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Effects of Brefeldin A on the Endocytic Route

REDISTRIBUTION 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 M_r 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)[1] is an isoprenoid 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 M_r 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-Man_4-BSA) was prepared from *Hansenula holstii* phosphomannan, 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. Märki, Ciba-Geigy, Basel; brefeldin A was kindly provided by Dr. Nespital, Sandoz, Nürnberg. Monoclonal antibodies (2C2) against the Man-6-P/IGF II receptor were those as described previously (15). Monoclonal antibodies (21D3) against the MPR 46 and recombinant arylsulfatase A were provided by Dr. A. Hille and H. J. Sommerlade of this institute, respectively. Disuccinimidyl suberate and IODOGEN were from Pierce Chemical Co., Rockford, IL. Man-6-P-Man_4-BSA was iodinated with the aid of IODO-GEN (15) to a specific activity of 163 μCi/μg, diferric transferrin, 2C2 and 21D3 antibodies and arylsulfatase A to specific activities of 1.6–5.8 μCi/μg. The cDNA for the human Man-6-P/IGF II receptor and mouse L^- cells deficient for the Man-6-P/IGF II receptor were kindly provided by Drs. W. S. Sly and S. Kornfeld (St. Louis, MO). Na-^{125}I- and L-[^{35}S]methionine were obtained from Amersham and ^{14}C-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% (v/v) fetal calf serum (GIBCO) plus penicillin and streptomycin (GIBCO). The baby hamster kidney (BHK) cells and mouse L^- fibroblasts stably transfected with the cDNA for the human Man-6-P/IGF II receptor were kindly provided by Dr. R. Pohlmann and M. Wendland of this institute. These cells were maintained in MEM supplemented with 5% (v/v) fetal calf serum and 5 μg/ml puromycin. The human hepatoma cell line Hep G2 and chinese hamster ovary (CHO) cells were grown in MEM containing 10% (v/v) fetal calf serum.

Binding and Endocytosis of ^{125}I-Man-6-P-Man_4-BSA—Detailed descriptions of the binding and endocytosis assays using ^{125}I-Man-6-P-

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[1] The abbreviations used are: BFA, brefeldin A; IGF, insulin-like growth factor; Man-6-P-Man_4-BSA, pentamannosyl 6-*o*-phosphate-substituted bovine serum albumin; BSA, bovine serum albumin; MEM, minimal essential medium; MPR 46, M_r 46,000 mannose 6-phosphate-specific receptor; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; SDS, sodium dodecyl sulfate; TGN, trans Golgi network; ER, endoplasmic reticulum.

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Man_4-BSA as a Man-6-P/IGF II receptor ligand have been published elsewhere (15, 18).

Cross-linking of ^{125}I-IGF II to Fibroblasts—^{125}I-IGF II 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-arylsulfatase A was performed on Percoll density gradient 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}S]methionine 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 M 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 μCi/μg) (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 surface bound ligands were displaced by two washes with 0.2 M acetic acid, 0.5 M NaCl at pH 2.5 for 5 min (22) with an efficiency of about 80%. The cells were solubilized in 1 N NaOH, and the acid wash-associated radioactivity was referred to cell protein.

Binding and Uptake of ^{125}I-Labeled Receptor Antibodies—The binding of ^{125}I-labeled 2C2 antibodies was measured as described (15). For the internalization assay the fibroblasts were pretreated with BFA and then incubated at 37 °C for the indicated times with the ^{125}I-labeled antibodies 21D3 or 2C2 in MEM containing 20 mM Hepes, pH 7.2, 0.1% BSA. After washing the cells with ice-cold Hanks' solution the cells were solubilized in 1 N NaOH, and cell-associated radioactivity was referred to cell protein.

Other Methods—Hep G2 cells that were used for immunoelectron microscopy were fixed in 1% acrolein and 0.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 h at 0 °C. Subsequent preparation of cryosections and immunogold labeling of Man-6-P/IGF II receptors were performed as described (23). Autoradiograms and fluorograms were quantified using a laser scan densitometer (Ultroscan XL, LKB, Bromma). Alternatively the polypeptide bands were excised from the gel and either directly counted in a γ counter (Berthold, bf 5300) or after solubilization (Tissue-sol-Roth M) by β-scintillation spectrometry (Packard Tri Carb 1900 TR). For the evaluation of K_D and B_{max} for Man-6-P-Man_4-BSA the nonlinear regression method by Scatchard was used (24).

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_4-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_4-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_4-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_4-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. 2*A*). Using a 30-min pretreatment, half-maximal stimulation was observed at 10^{-6} M BFA (Fig. 2*B*). In all further experiments the cells were pretreated for 30 min

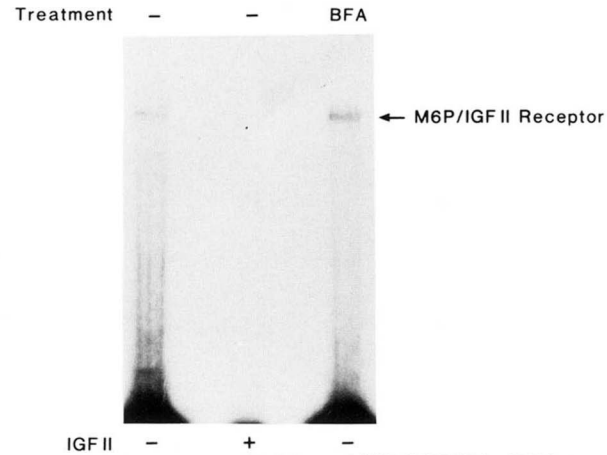

FIG. 1. **Affinity cross-linking of ^{125}I-IGF II in BFA-treated cells.** Fibroblasts were incubated for 30 min in the absence or presence of BFA (5 μg/ml). After chilling the cells to 4 °C the cells were incubated with ^{125}I-IGF II for 16 h at 4 °C in the absence or presence of unlabeled IGF II (0.25 μg), cross-linked with disuccinimidyl suberate, and analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions and visualized by autoradiography. The position of the Man-6-P/IGF II receptor is indicated. Counting 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

Effect of BFA on binding of Man-6-P/IGF II receptor ligands in various cell types

The cells were incubated with 5 μg/ml BFA for 30 min at 37 °C. Subsequently the binding of ^{125}I-Man-6-P-Man_4-BSA was determined. The values express the mean percentage ± S.D. in respect to untreated controls or the mean percentage of two independent experiments. Untreated human fibroblasts, Hep G2, CHO, and transfected BHK and mouse L$^-$ cells bound 0.19 ± 0.09, 0.11 ± 0.10, 0.08, 0.07, and 0.03 ng of Man-6-P-Man_4-BSA per mg of cell protein, respectively. Nontransfected mouse L$^-$ cells and BHK cells do not bind Man-6-P-Man_4-BSA. n = number of independent experiments.

Cell type	^{125}I-Man-6-P-Man_4-BSA
	% of control
Human fibroblasts	257 ± 43 (n = 31)
Hep G2	207 ± 30.5 (5)
Mouse L^{-a}	236 (219, 254)
BHK^a	311 (310, 313)
CHO	303 (275, 330)

[a] Overexpressing the human Man-6-P/IGF II receptor.

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 $t_{1/2}$ of about 30 min (Fig. 2*A*). In presence of BFA the increased binding of ^{125}I-Man-6-P-Man_4-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 cylcoheximide, 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

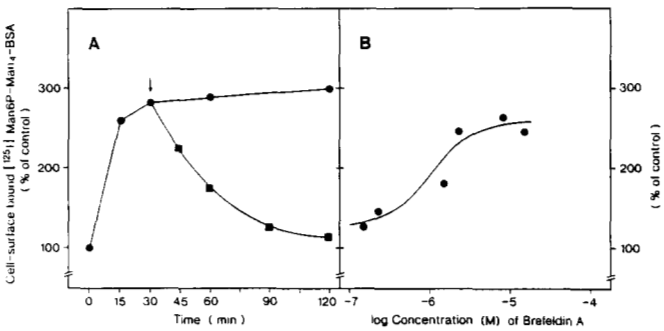

FIG. 2. **Time and concentration dependence and reversibility of BFA-induced redistribution of Man-6-P/IGF II receptor.** *A*, fibroblasts were either incubated for various times (●) with 5 μg/ml (1.8×10^{-5} M) BFA or for 30 min in the presence of BFA and recultured after removal of BFA (↓) for the indicated time (■). *B*, fibroblasts were incubated at various concentrations of BFA for 30 min at 37 °C. Subsequently the binding of ^{125}I-Man-6-P-Man$_4$-BSA was determined at 4 °C and expressed as percentages of untreated controls. The figure shows one of two experiments.

TABLE II

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

Fibroblasts were incubated with MEM containing 20 mM Hepes, pH 7.2, and 0.1% BSA in the absence or presence of 5 μg/ml BFA for 30 min. Subsequently the internalization of ^{125}I-labeled Man-6-P-Man$_4$-BSA (50 pM, 1×10^6 cpm), ^{125}I-arylsulfatase A (0.3×10^6 cpm) and ^{125}I-2C2 (0.4×10^6 cpm) was determined after 30 min at 37 °C in the presence or absence of BFA. The values are expressed as mean percentage ± S.D. of the respective controls measured in the absence of BFA. The controls internalized 0.27 ± 0.22 ng of Man-6-P-Man$_4$-BSA; 4.36 ± 0.26 ng of arylsulfatase A; and 3.59 ± 1.34 ng of 2C2 per mg of cell protein within 30 min.

Ligand	Uptake
	% of control
Man-6-P-Man$_4$-BSA	437 ± 74 (6)[a]
Arylsulfatase A	203 ± 13 (5)
2C2	223 ± 31 (5)

[a] 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$_4$-BSA, however, was 4.4-fold increased (Table II). It should be noted that the binding of Man-6-P-Man4-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$_4$-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$_4$-BSA at 37 °C. This specificity may be related to the polyvalency of Man-6-P-Man$_4$-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 with ^{125}I-Man-6-P-Man$_4$-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, $k_{int} \times [R]_{sur} = k_{ext} \times [R]_{int}$, where k_{int} represents the rate of internalization, $[R]_{sur}$ the concentration of receptors at the cell surface, 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$_4$-BSA or 2C2 antibody by BFA-treated cells was faster and more efficient (Fig. 3*B*). The initial rate of internalization and the relative amount of ligand internalized during the incubation for 30–50 min were 20–40% higher than in controls. The improved internalization was accompanied by a decreased release of prebound ligands into the medium (Fig. 3*C*), while the rate of disappearance of ligands from the cell surface was not affected by BFA (Fig. 3*A*).

The redistribution of Man-6-P/IGF II receptors to the cell surface of BFA-treated cells must therefore result from an increase of $k_{ext} \times [R]_{int}$, which overrides the effect of the higher k_{int}. The externalization of internalized Man-6-P-Man$_4$-BSA was measured to obtain information about the effect of BFA on k_{ext}. After binding of ^{125}I-Man-6-P-Man$_4$-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$_4$-BSA were then incubated for up to 15 min at 37 °C.

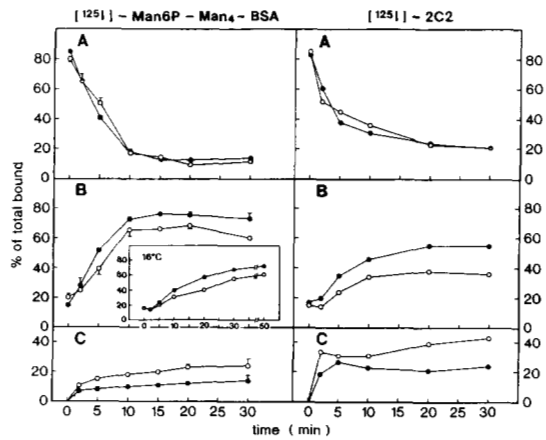

FIG. 3. **Effect of BFA on internalization and dissociation of ^{125}I-Man-6-P-Man$_4$-BSA and ^{125}I-2C2 prebound to the cell surface.** Fibroblasts were incubated for 30 min with or without BFA (5 μg/ml) prior to binding of ^{125}I-Man-6-P-Man$_4$-BSA or ^{125}I-2C2 for 4 h at 4 °C. After removal of unbound labeled ligands, the cells were incubated at 20 °C with MEM/BSA without (○) or with BFA (●). At various time points the cells were chilled to 4 °C and the radioactivity at the cell surface (*A*), in the cell extracts (*B*), and in the medium (*C*) were determined. The values for ^{125}I-Man-6-P-Man$_4$-BSA are given as the mean and range of two independent experiments. The values for ^{125}I-2C2 are the mean of duplicates of one experiment and are expressed as percentage of total bound radioactivity. *Inset*, the internalization of ^{125}I-Man-6-P-Man$_4$-BSA prebound to the cell surface is shown after warming to 16 °C in the absence (○) or presence (●) of BFA.

The incubation medium was supplemented with 2 mM Man-6-P to induce also the release of Man-6-P-Man_4-BSA returning to the cell surface in a receptor-bound form and to prevent re-uptake of secreted Man-6-P-Man_4-BSA. During the 15-min incubation 56% of the internalized Man-6-P-Man_4-BSA was released into the medium by control cells and 77% by BFA-treated cells (Fig. 4). This clearly indicates that k_{ext} is increased in BFA-treated cells. The concentration of intracellular Man-6-P/IGF II receptors $[R]_{int}$ 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 $k_{ext} \times [R]_{int}$ (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^{-8} 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.

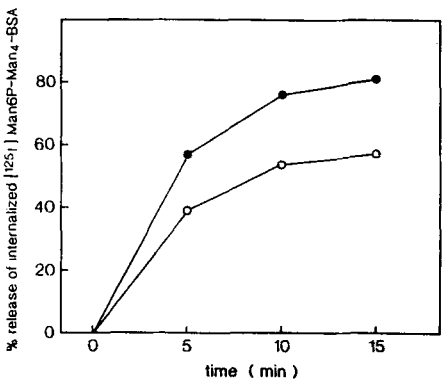

FIG. 4. **Effect of BFA on the externalization of ^{125}I-Man-6-P-Man_4-BSA.** Fibroblasts were incubated for 30 min with or without BFA (5 μg/ml) prior to binding of ^{125}I-Man-6-P-Man_4-BSA for 4 h at 4 °C. After removal of unbound ^{125}I-Man-6-P-Man_4-BSA the cells were warmed up to 37 °C for 3 min in the absence or presence of BFA. The cells were chilled to 4 °C, and cell surface-bound ^{125}I-Man-6-P-Man_4-BSA was displaced by Man-6-P. After washing, the cells were again warmed to 37 °C in the absence (○) or presence (●) of BFA in MEM containing 20 mM Hepes, pH 7.2, 0.1% BSA, and 2 mM Man-6-P to prevent re-uptake of ligands. At various time points the radioactivity in the medium was determined and expressed as percentage of internalized radioactivity.

TABLE III

Effect of growth factors on Man-6-P/IGF II receptor redistribution in BFA-treated cells

Fibroblasts were incubated for 30 min in the absence or presence of BFA (5 μg/ml). During the last 10 min IGF I or IGF II (10^{-8} M) were added. After chilling the cells to 4 °C the binding of ^{125}I-Man-6-P-Man_4-BSA was determined. The values are expressed as the mean of the percentage ± S.D. of untreated controls. The controls bound 0.22 ± 0.08 ng of ^{125}I-Man-6-P-Man_4-BSA. The number of independent experiments is $n = 6$.

Treatment (30 min)	Bound ^{125}I-Man-6-P-Man_4-BSA	
	None	BFA
	% of control	
Stimulation (10 min)		
None	100	215 ± 36.8
IGF I	195 ± 29.7	357 ± 55.6
IGF II	161 ± 19.3	268 ± 39.8

BFA Increases the Cell Surface Expression of the M_r 46,000 Mannose 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 M_r 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 MPR 46 at 37 °C under conditions analogous with 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 ^{125}I-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 ^{125}I-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 for 30 min with 5 μg/ml BFA, increased the expression of Man-6-P/IGF II receptor 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 of 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 cell surface were associated with coated-pit areas, this fraction increased to 30% in BFA-treated Hep G2 cells (Table IV). In spite of higher number of Man-6-P/IGF II receptors in coated-pit areas, the uptake of Man-6-P-Man_4-BSA in these cells was increased by BFA only 2-fold (not shown).

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_{int}, an increase of k_{ext}, and/or an increase of internal receptors that recycle. The increase of k_{ext} that was observed is likely to be compensated by the increase of k_{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

Cells were incubated at 37 °C for 2 h with 1 μg/ml BFA and fixed in 1% acrolein and 0.5% glutarladehyde in 0.1 M phosphate buffer, pH 7.4, for 1 h on ice. Cryosections were labeled with rabbit anti-Man-6-P/IGF II receptor immunoglobulin followed by protein A-gold (10 nm).

Treatment	None	BFA (2 h, 1 μg/ml)
Plasma membrane[a]	7	14
Coated pits[b]	8	30

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

[b] 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 dual exit from endosomes with the second Man-6-P-specific receptor, the M_r 46,000 mannose 6-phosphate receptor. 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 resialylation 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 coat is presented 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 at the cell surface 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 unkown 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 site 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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