Effects of Brefeldin A on the Endocytic Route

REGISTRATION OF MANNOSE 6-PHOSPHATE/INSULIN-LIKE GROWTH FACTOR II RECEPTORS TO THE CELL SURFACE*

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The effect of brefeldin A (BFA) on the trafficking of the mannose 6-phosphate/insulin-like growth factor II receptor within the endocytic route was analyzed. Treatment with BFA induced a redistribution of the receptor to the cell surface and increased both the binding and internalization of ligands 2-4 fold. The effect of BFA was dose- and time-dependent and reversible. Determinations of transport rates showed that BFA increases the internalization rate and the externalization rate of the receptor. This implies that the higher surface concentration is due to higher concentrations of receptor at the intracellular sites from where they recycle to the cell surface. The effect of BFA was additive to the redistribution induced by insulin-like growth factors I and II and was observed in all human and rodent cell lines analyzed. BFA increased also the cell surface expression of the M₄, 46,000 mannose 6-phosphate receptor but not of the transferrin receptor. The results indicate that BFA interferes with the transport of mannose 6-phosphate receptors and affects the endocytosis of lysosomal enzymes by increasing the number of receptors available for recycling to the cell surface.

Brefeldin A (BFA) is an isoprenoidal fungal metabolite that blocks the transport of secretory, membrane, and lysosomal proteins through the Golgi (1-5). The organization of the Golgi is rapidly and reversibly altered by a retrograde transport of proteins resident in the cis, mid, and trans Golgi to the endoplasmic reticulum (ER) (4, 6). Therefore, newly synthesized proteins accumulating in the ER can undergo carbohydrate processing reactions, which normally occur in the Golgi. The reports on the sensitivity of the trans Golgi network (TGN) to BFA are controversial and depend on the cell type and the TGN markers used (7-9).

In order to examine the effects of BFA on proteins which function in the secretory pathway as well as in the endocytic pathway, we studied the cellular distribution of the mannose 6-phosphate/insulin-like growth factor II (Man-6-P/IGF II) receptor. This receptor is primarily localized in the TGN, late endosomes and at the plasma membrane (10, 11). Receptors in these compartments are in equilibrium (12-14). In this study we report on the effects of BFA on the trafficking of the Man-6-P/IGF II receptor between the cell surface and internal membranes.

BFA leads to a specific and reversible redistribution of Man-6-P/IGF II receptors to the cell surface, which is ascribed to an increase of the receptor pool available for the recycling to the plasma membrane. A similar redistribution to the cell surface was seen for the M₄, 46,000 mannose 6-phosphate receptor (MPR 46), which shares with the Man-6-P/IGF II receptor the recycling from endosomes to the Golgi as well as to the plasma membrane (11).

MATERIALS AND METHODS

Pentamannosyl 6-o-phosphate-substituted bovine serum albumin (Man-6-P-Man₄-BSA) was prepared from獐门ala holami pharmophomannan, provided by Dr. M. Slodki (United States Dept. of Agriculture, Northern Regional Research Center, Peoria, IL) as described (15). Man-6-P (disodium salt) and human transferrin were obtained from Sigma and Pansorbin from Calbiochem. Percoll was purchased from Pharmacia LKB Biotechnology Inc. Recombinant insulin-like growth factors (IGF) I and II were a kind gift by Dr. M. A. Fischli, Ciba-Geigy, Basel; brefeldin A was kindly provided by Dr. R. S. S. Sly and S. Kornfeld (St. Louis, MO). Na₁₂⁵I- and [¹³⁵S]methionine were obtained from Amersham and [¹⁴C]-labeled molecular weight standards were from Du Pont-New England Nuclear. Antisera against the human placental arylsulfatase A and the Man-6-P/IGF II receptor from human liver were those as described (16, 17).

Cell Culture—Human skin fibroblasts were grown in 35-mm dishes in minimal essential medium (MEM, Seromed) containing 5% fetal calf serum (GIBCO) penicillin and streptomycin (GIBCO), the baby hamster kidney (BHK) cells and mouse L cells fibroblasts stably transfected with the cDNA for the human Man-6-P/IGF II receptor were kindly provided by Drs. W. S. S. T. S. and Kornfeld (St. Louis, MO). Na₁₂⁵I- and [¹⁴C]-labeled molecular weight standards were from Du Pont-New England Nuclear. Antisera against the human placental arylsulfatase A and the Man-6-P/IGF II receptor from human liver were those as described (16, 17).

Binding and Endocytosis of [¹²⁵I-Man-6-P-Man₄-BSA—Detailed descriptions of the binding and endocytosis assays using [¹²⁵I-Man-6-P-
Man-6-P/IGF-I receptor ligand have been published elsewhere (15, 18). Cross-linking of $^{125}$I-IGF-I to fibroblasts—$^{125}$I-IGF-I was cross-linked to cell surface Man-6-P/IGF-II receptors as described (18).

Internalization of $^{125}$I-Labeled Arylsulfatase A—The internalized $^{125}$I-labeled arylsulfatase A, which binds to more than 85% to a Man-6-P/IGF-II receptor matrix in a Man-6-P dependent manner, was determined by incubation of fibroblasts for 60 min at 37°C. The cells were chilled to 4°C, washed, and the cell surface-bound ligand displaced by Man-6-P. The cells were solubilized in 1 N NaOH, and the cell-associated radioactivity referred to cell protein (19). The presence of 5 mM Man-6-P during the incubation period inhibited the endocytosis by 97%. Subcellular fractionation of internalized $^{125}$I-aryl sulfate A was performed on Percoll density centrifugation (20).

$[^{35}]$S]methionine Incorporation in Man-6-P/IGF-II Receptors—Immunoprecipitation of Man-6-P/IGF-II receptors metabolically labeled with $[^{35}]$Smethionine for 3 h (0.05 mCi/ml) was carried out as described (15) but using a 10 mM Tris-HCl buffer, pH 7.5, containing 1.5% (w/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% SDS, 1% (w/v) BSA, and 0.5 mM NaCl.

Binding of $^{125}$I-Transferrin—For determination of the cell surface expression of transferrin receptor cells were incubated with $^{125}$I-labeled diferric transferrin (5 nM; 4.6 pCi/pg) (21) in MEM containing 0.1% BSA, 20 mM Hepes, pH 7.2, at 4°C for 4 h. Unbound ligands were removed by five washes with ice-cold Hanks' solution and then incubated at 37°C for the indicated times with the $^{125}$I-labeled 2C2 antibodies. For the evaluation of excised receptor bands from the gel showed that the receptor cross-linked $^{125}$I-IGF-II was 1.6-fold higher in BFA-treated cells.

Table I

| Cell type                  | $^{125}$I-Man-6-P-Man$_{6}$-BSA | % of control |
|----------------------------|-------------------------------|-------------|
| Human fibroblasts          | 257 ± 43 (n = 31)             |             |
| Hep G2                     | 207 ± 30.5 (5)                |             |
| Mouse L$^*$                | 236 (219, 254)                |             |
| BHK                        | 311 (310, 319)                |             |
| CHO                        | 305 (275, 330)                |             |

*Overexpressing the human Man-6-P/IGF-II receptor.

RESULTS

BFA Increases the Cell Surface Expression of Man-6-P/IGF-II Receptors—Fibroblasts were incubated for 30 min with 5 µg/ml BFA before determining the cell surface expression of the Man-6-P/IGF-II receptor. The binding of two high affinity ligands for the IGF-II and Man-6-P binding sites of the receptor, $^{125}$I-IGF-II and $^{125}$I-Man-6-P-Man$_{6}$-BSA, was 1.6- and 2.6-fold increased (Fig. 1 and Table I). The binding of the monoclonal receptor antibody $^{125}$I-2C2 was also 1.7-fold increased in BFA-treated cells. Presence of 5 µg/ml BFA during the binding at 4°C had no effect on the binding of Man-6-P-Man$_{6}$-BSA, indicating that the observed changes are not due to a direct effect of BFA on the Man-6-P/IGF-II surface receptors. Dose-dependent binding of Man-6-P-Man$_{6}$-BSA at concentrations ranging from 1 to 200 pM revealed that BFA did not change the affinity of the receptor for Man-6-P-Man$_{6}$-BSA ($K_d$ 13.8 pM in BFA-treated cells vs. 14.2 pM in controls).

The effect of 5 µg/ml BFA reached half of its maximum within 5 min (Fig. 2A). Using a 30-min pretreatment, half-maximal stimulation was observed at $10^{-6}$ M BFA (Fig. 2B). In all further experiments the cells were pretreated for 30 min at 37°C with 5 µg/ml BFA (1.8 × 10$^{-5}$ M). Removal of BFA from the medium showed that the effect was reversible with a t1/2 of about 30 min (Fig. 2A). In presence of BFA the increased binding of $^{125}$I-Man-6-P-Man$_{6}$-BSA persisted for at least 15 h. When fibroblasts were incubated for 1 and 4 h with 0.5 mM cycloheximide and then treated for 30 min with 5 µg/ml BFA and cycloheximide, a 2.6-fold increase of cell surface Man-6-P/IGF-II receptors was observed (not shown). The increased expression of Man-6-P/IGF-II receptors is therefore due to redistribution of preexisting receptors and not dependent on the synthesis of new receptors.

About 20% of the Man-6-P/IGF-II receptors at the cell surface of fibroblasts are occupied with endogenous Man-6-P-containing ligands (15). In BFA-treated cells only 9% (n = 5) of the cell surface receptors were occupied, indicating that largely unoccupied receptors are redistributed to the cell surface.

BFA Stimulates the Endocytosis of Man-6-P/IGF-II Receptor Ligands—To examine whether the increase of cell surface expression results in an increased endocytosis of receptor ligands the uptake of the lysosomal enzyme arylsulfatase A
with $^{125}$I-Man-6-P-Man$_6$-BSA, BFA increased the amount of internalized and of degraded ligands 4-fold (not shown). These results indicate that the transport of Man-6-P-containing ligands to lysosomes and the degradation therein is not affected by BFA.

Effect of BFA on the Internalization and Recycling of Man-6-P/IGF II Receptor—At steady state the number of receptors that is internalized per unit of time equals that which is recycled to the cell surface. Thus, $k_{int} = k_{ext} + k_{int}$ where $k_{int}$ represents the rate of internalization, $k_{ext}$ the rate of externalization, and $[R]_{int}$ the concentration of receptor available for recycling to the cell surface. An increase of the Man-6-P/IGF II receptor concentration at the cell surface can result from a decrease of $k_{int}$, an increase of $k_{ext}$, or an increase of $[R]_{int}$.

To examine whether BFA affects $k_{int}$, we measured the uptake of prebound (4 °C) ligands upon warming the cells to 16 or 20 °C. The uptake of prebound Man-6-P-Man$_6$-BSA or 2C2 antibody by BFA-treated cells was faster and more efficient (Fig. 3B). The initial rate of internalization and the relative amount of ligand internalized during the incubation for 30 or 60 min were 20–40% higher than in controls. The improved internalization was accompanied by a decreased release of prebound ligands into the medium (Fig. 3C), while the rate of disappearance of ligands from the cell surface was not affected by BFA (Fig. 3A).

The redistribution of Man-6-P/IGF II receptors to the cell surface of BFA-treated cells must therefore result from an increase of $k_{int}$, which overrides the effect of the higher $k_{ext}$. The externalization of internalized Man-6-P-Man$_6$-BSA was measured to obtain information about the effect of BFA on $k_{ext}$. After binding of $^{125}$I-Man-6-P-Man$_6$-BSA at 4 °C the cells were shifted to 37 °C for 3 min, and the surface bound ligands were stripped by washing with 2 mM Man-6-P. The cells containing the internalized Man-6-P-Man$_6$-BSA were then incubated for up to 15 min at 37 °C.

**TABLE II**

Endocytosis of Man-6-P/IGF II receptor ligands in BFA treated fibroblasts

| Ligand                  | Uptake % of control |
|-------------------------|---------------------|
| Man-6-P-Man$_6$-BSA     | 437 ± 74 (6)*       |
| Arylsulfatase A         | 203 ± 13 (5)        |
| 2C2                     | 223 ± 31 (5)        |

* The numbers in parentheses represent the number of independent experiments.

was measured. Cells were pretreated for 30 min at 37 °C in the presence of 5 μg/ml BFA and then incubated for additional 30 min at 37 °C with $^{125}$I-arylsulfatase A and BFA. The uptake of $^{125}$I-arylsulfatase A was 2.0-fold increased in BFA-treated cells compared to untreated cells (Table II). Also the uptake of the 2C2 antibody was 2.2-fold increased (Table II) and linear for at least 120 min in BFA-treated cells (not shown). The endocytosis of $^{125}$I-Man-6-P-Man$_6$-BSA, however, was 4.4-fold increased (Table II). It should be noted that the binding of Man-6-P-Man$_6$-BSA was also increased only 2-fold as that of 2C2 (see Table I). In Hep G2 cells (see below) binding and uptake of Man-6-P-Man$_6$-BSA was increased 2-fold. BFA may induce conformational changes of Man-6-P/IGF II receptors in BFA-treated fibroblasts which specifically favor the interaction with Man-6-P-Man$_6$-BSA at 37 °C. This specificity may be related to the polyvalency of Man-6-P-Man$_6$-BSA (≥30 mol of Man-6-P/mol BSA).

To examine whether BFA affects the transport from endosomes to lysosomes Percoll density centrifugation was performed with postnuclear supernatants obtained from cells which had internalized $^{125}$I-arylsulfatase A in the absence and presence of BFA for 15 and 45 min. In cells incubated for 15 min with $^{125}$I-arylsulfatase A 12% of the ligand was associated with the dense lysosomal fraction. This fraction increased to 38% after an internalization period for 45 min and was not affected by BFA. When cells were incubated for up to 3 h
The incubation medium was supplemented with 2 mM Man-6-P to induce the release of Man-6-P-Man, BSA. During the 15-min incubation, 66% of the internalized Man-6-P-Man, BSA was released into the medium by control cells and 77% by BFA-treated cells (Fig. 4). This clearly indicates that \( k_{\text{ext}} \) is increased in BFA-treated cells. The concentration of intracellular Man-6-P/IGF II receptors \( [R]_{\text{int}} \) recycling to the cell surface cannot be determined by available methods.

**Additive Effect of IGF I, IGF II, and BFA on the Redistribution of Man-6-P/IGF II Receptors—IGF I and IGF II are known to induce a redistribution of Man-6-P/IGF II receptors to the cell surface by increasing the externalization \( k_{\text{ext}} \times [R]_{\text{int}} \) (18). It was therefore of interest whether IGF I and IGF II induce the redistribution through a mechanism which is shared with or independent of the BFA-induced mechanism.

Incubation of fibroblasts for 10 min with saturating amounts (10^{-6} M) of IGF I and IGF II resulted in a 2- and 1.6-fold increase of the Man-6-P/IGF II receptor expression at the cell surface (26). When the cells had been treated for 30 min with BFA, IGF I and IGF II led to a 3.6- and 2.7-fold increase of Man-6-P/IGF II receptor expression (Table III). The effects of IGF I and IGF II are therefore largely additive to that of BFA, suggesting that BFA and the growth factors induce the receptor redistribution through independent mechanisms.

**BFA Increases the Cell Surface Expression of the Man, 46,000 Manose 6-Phosphate Receptor, but Not of Transferrin Receptors—** The effect of BFA on two other recycling receptors in fibroblasts was examined. The surface expression of the Man, 46,000 mannose 6-phosphate receptor (MPR 46) cannot be followed directly by binding of ligands since this receptor does not bind Man-6-P-containing ligands when expressed at the cell surface (11, 27). Furthermore, its expression is too low to be measured by binding of receptor antibodies. We, therefore, measured the continuous uptake of the monoclonal antibody 21D3 against the Man-6-P/IGF II receptor (see Table II). Treatment with BFA for 30 min increased the uptake of 125I-labeled 21D3 to 163 ± 22% \( (n = 4) \) of control indicating that MPR 46 expression at the cell surface is also increased by BFA.

The surface expression of transferrin receptors measured by binding of 125I-labeled transferrin decreased to 85 and 83% after treating cells for 0.5 and 1 h with BFA.

**BFA Induces the Redistribution of Man-6-P/IGF II Receptors in a Variety of Cell Types—** Treatment of the human hepatoma cell line Hep G2, of Chinese hamster ovary cells, and of transfected mouse L+ cells and baby hamster kidney cells, which overexpress the human Man-6-P/IGF II receptor, revealed that BFA increases the concentration of the receptor at the cell surface 2.1-3.1-fold (Table I).

In Hep G2 cells the distribution of Man-6-P/IGF II receptor was also analyzed by indirect immunogold labeling. The fraction of protein A-gold particles present at the plasma membrane of BFA-treated cells was twice that in controls. Interestingly, also the distribution of Man-6-P/IGF II receptors at the cell surface was affected by BFA. While in controls 8% of immunogold particles at the plasma membrane of BFA-treated cells was twice of that in controls, the uptake of 125I-labeled 21D3 against the MPR 46 at 37 °C under conditions analogous to that for the 2C2 antibody directed against the Man-6-P/IGF II receptor (see Table II). Treatment with BFA for 30 min increased the uptake of 125I-labeled 21D3 to 163 ± 22% \( (n = 4) \) of control indicating that MPR 46 expression at the cell surface is also increased by BFA.

**DISCUSSION**

The present study describes the redistribution of Man-6-P/IGF II receptors induced by BFA in a variety of cell types. BFA increases the concentration of the receptor at the cell surface 2-fold. Formally, such an effect could result from a decrease of \( k_{\text{int}} \), an increase of \( k_{\text{ext}} \), and/or an increase of internal receptors that recycle. The increase of \( k_{\text{ext}} \) that was observed is likely to be compensated by the increase of \( k_{\text{int}} \). We assume, therefore, that the number of internal receptors

**TABLE IV**

**Distribution of the Man-6-P/IGF II Receptor in Hep G2 cells**

| Treatment | None | BFA (2 h, 1 μg/ml) |
|-----------|------|-------------------|
| Plasma membrane | 7 | 14 |
| Coated pits | 8 | 30 |

* Percentage of gold particles at the plasma membrane. The data were calculated from 900 gold particles counted in 125 cells.

* Gold particles in coated-pit areas expressed as percentage of the plasma membrane bound gold particles. In controls 110 and in BFA-treated cells 143 gold particles at the cell surface were counted.
that are available for recycling to the cell surface is increased in BFA-treated cells.

Man-6-P/IGF II receptors can recycle from endosomes either to the cell surface or to the TGN (11). They share this secretory pathway. Interestingly, BFA induces an increase of both Man-6-P receptors at the cell surface, while the surface concentration of the transferrin receptor, which recycles largely between early endosomes and the plasma membranes (28, 29) is slightly decreased in BFA-treated cells. The possibility should therefore be considered that BFA impairs the recycling of receptors to the TGN and thereby increases the number of receptors that can recycle from endosomes to the cell surface. The resylation of Man-6-P/IGF II receptors in BFA-treated cells that was observed by Chege and Pfeffer (7) would imply that transport to the TGN is only impaired but not abolished. Alternatively, BFA could facilitate the recycling of receptors between the cell surface and intracellular compartments that normally are excluded from recycling.

BFA not only increases the surface expression of Man-6-P/IGF II receptors, but also alters the distribution at the cell surface. In Hep G2 cells the fraction of surface receptors associated with coated pits was 4-fold higher than in controls. It remains to be shown whether the present level is maintained by clathrin and whether the coated pits are altered in size or number. It is conceivable that the effect of BFA on the distribution of Man-6-P/IGF II receptors is related to its stimulatory effect on the rate of internalization.

While the dramatic effect of BFA on the morphology of the Golgi and the redistribution of Golgi components to the ER are well-described (2, 6, 30), it is still unknown how BFA acts on the molecular level. The earliest effect of BFA noted so far is the rapid (within 30 s) displacement of the 110-kDa β-COP subunit from the Golgi membranes (31). β-COP is a peripheral protein on the cytoplasmic side of the transitional ER, Golgi stack, and TGN and a major component of the nonclathrin coat of vesicles that are thought to mediate intercisternal transport in the Golgi (32, 33). BFA is supposed to prevent the assembly of the nonclathrin coat required for the formation of vesicles mediating anterograde transport from the ER to the Golgi and across the Golgi (34).

The effect of BFA on the redistribution of Man-6-P receptors could result from the interaction of BFA (or its receptor) with a component required for vesicular transport within the endocytic route or the transport from endosomes to the TGN. It is also conceivable however, that the redistribution of Man-6-P receptors is secondary to the effects of BFA on the secretory route, e.g. the acceptor properties of the TGN for vesicles arriving from endosomes may be altered in BFA-treated cells. The accumulation of such vesicles may impair their formation. Notwithstanding the redistribution of Man-6-P receptors to the cell surface is a direct or an indirect effect of BFA on the endocytic route, our findings clearly demonstrate that the biological effects of BFA are not restricted to the secretory pathway.

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