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TRANSPORT OF NEWLY SYNTHESIZED ARYLSULFATASE A TO THE LYSOSOME VIA TRANSFERRIN RECEPTOR-POSITIVE COMPARTMENTS

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We have studied the convergence of the biosynthetic lysosomal route marked by the newly synthesized lysosomal enzyme arylsulfatase A (ASA) with the endosomal/ prelysosomal compartment in ASA overexpressing baby hamster kidney (BHK) cells. A monoclonal antibody against ASA conjugated to transferrin (Tf-αASA) was used to load the endocytic pathway via the transferrin receptor. Subsequent internalization of [^{125}I]labeled ASA and Tf-αASA conjugates at 18°C followed by rewarming to 37°C showed that immunocomplexes were formed within the recycling pathway and released into the medium. Furthermore, in cells labeled with [^{35}S]methionine for 10 min about 54% of newly synthesized ASA passed into Tf-αASA accessible compartments during a 4 hour chase period and accumulated in the medium. These data indicate that in overexpressing BHK cells the majority of newly synthesized ASA is transported to the lysosome via transferrin receptor-containing early endosomes.

Two mannose 6-phosphate receptor with M_r values of 46,000 (MPR 46) and 300,000 (MPR 300) mediate the transport of newly synthesized lysosomal enzymes equipped with mannose 6-phosphate (M6P) residues from the trans Golgi network (TGN) to an endosomal/ prelysosomal compartment. Due to the acidic endosomal pH the MPR-enzyme complexes dissociate and the enzymes are subsequently delivered to dense lysosomes while the receptors are recycled back to the Golgi or to the cell surface. The MPR 300 is able to bind and internalize exogenous M6P-containing ligands and the insulin-like growth factor II. MPRs in the TGN, endosomes and at the plasma membrane are in equilibrium, indicating that the biosynthetic and the endocytic pathways share at least one compartment (1, 2).

Receptors for a variety of ligands including nutrients, growth factors and toxins enter the cell via the same endocytic route as the MPRs but then become sorted selectively to a variety of destinations (3, 4). These sorting processes are believed to occur at various stages along the endocytic pathway

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Abbreviations: ASA, arylsulfatase A; Tf-αASA, transferrin coupled to antibodies against ASA; TfR, transferrin receptor; MPR 300, 300 kDa mannose 6-phosphate receptor; M6P, mannose 6-phosphate; TGN, trans Golgi network.

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(early, intermediate, late endosomes) (5-13). It is clear therefore that within the endocytic pathway there are several points of entry and exit for trafficking receptors.

To study whether the biosynthetic lysosomal pathway converges with the endocytic pathway already at the early endosomal stage a new experimental approach was used. Antibodies against the lysosomal enzyme arylsulfatase A (ASA) conjugated to transferrin (Tf-αASA) were introduced by endocytosis in ASA-overexpressing cells. Their ability to associate with metabolically labeled ASA arriving from the biosynthetic route was detected by the release of ASA-Tf conjugate immunocomplexes into the medium. We have shown that at least 54 % of the newly synthesized ASA are transported via transferrin receptor-positive compartments to the lysosome.

Materials and Methods

Na-[^{125}I] and L-[^{35}S]methionine were obtained from Amersham and [^{14}C]-labeled molecular weight standards were from DuPont New England Nuclear. M6P, human transferrin and mouse immunoglobulins were obtained from Sigma and Pansorbin from Calbiochem. Protein G-Sepharose were purchased from Pharmacia. IODO-GEN and N-succimidyl-3-2-pyridyldithio propionate (SPDP) were from Pierce Chemical Co. Recombinant ASA was affinity purified from BHK-HTCP3 cells as described (14). ASA and Tf conjugates were iodinated with IODO-GEN to specific activities of 1.6 - 5.8 μCi/μg.

Antibodies: A monoclonal antibody, 11B5 against the human ASA was produced and shown to be a member of the IgG2a subclass. The antibody was purified from ascitic fluids on protein G-Sepharose affinity chromatography according to manufacturer's instruction. The antibody binds to an epitope between amino acid residues 244 and 280 of ASA and precipitates more than 90% of total ASA activity and [^{35}S]methionine metabolically labeled ASA (14). Polyclonal goat antisera directed against human placenta ASA were previously described (15).

Preparation of transferrin conjugates: Human apotransferrin (Tf, 10 mg) was coupled to the antibody 11B5 (7 mg) using SPDP as a coupling reagent as described (16). The Tf-conjugates were separated from free antibodies and Tf by FPLC (Pharmacia; Hiload 16/60 Superdex 200) in 10 mM phosphate buffered saline, pH 7.4. The fractions, analyzed by SDS-PAGE and silver staining, containing low molecular weight Tf-antibody conjugates (M_r 250,000) were pooled and saturated with Fe^{3+} as described (17). Internalization studies in BHK cells showed that the equilibrium between surface bound (acid wash-sensitive) and internalized (acid wash-resistent) [^{125}I]Tf-αASA was reached after 60 min. A 200-fold excess of Tf inhibited the uptake of the Tf-conjugate by about 80%. The externalization kinetics of [^{125}I]Tf-αASA was also quiet similar to [^{125}I]Tf with a half time of about 5 min. Under conditions described below, saturating amounts of the Tf-αASA conjugate precipitated 63% of metabolically [^{35}S]methionine labeled ASA from cellular extracts compared with a polyclonal antibody against ASA.

Cell culture: Baby hamster kidney cells transfected with the cDNA of human arylsulfatase A (BHK HTCP3; 18) were maintained in MEM supplemented with 5% (v/v) fetal calf serum and 0.7 mg/ml neomycin.

Detection of newly synthesized ASA within the endosomal recycling pathway: BHK HTCP3 cells growing on 35 mm dishes were labeled for 10 min with [^{35}S]methionine (50-100 μCi/ dish) and chased for various time periods (30-240 min) in the presence or absence of Tf-αASA conjugates (14 μg/0.6 ml). The formed apotransferrin immune complexes were collected by exposure to Pansorbin from the media, adjusted to 0.4% Triton X-100, 0.2% sodium deoxycholate, 0.2% SDS, 0.9% BSA, 1 mM phenylmethylsulfonyl fluoride, 5 mM iodoacetic acid and 1 mM EDTA. The residual cell-associated ASA was immunoprecipitated, analyzed by SDS-PAGE (10% polyacrylamide) and fluorography (19). The fluorograms were quantified by laser scan densitometry (Ultra scan, LKB) or by excision of polypeptide bands from the gel, solublization and ß-scintillation spectrometry (Packard Tricarb 1900 TR). The accumulated [^{35}S]ASA complexes in the media were expressed as percentage of the sum of newly synthesized ASA in the medium and the cells.

Results

To test the ability of the Tf conjugate to bind ASA in endosomes and to recycle to the cell surface, non-transfected BHK cells were incubated for 2 h at 18°C with [^{125}I]ASA. At this temperature transport to the lysosomes is greatly inhibited (8, 11) but recycling to the surface continues. After removal of cell surface bound [^{125}I]ASA, the cells were incubated for an additional 2 h at 18°C with fresh medium in the absence or presence of Tf-αASA. During this period about 19% of the internalized [^{125}I]ASA appeared in the medium under both conditions (Fig. 1). However, the amount of [^{125}I]ASA accumulating in the medium during a subsequent incubation at 37°C for 30 min increased by 28% of total (internalized radioactivity) in the presence of Tf-αASA compared to 10% in the control medium. Due to separation of the route for lysosomal enzymes and the TfR pathway, the amount of [^{125}I]ASA accumulating in the medium decreased during a further 30 min period at 37°C. At the end of incubation, 43% of the [^{125}I]ASA internalized at 18°C remained cell associated in

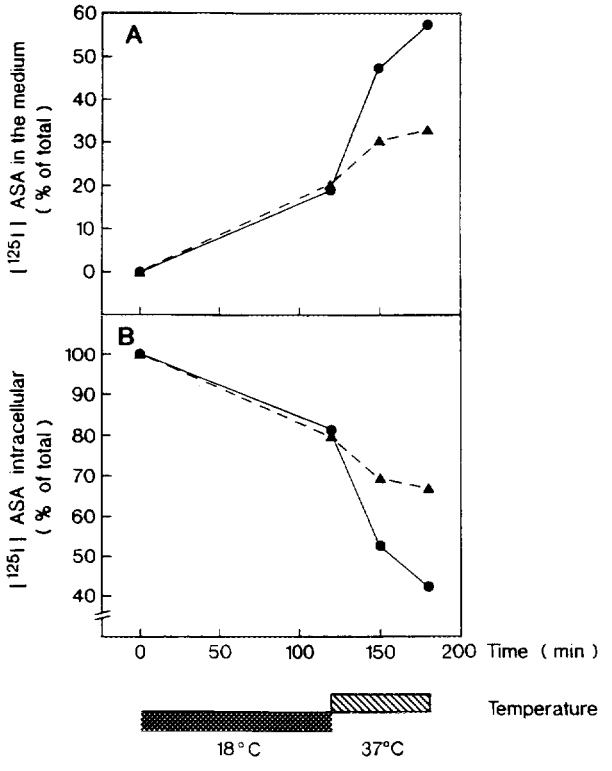

Fig.1. Tf-αASA induced secretion of endosomal [^{125}I]ASA. Two plates with BHK21 cells were incubated with [^{125}I]ASA in MEM, 0.1% BSA for 2 h at 18°C. After removal of the labeling medium and washing the cells were further incubated for 2 h in the absence (▲) or presence (●) of Tf-αASA at 18°C. Thereafter the media were removed and the cells were warmed up to 37°C for two times 30 min with prewarmed medium in the absence or presence of Tf-αASA. The media were collected after each incubation and the released radioactivity (A) and the remaining cell associated radioactivity (B) was determined and expressed as percentage of total prior internalized [^{125}I]ASA. The data are from a representive experiment out of three.

Tf-αASA treated cells compared to 67% in controls. These data indicate that Tf-αASA can form immunocomplexes with ASA molecules within the endosomal compartment which recycle to the plasma membrane and become released into the medium.

BHK-HTCP3 cells were then labeled for 10 min with [^{35}S]methionine and chased for 4 h in the absence or presence of increasing amounts (1.5 - 28 µg/0.6 ml) of Tf-αASA. The cell extracts and collected media were subjected for immunoprecipitation of ASA. About 6% of newly synthesized ASA (range 3 to 11%, n = 7) was secreted during the 4 h chase period in the absence of Tf-αASA (Fig. 2). The addition of increasing amounts of Tf-αASA conjugate during the 4 h chase period led to an accumulation of [^{35}S]labeled ASA in the medium reaching maximal values between 14 to 28 µg of conjugate per plate (mean 54%, range 46 - 65 % of the newly synthesized ASA; n = 7 at 14 µg Tf-αASA/plate). All further experiments were carried out with 14 µg Tf-αASA conjugate added to 0.6 ml chase medium. To examine the specificity of the Tf-αASA mediated accumulation of ASA in the medium, various control experiments were performed: 1) an excess of Tf during the chase in the presence of Tf-αASA abolished the Tf-αASA mediated ASA accumulation in the medium completely (Fig. 3, lane 2); 2) the incubation of cells with Tf conjugated to non-specific mouse IgG or with Tf alone did not increase the amount of ASA in the medium (lane 4 and 5); 3) to exclude the possibility that the ASA accumulation in the medium is due to the binding of Tf-αASA to ASA trafficking via the cell surface, the cells were chased in the presence of 5 mM M6P to displace ASA on the cell surface and inhibit the reinternalization by cell surface receptors. The newly synthesized ASA precipitated from the medium under these conditions did not exceed the level of untreated control cells (Fig. 3, lane 1 and 6). Together, these results show that newly synthesized ASA passes through TfR-containing compartments en route to the lysosome. Within the TfR-containing compartments the newly synthesized ASA binds Tf-αASA conjugates, recycles back to the cell surface and then becomes released into the extracellular medium.

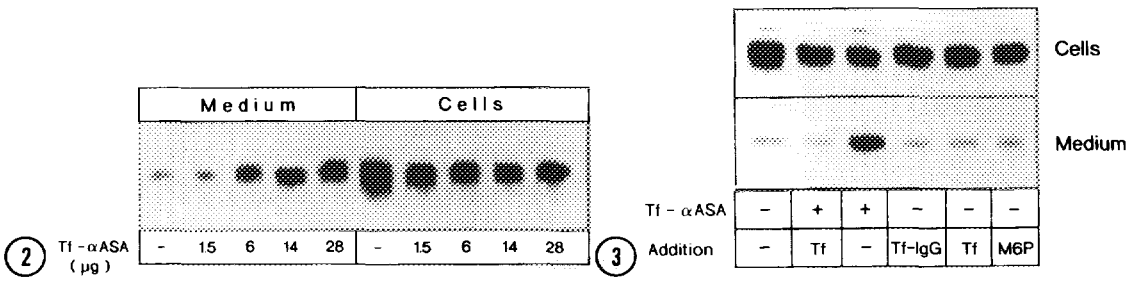

Fig.2. Tf-αASA mediated accumulation of newly synthesized ASA in the medium. BHK-HTCP3 cells were labeled for 10 min with [^{35}S]methionine and then chased for 4 h in the absence or presence of increasing amounts of Tf-αASA. The ASA was immunoprecipitated from the media and cells and analyzed by SDS-PAGE and fluorography.

Fig.3. Specificity of the Tf-αASA mediated accumulation of ASA in the medium. BHK-HTCP3 cells were labeled for 10 min and chased (0.6 ml/plate) for 4 h in the absence (lane 1) or presence of 14 µg Tf-αASA (lanes 2, 3), 47 and 9 µg Tf (lanes 2 and 5, respectively), 21 µg of an unspecific TfIgG conjugate (lane 4) or 5 mM M6P (lane 6). Subsequently ASA was immunoprecipitated from the media and cell extracts and analyzed by SDS-PAGE and fluorography.

To determine the total amount of formed ASA-Tf conjugate complexes as a function of chase time one plate with BHK-HTCP3 cells was labeled for 10 min with [^{35}S]methionine. The cells were then successively chased eight times of each 30 min in the presence of Tf-αASA followed by precipitation of the complexes from the media (Fig.4). The sum of [^{35}S]ASA in the media accounted for 52% of total newly synthesized ASA. The maximum of [^{35}S]ASA accumulating in the medium was reached 105 to 135 min after synthesis.

Discussion

The new experimental approach presented here supports a model in which the biosynthetic lysosomal pathway converges with the endocytic route in early endosomes open to efficient recycling. Antibodies against human ASA conjugated to Tf were used to load the endocytic receptor recycling pathway. We found that the internalized antibody conjugates formed immunocomplexes with newly synthesized ASA which accumulated in the culture medium. More than 50% of the newly synthesized ASA in BHK cells accumulated in the medium within 4 h.

The efficient binding of the Tf antibody conjugate to ASA followed by the recycling to the plasma membrane indicates that the ASA dissociates from the MPR 300 in early endosomes. These early endosomes have a pH of 6 to 6.3 (20) which is unlikely to be sufficiently acidic to cause the dissociation of MPR 300 ligands (21). On the other hand, Borden et al. (22) reported that in CHO cells immediately following a 2 min internalization period, 23% of the [^{125}I]M6P-containing ligands are already dissociated from MPR 300 reaching half maximal values within 11 min. It is also possible that the Tf antibody conjugate binds to the ASA-MPR 300 complex in early endosomes and as reaching later more acidic stages of the pathway ("sorting endosomes", 6), the ASA dissociates from the MPR 300 and recycles as Tf immunocomplexes to the plasma membrane. Whichever occurs, our data clearly show that newly synthesized ASA transported by MPR 300 enter the endocytic pathway in the TfR-containing endosome.These data are in agreement with recent studies detecting i) in steady state about 10 % of MPR 300 in HepG2 cells in Tf-horseradish peroxidase positive early endosomes (23), and ii) newly synthesized cathepsin D in early endosomal structures of NRK cells (24). The

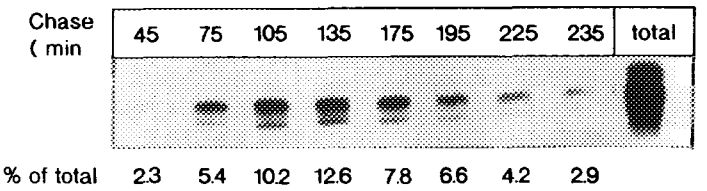

Fig.4. Time course of Tf-αASA induced accumulation of ASA in the medium. BHK HTCP3 cells were labeled with [^{35}S]methionine followed by a chase for 15 min to release the ASA from the endoplasmic reticulum. Subsequently, the cells were chased (0.6 ml/plate) 8 times for each 30 min with fresh medium containing Tf-αASA (10 µg). ASA was precipitated from all media and analyzed by SDS-PAGE and fluorography. The accumulated ASA in the media was quantified by determination of the radioactivity from the excised polypeptide bands and expressed as percentage of the total ASA (at the bottom of the lanes).

authors showed that cathepsin D accounted for 20 - 30% of total M6P-containing hydrolases found in the entire endocytic pathway.

The time course analysis of Tf immunocomplex formation revealed a maximum of newly synthesized ASA accumulated in the medium about 2 hours after synthesis. Thereafter, the amount of ASA accumulating in the medium decreased. These data are best explained if newly synthesized ASA passes transiently into TfR-containing endosomal compartments.However, we cannot exclude completely the formation of immunocomplexes in the TGN but it is unlikely to account for the major fraction because the passage of TfR through this compartment is very slow (25-27).

It is noteworthy to mention that due to the low expression of ASA, e.g. in human fibroblasts, we used BHK cells which overexpress the human ASA about 120-fold by activity (18).Therefore, it might be possible that MPR transferring newly synthesized lysosomal enzymes from the TGN to late endosomes become oversaturated resulting in an artificial opening of new transport routes, e.g. between the TGN and early endosomes. Thus, the overexpression of human α galactosidase in CHO-cells results in selective secretion of about 65 % of total enzyme synthesized (28). Even if a very low percentage (6 %) of newly synthesized ASA is secreted while the majority is transported via early endosomes to the lysosomes in BHK cells used here, the data can not extrapolated to other cell types. However, the use of antibodies coupled to Tf may offer the possibility also to quantify biochemically the passage of other more prominent soluble lysosomal enzymes through early endosomes in non-overexpressing cells.

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