4 h with [^{125}I]transferrin, washed and then warmed to 37°C for up to 30 min. The internalized radioactivity (acid wash resistant) was determined and expressed as percentage of total radioactivity (internalized, cell surface-bound and radioactivity released into the medium) (18).

Externalization of [^{125}I]transferrin: HepG2 cells were incubated for 15 min at 37°C with [^{125}I]transferrin (5 nM) in the absence or presence of BFA. After chilling to 4°C the cells were washed and cell surface bound [^{125}I]transferrin gently removed by six

alternating washes at pH 5.4 and pH 7.4 containing 0.8 mM deferroxamine mesylate (20). The cells were then warmed to 37°C in MEM containing 0.1 % BSA and 10 μg/ml transferrin for various time periods. The radioactivity released into the medium and remaining at the cells was determined.

Cell surface iodination and immunoprecipitation of receptors: Hep G2 cells were incubated for 30 min at 37°C in the absence and presence of BFA (5 μg/ml), then rapidly chilled to 4°C and iodinated by the addition of 0.6 ml 10 mM phosphate buffered saline (PBS), pH 7.4 containing 10 mM glucose, 2 U lactoperoxidase, 0.5 mCi Na[^{125}I] and 1 U glucose oxidase. The cells were washed to remove free [^{125}I] and lysed in 10 mM PBS containing 1 % Triton X-100, 0.5 % SDS, 0.25 % deoxycholate, 0.5 % BSA, 0.02 % azide and 1 mM phenylmethylsulfonylfluoride. Because of incomplete solubilization of Man6P/IGF II receptors under these conditions the receptors were immunoisolated from dishes labeled in parallel and solubilized as described (9). Immunoprecipitation was carried out sequentially with each 2 μl antiserum against the ASGPR, and transferrin receptor. Pansorbin was used to collect the antibody receptor complexes which were subsequently solubilized at 95°C and analyzed by SDS-PAGE (10 % polyacrylamide for ASGPR and TfR and 5 % polyacrylamide for Man6P/IGF II receptor) and autoradiography of dried gels at - 70°C.

RESULTS

Different effects of BFA on the cell surface expression of endocytic receptors

Incubation of Hep G2 cells for 30 min with 5 μg/ml BFA increased the binding of the Man6P/IGF II receptor ligand [^{125}I]PMP-BSA 2.1fold compared with untreated controls (Table 1; (9)). Parallel determinations of the ligand binding to the ASGPR and TfR revealed a decrease in binding by 40 % after BFA treatment. Scatchard analysis of dose dependent binding data showed that BFA did not reduce the affinity of the ASGPR (30 nM in both control and BFA treated cells) and the TfR (2.63 nM in control cells and 2.45 nM in BFA treated cells) but the number of surface associated receptors. These data were confirmed by determination of receptors accessible to iodination at the cell surface of control and BFA treated Hep G2 cells. After iodination at 4°C the different receptors were sequentially immunoprecipitated and analyzed by SDS-PAGE and autoradiography. Counting of the excised [^{125}I]-labeled receptor bands or densitometric evaluation showed that in BFA treated cells the amount of ASGPR and TfR at the plasma membrane was decreased by 50 and 44 % compared with controls (Fig. 1). In a parallel experiment using conditions optimized for solubilization of Man6P/IGF II receptors (9) BFA increased the amount of cell surface iodinatable receptors 3.4fold.

Table 1: Effect of BFA on binding and endocytosis of different receptor ligands in Hep G2 cells

Ligand	Binding (% of control)	Uptake (% of control)
[^{125}I]PMP-BSA	214 ± 35 (6)	247 ± 52 (5)
[^{125}I]ASOR	60 ± 21 (4)	60 ± 21 (6)
[^{125}I]transferrin	60 ± 4 (6)	78 ± 20 (4)

Hep G2 cells were incubated in the presence of 5 μg/ml BFA for 30 min at 37°C. Subsequently the binding and uptake of [^{125}I]-labeled receptor ligands were determined. The values are expressed as mean ± SD of bound and internalized ligand in percent of controls. Controls bound 83 ± 51 pg [^{125}I]PMP-BSA, 3.0 ± 0.5 ng [^{125}I]ASOR, and 5.9 ± 0.2 ng [^{125}I]transferrin per mg cell protein and internalized 150 ± 77 pg PMP-BSA, 4.2 ± 1.0 ng ASOR and 4.7 ± 0.4 ng transferrin per mg cell protein within 30 min. The numbers in parentheses indicate the number of independent experiments.

Ligand uptake by different endocytic receptors

In human fibroblasts the increased number of Man6P/IGF II receptors at the cell surface induced by BFA treatment is

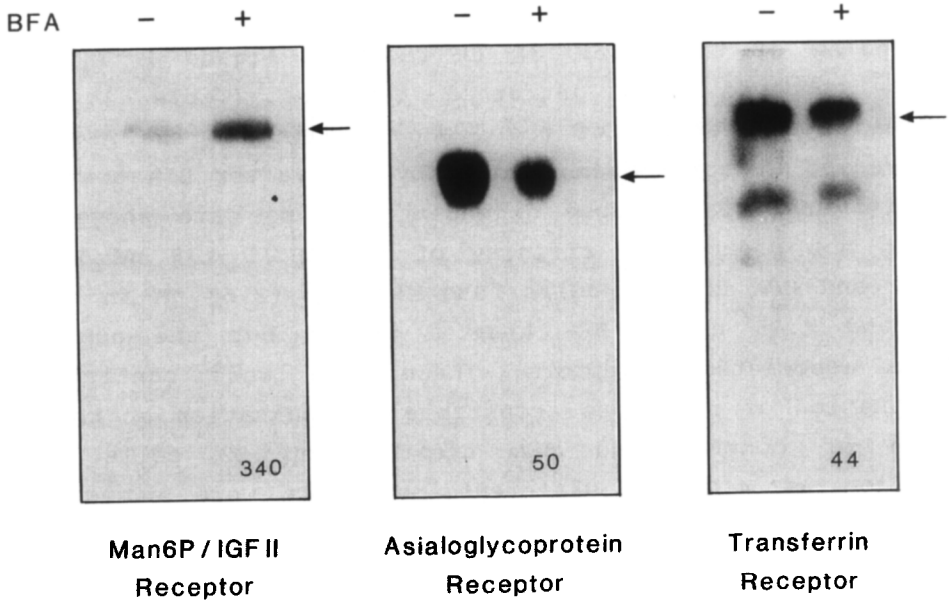

Fig. 1. Effect of BFA on endocytic receptor expression in surface iodinated Hep G2 cells Hep G2 cells incubated in the absence or presence of BFA were iodinated at the cell surface and the Man6P/IGF II receptor, the ASGPR and TfR were immunoprecipitated and analyzed by SDS-PAGE and autoradiography. The values at the bottom give the relative amount of receptors at the cell surface of BFA-treated cells as a percentage of receptors detected in untreated cells. Similar results were obtained in a second experiment.

paralleled by an increased uptake of lysosomal enzymes (9). To examine whether in BFA treated Hep G2 cells the different cell surface expression of endocytic receptors led to an altered uptake of receptor ligands the uptake of PMP-BSA, ASOR and transferrin was measured. Hep G2 cells were pretreated for 30 min at 37°C with BFA (5 μg/ml) and then incubated for further 30-60 min with the [^{125}I]-labeled ligands in the presence of BFA. The uptake of [^{125}I]PMP-BSA was 2.5fold increased whereas the endocytosis of [^{125}I]ASOR and [^{125}I]transferrin was inhibited by 40 % in BFA treated cells compared with untreated cells (Table 1). The examination of [^{125}I]-labeled ligand uptake showed that in BFA treated Hep G2 cells the internalization was either increased (PMP-BSA) or decreased (ASOR, transferrin) corresponding to the changes of the receptor number at the cell surface.

For fibroblasts it was shown that BFA induces the increased expression of Man6P/IGF II receptors at the cell surface by increasing the pool of recycling receptors, rather than by affecting the cycling kinetics (9). The same mechanism may account for the redistribution of Man6P/IGF II receptors in Hep G2 cells, since the effects of BFA in both cell types resembled each other. In order to determine whether in BFA treated Hep G2 cells the redistribution of transferrin receptors is regulated by similar mechanisms, the internalization and externalization rate of transferrin receptors were measured. The internalization of prebound [^{125}I]transferrin was determined upon warming to 37°C. The internalization rate and the fraction of internalized [^{125}I]transferrin were 10 % lower in BFA treated cells than in controls (Fig. 2 A). In a second approach the externalization of prior internalized [^{125}I]transferrin was followed in the presence and absence of BFA. The data in Fig. 2 B show that after a lag phase of 2 min the externalization of transferrin was decreased by BFA and that the fraction of externalized transferrin was about 25 % lower than in control cells. The lag phase may be due to heterogeneity of recycling vesicles or to incomplete removal of surface associated [^{125}I] diferric transferrin by the washing with deferroxamine.

DISCUSSION

Recent studies have demonstrated that in certain cell types BFA treatment not only leads to the fusion of the Golgi with the ER

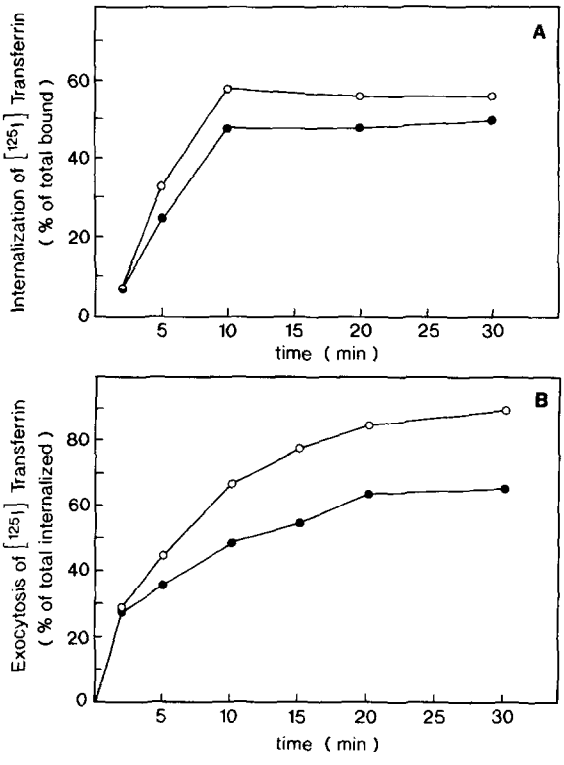

Fig. 2. Effect of BFA on internalization and externalization of [^{125}I]transferrin
A: Hep G2 cells were incubated for 30 min with or without BFA prior to binding of [^{125}I]transferrin for 4 h at 4°C. After removal of unbound ligands, the cells were incubated at 37 °C with MEM/BSA without (o) or with BFA (●). At various time points the cells were chilled to 4°C and the internalized radioactivity was determined.
B: The BFA pretreated and nontreated Hep G2 cells were incubated with [^{125}I]transferrin with or without BFA for 15 min at 37°C. Thereafter the cells were chilled to 4°C and cell surface bound [^{125}I]transferrin removed and subsequently the cells rewarmed to 37°C for various times without (o) and with (●) BFA. The externalized [^{125}I]transferrin was determined in the medium. The results are representative for two independent experiments.

but also to the formation of a TGN/early endosome network that associates with microtubules (7-9). In the fused system membrane traffic still continues and may allow Man6P/IGF II receptors and MPR 46 to be rapidly transported to the cell surface (8, 9). In this study we show that BFA affected the cellular distribution of three endocytic receptors differently. The amount of Man6P/IGF II receptors at the cell surface was increased 2fold while that of transferrin and asialoglycoprotein receptors was decreased by about 50 %. The BFA induced changes in cell surface receptor expression affected also the respective uptake of receptor ligands.

All three receptors are localized in the TGN, endosomes and at the plasma membrane but differ in their relative distribution. Using a cross-linking assay based on internalized transferrin-horseradish peroxidase conjugates Stoorvogel et al. (10) concluded that asialoglycoprotein and transferrin receptors have a common pathway for cycling between plasma membrane and endosomes, whereas 70 % of surface derived Man6P/IGF II receptors were sorted from this route. Furthermore, 20-30 % of the intracellular asialoglycoprotein receptor pool is not involved in endocytosis and devoid of recycling transferrin receptors (10, 21, 22). The decrease of surface receptors for asialoglycoproteins and transferrin induced by BFA can be explained in different ways. First, a fraction of both receptors may become sequestered into a non-recycling pool. Second, in the presence of BFA the internalization rate may be increased or the externalization rate decreased. Thirdly, BFA may abolish receptor specific equilibria between the cell surface and internal membranes. This is suggested by the observation that in the presence of BFA 20 % of the receptors for Man6P/IGF II, transferrin and asialoglycoprotein are localized at the cell surface. This third possibility, however, is unlikely, since in preliminary experiments BFA did not affect the surface expression of receptors for LDL and EGF, of which more than 80 % are localized at the cell surface. The present data do not allow to discriminate between the two former possibilities. The transport kinetics of the transferrin receptor was slightly affected. The decreased externalization, which would reduce the surface expression, was at least partly compensated by the decreased internalization. The most likely explanation is therefore that the redistribution is due to the sequestration of transferrin and asialoglycoprotein receptors into a non-recycling pool.

It has been shown earlier that BFA induces the increase in Man6P/IGF II receptors in fibroblasts by increaseing the number of recycling receptors (9). Furthermore both the internalization and the externalization of Man6P/IGF II receptors were accelerated. A possible explanation for the apparent differential effects of BFA on the distribution of transferrin and asialoglycoprotein receptors versus Man6P/IGF II receptors could be that BFA interacts with different target molecules that regulate specific transport steps within the endosomal system. In agreement with this assumption, it has been shown that in

polarized MDCK cells BFA does not affect the internalization, recycling and intracellular degradation of the polymeric immunoglobulin while the formation of IgA containing transcytotic vesicles and their release from the basolateral endosomal system was blocked (23). The rapid effects of BFA on the disassembly of the Golgi complex might involve an inhibition of the binding of the ß-COP protein to Golgi membranes (4, 24). The absence of ß-COP on endosomes, the sensitivity of endosomes in MDCK- and PtK_1-cells to BFA while their Golgi complex is BFA resistant (23, 25) strongly suggests the presence of different related BFA sensitive proteins. Other coat proteins or low molecular weight guanine nucleotide binding proteins involved in the association/dissociation of coat proteins (26) localized in specific intracellular organelles (27) might be such targets for BFA.

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