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REGULAR ARTICLE

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Transferrin recycling and dextran transport to lysosomes is differentially affected by bafilomycin, nocodazole, and low temperature

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Abstract The effects of bafilomycin, nocodazole, and reduced temperature on recycling and the lysosomal pathway have been investigated in various cultured cell lines and have been shown to vary dependent on the cell type examined. However, the way in which these treatments affect recycling and transport to lysosomes within the same cell line has not been analyzed. In the current study, we used fluorophore-labeled transferrin and dextran as typical markers for the recycling and the lysosomal pathways, respectively, to explore the morphology and the intravesicular pH of endocytic compartments in HeLa cells. The V-ATPase inhibitor bafilomycin selectively inhibited the transport of marker destined for lysosomal degradation in early endosomes, whereas the transport of transferrin to the perinuclear recycling compartment (PNRC) still occurred. The kinetics of transferrin acidification was found to be biphasic, indicative of fast and slow recycling pathways via early endosomes (pH 6.0) and PNRC (pH 5.6), respectively. Furthermore, the disruption of microtubules by nocodazole blocked the transport of transferrin to the PNRC in early endosomes and of lysosome-directed marker into endosomal carrier vesicles. In contrast, incubation at 20°C affected the lysosomal pathway by causing retention of internalized dextran in late endosomes and a delay in transferrin recycling. Taken together, these data clearly demonstrate, for the first time, that the transferrin recycling pathway and transport of endocytosed material to lysosomes are differentially affected by bafilomycin, nocodazole, and low temperature in HeLa cells. Consequently, these treatments can be applied to investigate whether internalized macromolecules such as viruses follow a recycling or degradative pathway.

Keywords Endocytic pathways · Bafilomycin · Nocodazole · Low temperature · Endosomal pH · HeLa Ohio cells (Wisconsin strain)

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Introduction

Endocytosis is of major importance for the transport of nutrients, hormones, growth factors, and plasma proteins from the exterior into the interior of a cell. Internalized material is rapidly delivered to early or sorting endosomes, a compartment with tubulovesicular shape at the cell periphery (Conner and Schmid 2003). The interior of early endosomes and of other endocytic and some exocytic compartments is acidified by an ATP-dependent proton pump (the so-called vacuolar H^+-ATPase or V-ATPase; Nishi and Forgac 2002). Because of the activity of this pump, ingested material is exposed to an increasingly acidic pH during transport through endocytic compartments en route to lysosomes (Mellman et al. 1986; Murphy et al. 1984; Sonawane and Verkman 2003). The control of the acidification of endosomes plays an important role in the trafficking of macromolecules through endocytic organelles. As a result, the internalized material can follow various intracellular pathways after arrival in early endosomes (Mukherjee et al. 1997): (1) transport to lysosomes, by which ligands are degraded in lysosomes; (2) recycling, by which recycling proteins such as the transferrin receptor (TfnR) are recycled to the cell surface, and (3) in polarized

cells, transport from one plasma membrane domain to the opposite plasma membrane (transcytosis). The mildly acidic pH in early endosomes causes the dissociation of many receptors and ligands, and the membrane-bound components (i.e., the receptors) are efficiently recycled. Recycling receptors selectively accumulate in the tubular extensions of early endosomes, which then bud off to yield a population of transport vesicles that mediate recycling (Geuze et al. 1987), whereas most ligands are concentrated in the vesicular parts of early endosomes. The bulk volume containing the released ligands is then routed through late endosomes to lysosomes in which rapid degradation takes place.

The serum protein transferrin is of major importance for the cellular iron uptake mechanism (Chung and Wessling-Resnick 2003). The (iron-free) apotransferrin has the ability to bind two Fe^{3+} ions. Iron-loaded transferrin then binds with high affinity to the TfnR, which has a tyrosine motif (YXRF) in its cytoplasmic tail (Jing et al. 1990). The receptor-ligand complex is efficiently internalized via clathrin-coated pits and vesicles into acidic early endosomes, in which both Fe^{3+} ions are released, with apotransferrin remaining bound to the receptor. The free Fe^{3+} is reduced to Fe^{2+}, which is subsequently transported from the endosome interior into the cytosol by the divalent metal transporter DMT1 (Tabuchi et al. 2000). The apotransferrin-TfnR complex is recycled to the plasma membrane where the neutral pH of the extracellular environment causes its dissociation. Thus, apotransferrin can bind two other Fe^{3+} ions and reinitiate the cycle. After endocytosis into early endosomes, transferrin rapidly leaves this compartment ($t_{1/2}$ ≈2.5 min) and enters a perinuclear recycling compartment (PNRC) from which it is recycled ($t_{1/2}$ ≈7 min). The recycling of transferrin is typically biphasic with distinct fast (from early endosomes) and slow components (via the PNRC; Ghosh et al. 1994; Gruenberg and Maxfield 1995; Sheff et al. 1999). The PNRC is concentrated near the microtubule organizing center (MTOC) in most cell types. In Chinese hamster ovary (CHO) cells and in HEp-2 cells, this compartment is less acidic than early endosomes and contains sorted recycling material, such as the TfnR and low density lipoprotein (LDL) receptors, but no material targeted to lysosomes (Ghosh et al. 1994; Mayor et al. 1993; McGraw et al. 1993; Yamashiro et al. 1984). Moreover, the PNRC is biochemically distinct from early endosomes by the absence of the GTPase Rab5 and enrichment of Rab11 and VAMP3/cellubrevin and is also clearly distinct from other endocytic organelles in the perinuclear region, such as late endosomes and lysosomes (Daro et al. 1996; Miaczynska and Zerial 2002; Ullrich et al. 1996).

The acidic pH in endocytic and exocytic compartments can be raised to neutrality by the V-ATPase inhibitor bafilomycin, depending on the concentration applied (Bayer et al. 1998; Bowman et al. 1988). As a consequence, reduced rates of internalization and recycling of transferrin have been observed in CHO, TRVb-1, and Hep G2 cells (Johnson et al. 1993; Presley et al. 1997; van Weert et al. 1995). Nevertheless, transferrin transport to the PNRC is not inhibited and therefore results in its accumulation in this compartment (Presley et al. 1997). Thus, low endosomal pH per se appears not to be required for transferrin transport to the PNRC. Because of the requirement for low endosomal pH for iron release from apotransferrin, bafilomycin additionally prevents the release of recycled transferrin (van Weert et al. 1995).

The microtubule disrupting agent nocodazole also causes reduced transferrin uptake in hepatocytes (Runnegar et al. 1997), whereas decreased formation of or transit to the PNRC in CHO cells has been observed (Daro et al. 1996). In addition, nocodazole leads to the dispersion of the PNRC throughout the cytoplasm, although the kinetics of transferrin endocytosis and recycling remain unaffected (McGraw et al. 1993). This suggests that translocation along microtubules is not the rate-determining step in transport from the PNRC to the plasma membrane. Presumably, a process including the minus-end-directed motor protein, cytoplasmic dynein, moves recycling membranes towards the MTOC (Burkhardt et al. 1997). Microinjection of function-blocking antibodies directed against detyrosinated stable microtubules (Glu-MT) or kinesin, a plus-end-directed motor protein, does not prevent the delivery of transferrin to the PNRC but slows its recycling to the cell surface (Lin et al. 2002). This strongly supports a role for stable Glu-MTs and kinesin in plus-end-directed transport in membrane recycling.

Furthermore, low temperature can affect the perinuclear recycling pathway and the kinetics of endosome acidification. Temperatures below 20°C cause a delay in endosome acidification in A549 and K562 cells, and transferrin recycling is completely blocked (Sipe et al. 1991; Sipe and Murphy 1987).

In addition to receptor-mediated endocytosis, uptake via non-specific fluid-phase endocytosis is of major importance for the internalization of various serum constituents. Material endocytosed by fluid-phase mechanism is preferentially destined for degradation in lysosomes by passing through distinct endosomal subcompartments, such as early endosomes, endosomal carrier vesicles (ECV; vesicular intermediates between early and late endosomes), and late endosomes (Gruenberg et al. 1989). Inhibition of vesicle acidification by bafilomycin results in reduced uptake of the fluid-phase markers horseradish peroxidase (HRP) and dextran in rat liver endothelial cells (Harada et al. 1997). In addition, this drug blocks distinct endocytic transport steps in various cell types. In BHK cells, bafilomycin completely inhibits the delivery of internalized HRP to ECV without significantly affecting the internalization step or recycling to the plasma membrane (Clague et al. 1994). Moreover, early endosomes become highly tubular in the presence of bafilomycin (D'Arrigo et al. 1997). Similarly, we have demonstrated that human rhinovirus serotype 2 (HRV2), which is taken up via receptors of the LDL receptor family, and the fluid-phase marker dextran are trapped in early endosomes in HeLa cells in the presence of the drug (Bayer et al. 1998). However, in HEp2 cells, the transport between late endosomes and lysosomes is inhibited by bafilomycin (van Deurs et al. 1995).

In contrast to bafilomycin, nocodazole treatment of BHK and HeLa cells results in the accumulation of fluid-

phase marker in ECV (Bayer et al. 1998; Gruenberg et al. 1989). In polarized MDCK cells, fluid-phase transport from apical early to late endosomes is microtubule-dependent, whereas endocytosis per se is not influenced by nocodazole (Apodaca 2001). In HEp-2 cells, microtubules play a role in preventing the formation of large lysosome aggregates (van Deurs et al. 1995) by separating endosomes and lysosomes and by moving them toward the cell periphery. Furthermore, pretreatment of CHO, 3T3, HeLa, and NRK cells with nocodazole inhibits the content exchange of lysosomes in cell fusion experiments (Deng and Storrie 1988). Thus, microtubules appear to be required for the normal positioning and motility of endocytic compartments, for transport between the various endocytic compartments, and for lysosome-lysosome exchange.

Moreover, the uptake and transport of fluid-phase markers through the endocytic pathway to lysosomes is also temperature-dependent (Roederer et al. 1987). Although uptake at 20°C is reduced, HRP is internalized into early endosomes in AtT20 cells (Tooze and Hollinshead 1991), and fluid-phase markers and HRV2 reach late endosomes but not lysosomes in HeLa cells (Kronenberger et al. 1997; Prchla et al. 1994). In rat hepatocytes, two temperature-sensitive steps along the degradative route have been identified: 1) between early and late endsomes, and 2) between late endosomes and lysosomes (Dunn et al. 1980; Ellinger et al. 2002; Mueller and Hubbard 1986).

Therefore, the effects of bafilomycin, nocodazole, and reduced temperature on endocytic pathways are cell-type-specific. So far, the influence of both types of drug and low temperature on the transferrin recycling pathway and on the transport of fluid-phase markers to lysosomes has not been investigated in the same cell type. We have therefore analyzed the effects of these treatments on HeLa cells by using fluorescence microscopy for morphological studies and flow cytometry to determine endosome acidification. Our results clearly indicate, for the first time, that, at least in HeLa cells, the inhibitor of the ATP-dependent H^+-pump bafilomycin, the microtubule disrupting agent nocodazole, and the influence of low temperature differentially affect the transferrin recycling pathway and the transport of endocytosed materials to lysosomes.

Materials and methods

All chemicals were purchased from Sigma (Sigma, St. Louis, Mo., USA), unless stated otherwise. Tissue culture media and supplements were from Gibco (Invitrogen, Paisley, UK). Tissue culture media for the growth of suspension-cultured cells was from Sigma. Tissue culture plates and flasks were from Iwaki (Bibby Sterilin, Staffordshire, UK). Alexa Fluor-568-conjugated transferrin (Alexa568-transferrin), dissolved in phoshate-buffered saline (PBS) at 5 mg/ml, was obtained from Molecular Probes (Eugene, Ore., USA). Fluorescein isothiocyanate (FITC)-dextran (Sigma) was dissolved in PBS at 50 mg/ml and then extensively dialyzed against TRIS-buffered saline (pH 7.4) and finally against PBS before use. FITC on celite was coupled to transferrin following the protocol of van Renswoude et al. (1982). Cy5.18-OSu (Cy5) was obtained from Amersham Biosciences (Buckinghamshire, UK) and coupled to dextran (M_r: 70 kDa) and transferrin as described (Rybak and Murphy 1998). Bafilomycin A1 (Alexis Biochemicals, San Diego, Calif., USA) and nocodazole (Sigma) were dissolved in dimethyl sulfoxide (DMSO) at 20 μM and 20 mM, respectively, and stored at −20°C.

Preparation of transferrin conjugates

Human differic transferrin (10 mg) was resuspended in 2.4 ml 0.1 M Na-bicarbonate buffer (pH 9.5), and Cy5.18-OSu (1.0 mg) was dissolved in 140 μl dry dimethylformamide. Cy5-solution (100 μl) was mixed with the transferrin solution and incubated for 1 h at room temperature. Subsequently, free Cy5 was removed by passing the solution over a Sephadex G-25 column. The dye:protein ratio of Cy5-transferrin as determined by spectroscopy was 1:4.

Cell culture

HeLa Ohio cells (Wisconsin strain, kindly provided by R. Rueckert, University of Wisconsin) were grown in monolayers in minimal essential medium (MEM) supplemented with 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin G sodium salt, and 100 μg/ml streptomycin sulfate. For suspension-cultured HeLa Ohio cells, MEM modified (Joklik) for suspension (S-MEM) was used containing 7% horse serum and the same additives.

Co-internalization of Alexa568-transferrin and FITC-dextran

HeLa cells were plated on coverslips and grown overnight until half-confluent, washed, and preincubated in serum-free MEM for 30 min at 37°C to deplete endogenous transferrin. Afterwards, cells were incubated for 5 min (pulse) at 37°C in 200 μl pre-warmed MEM containing 25 μg/ml Alexa568-transferrin and 10 mg/ml FITC-dextran. Cells were washed three times with pre-warmed MEM and either cooled to 4°C to halt endocytosis or further incubated for 15, 35, or 55 min (chase) at 37°C with 500 μl MEM in the absence of Alexa568-transferrin and FITC-dextran and subsequently cooled. Cells were immediately washed with ice-cold PBS^{++} (PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$) and incubated (2×5 min) with acid wash buffer (150 mM NaCl, 25 mM acetic acid in twice distilled water, pH 3) at 4°C to release Fe^{3+} from plasma-membrane-bound transferrin. Thereafter, cells were incubated (2×5 min) with PBS^{++} to release apotransferrin. To investigate the effect of bafilomycin and nocodazole, 200 nM bafilomycin A1 (final concentration) or 20 μM nocodazole (final concentration) was included in the medium (MEM) throughout the experiment (pre-incubation, internaliza-

tion), and the cells were processed as above. In experiments in which the effect of reduced temperature (20°C) was examined, all incubations in MEM were performed at 20°C. Otherwise, the conditions and concentrations of markers were identical to those described above.

Fluorescence microscopy

Cells were fixed with 4% paraformaldehyde for 30 min at 4°C, quenched with 50 mM NH_4Cl in PBS for 10 min at room temperature, and washed (3×) with PBS. Cells were mounted in Immuno Floure Mounting Medium (ICN Pharmaceuticals, Costa Mesa, CA,USA) and viewed with a Zeiss Axioplan 2 microscope (Carl Zeiss, Jena, Germany). Images were acquired by using a multiband filter set from Carl Zeiss (excitation filters F84-490 for FITC, and F83-570 for Alexa568; polychroic filter F83-100 for DAPI/FITC/Alexa568, with tripleband emission filter F83-101). In order to compare the control condition (Fig. 1) with the respective treatments (Figs. 2, 4, 8), all samples were photographed with the same exposure time, and images were processed identically by using Zeiss AxioVision and Adobe Photoshop software. Experiments were repeated 4–10 times, and about 100 cells under each condition were inspected; one representative experiment is shown in each of the figures.

Transferrin internalization and determination of endosomal pH

HeLa Ohio cells (2×10^8) grown in suspension were washed with PBS and pre-incubated for 30 min at 37°C or 20°C in serum-free MEM and, when indicated, for an additional 30 min in the presence of 20 μM nocodazole. Cells were washed twice and pelleted by centrifugation, and the cell pellet was resuspended in 2 ml PBS and divided into three samples (0.3, 0.7, and 1 ml). (1) The 0.3-ml aliquot was used as an "unlabeled sample" to determine the autofluorescence at the respective pH (see below). Cells were therefore incubated in 2 ml MEM without transferrin for 1 h at 4°C. Cells were washed and pelleted, and the pellet was resuspended in 1.2 ml PBS and then divided into six aliquots (200 μl each). Cells were centrifuged and the pellet was resuspended in 200 μl buffer pH 5.0–7.4 at 4°C (see below). (2) The 1-ml aliquot was used to determine the amount of "internal transferrin", and the endosomal pH and was incubated for 1 h at 4°C in 2 ml MEM (with or without nocodazole) containing 3 μg/ml FITC-transferrin and 0.5 μg/ml Cy5-transferrin. After extensive washes, cells were pelleted, and the pellet was resuspended in 1 ml PBS; a 0.3-ml aliquot of this suspension was used to generate the pH standard curve (see below). The remaining 0.7 ml was centrifuged and the cell pellet was resuspended in 3,000 μl pre-warmed MEM (37 or 20°C; with or without nocodazole). Cells were then incubated at 37°C or 20°C in a waterbath and at the times indicated in the figure legends; 100 μl suspension was removed, placed in a flow cytometry tube, and mixed with 400 μl ice-cold PBS to halt endocytosis. Thereafter, cells were washed twice and analyzed immediately by flow cytometry (eight parallel measurements were carried out). (3) For determination of the amount of "external transferrin", the 0.7-ml aliquot was incubated for 1 h at 4°C in 2 ml MEM (with or without nocodazole) containing 0.5 μg/ml Cy5-transferrin but no FITC-transferrin. After extensive washes and centrifugation, the cell pellet was resuspended in 3,000 μl pre-warmed MEM (37°C or 20°C; with or without nocodazole). At the indicated times, internalization was halted by removing 100 μl of the cell suspension and mixing it with 400 μl ice-cold PBS. Cells were washed (3×) and resuspended in 200 μl ice cold PBS. Subsequently, 50 μl FITC-labeled anti-human transferrin antibody (1:100; Binding Site, UK) was added and incubated for 30 min on ice to determine the percentage of

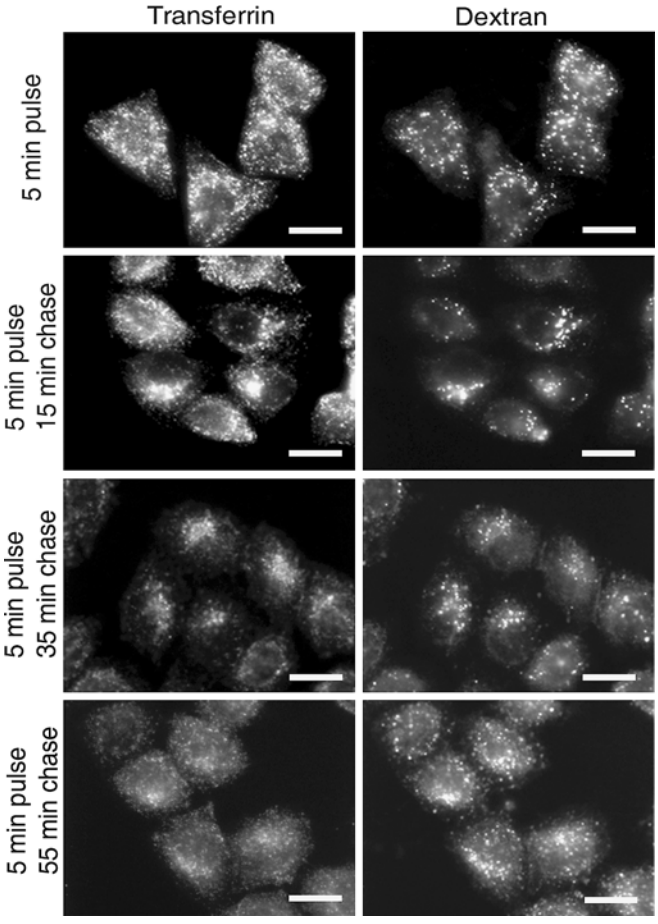


Fig. 1 Transferrin and dextran co-internalization at 37°C. HeLa cells were incubated for 5 min (*pulse*) at 37°C with 25 μg/ml Alexa568-transferrin and 10 mg/ml FITC-dextran, and then cells were further incubated in MEM without markers for 15, 35, and 55 min (*chase*). Thereafter, membrane-bound transferrin was removed by low pH followed by pH 7.4 treatment. Cells were then fixed and processed for fluorescence microscopy. To allow for comparisons of the control experiment shown here and the various treatments (Figs. 2, 4, 8), images were taken with the same exposure time and processed identically in AxioVision and Adobe Photoshop. Approximately 100 cells were inspected under each experimental condition; one typical experiment out of 10 is shown. *Bars* 20 μm

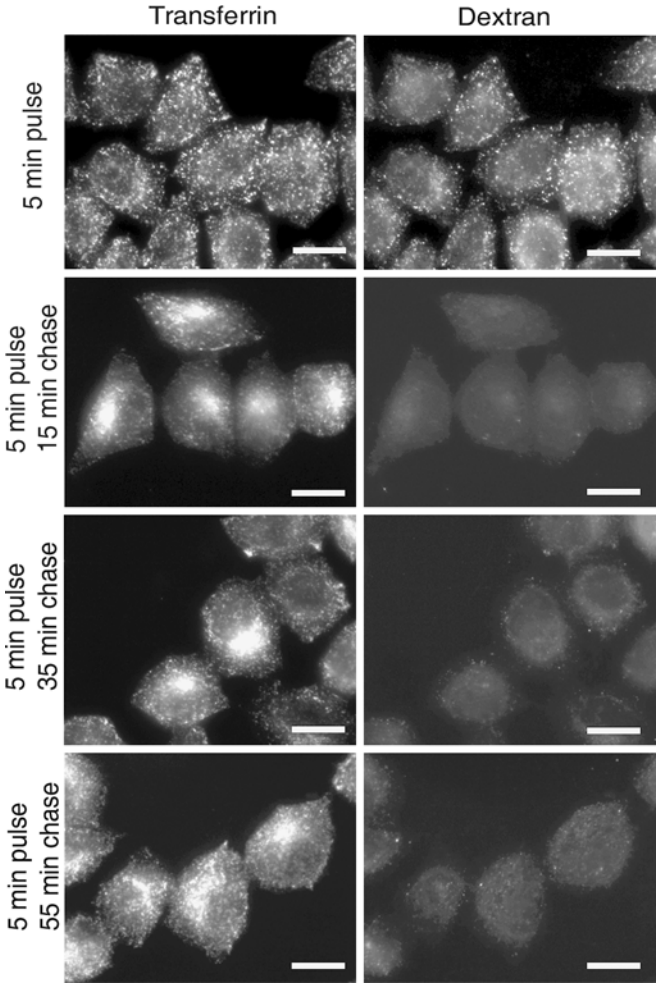


Fig. 2 Effect of bafilomycin on transferrin and dextran internalization. HeLa cells were depleted of endogenous transferrin by pre-incubation in MEM in the absence or presence of 200 nM bafilomycin for 30 min. Cells were then incubated for 5 min (*pulse*) at 37°C with 25 μg/ml Alexa568-transferrin and 10 mg/ml FITC-dextran followed by incubation in MEM without markers for 15, 35, and 55 min (*chase*). During the entire experiment, 200 nM bafilomycin was included in the medium. Plasma-membrane-bound transferrin was removed, and cells were fixed and processed for fluorescence microscopy. The corresponding control experiments are shown in Fig. 1. To allow for comparison, images were taken with the same exposure time and processed identically in AxioVision and Adobe Photoshop. Approximately 100 cells were inspected under each experimental condition; one typical experiment out of 10 is shown. *Bars* 20 μm

transferrin still bound to the plasma membrane (% of "external transferrin") for the indicated time points. Cells were subsequently washed with PBS and pelleted, and the cell pellet was resuspended in 400 μl PBS and immediately analyzed (eight parallel measurements).

Generation of a pH-standard curve of transferrin-labeled cells

For the generation of a pH standard curve, the 0-min sample of FITC- and Cy5-transferrin-labeled cells (see "internal transferrin" above; 1 h labeling at 4°C) was used. Cells were pelleted and divided into six aliquots. Each aliquot was resuspended in 300 μl buffer pH 5.0–7.5 at 4°C. Buffers (pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5) were obtained by mixing 50 mM HEPES pH 7.5 with 50 mM MES pH 4.5 (both containing 50 mM NaCl, 30 mM ammonium acetate, 40 mM sodium azide) to give the required pH. The samples were left on ice for 5 min for ATP-depletion and for pH equilibration. The mean fluorescence value of FITC- and Cy5-transferrin, respectively, was determined at the respective pH, and the autofluorescence of unlabeled samples was subtracted. Subsequently, the ratio of the FITC and Cy5 mean values at the respective pH was calculated and normalized to the value 1.0 at pH 7.4. These values were used to generate a pH standard curve to determine the endosomal pH of FITC/Cy5-transferrin-labeled compartments at the stated time intervals.

Calculation of the pH of transferrin-labeled compartments

Since the absolute FITC and Cy5 fluorescence signals varied from day to day, pH calibration curves were performed in parallel with all experiments. To allow comparisons between experiments, mean values for FITC and Cy5 fluorescence for each experiment were divided by the mean initial fluorescence (time point 0) and averaged for all experiments at a given temperature. Fluorescence values from "external transferrin" samples (see above) were also averaged and interpolated at the same intervals, allowing the estimation of the amount of total FITC and Cy5 fluorescence attributable to transferrin present on the plasma membrane. This amount was subtracted from the mean fluorescence signals for each interval. The ratio of FITC and Cy5 fluorescence for each of these intervals was then interpolated onto the normalized and averaged calibration curve. This allowed the calculation of average pH values for the fraction of transferrin contained within the internal cellular compartments only (Sipe and Murphy 1987).

Dextran internalization and determination of the endosomal pH

HeLa cells (10^8) grown in suspension were preincubated in 4 ml S-MEM for 30 min with or without 20 μM nocodazole at 37°C or 20°C. Cells were pelleted by centrifugation, and the pellet was resuspended in 2 ml MEM (pre-warmed at 20 or 37°C) containing 10% FCS, 12 mg FITC-dextran, 2 mg Cy5-dextran. When indicated, 20 μM nocodazole was present throughout the experiment. Endosomes were labeled for 5 min (pulse) at 37°C or 20°C, followed by incubation in 2 ml marker-free MEM for the times indicated (chase) in the figure legends. Endocytosis was stopped by the addition of ice-cold PBS (pH 7.4). Cells were washed (2×) with PBS and pelleted, and the pellet was resuspended in PBS and analyzed immediately by flow cytometry.

Generation of a pH-standard curve of dextran-labeled compartments

An aliquot (300 μl) of the cells labeled for 5 min (pulse) was divided into six aliquots (50 μl each). Cells were pelleted and resuspended in buffer pH 5.0–7.5 obtained as described above. For ATP depletion and for equilibration of intravesicular pH (pH clamped), 1 μl monensin (stock solution: 200 μM) was added, and the samples were left on ice for 5 min.

Determination of the pH of dextran-labeled endosomes

Each sample was analyzed eight times. The mean fluorescence values of FITC-dextran and Cy5-dextran were calculated for each sample, and the autofluorescence from unlabeled samples was subtracted. The ratio of FITC to Cy5 was determined, and the intravesicular pH was calculated from the standard curve.

Flow cytometry

A dual laser FACS Calibur (Becton Dickinson Immunocytometry Systems) equipped with argon-ion and red-diode lasers was used. FITC-fluorescence (488 nm excitation) was measured by using a 530-nm band-pass filter (30 nm band width), and Cy5-fluorescence (635 nm excitation) was measured by using a 661-nm band-pass filter (16 nm band width). Forward light scatter and 90° (side)-scatter, together with both fluorescence values, were collected in list mode by using 256-channel resolution. Data for 10,000 cells were collected for flow cytometry of cell suspensions.

Results

Effect of bafilomycin on transferrin and dextran endocytosis

The influence of bafilomycin A1 on endocytic pathways was examined by using transferrin, a well-known marker of the recycling pathway, and the fluid phase marker dextran (70 kDa) preferentially to follow transport to lysosomes. Alexa568-transferrin and FITC-dextran were used to visualize these markers by fluorescence microscopy. After depletion of endogenous transferrin, Alexa568-transferrin and FITC-dextran were co-internalized into HeLa cells in the absence or presence of 200 nM bafilomycin by application of a pulse-chase protocol (5 min pulse followed by a chase of up to 55 min). After internalization, transferrin still bound to the plasma membrane was removed by acid and neutral pH wash. Under control conditions, both markers mainly accumulated in small vesicles located in the periphery of the cells (early endosomes) immediately after the pulse (Fig. 1). After a 15-min chase, Alexa568-transferrin showed accumulation in large vesicles in the center of the cell near the nucleus, the PNRC. After a 35-min and 55-min chase, staining seemed to be less intense. Similarly, FITC-dextran also accumulated in perinuclear compartments after the chase. These compartments had been identified as late endosomes and lysosomes by subcellular fractionation of HeLa cells in our previous studies (Schober et al. 1998). Although a control condition was included in each experiment and treatment, we only show one such control (Fig. 1). To allow for comparisons, all images shown were taken with the same exposure time and identical processing in AxioVision and Adobe Photoshop.

In the presence of bafilomycin, Alexa568-transferrin was still delivered to the PNRC after a 15-min chase (Fig. 2). The same fluorescence pattern as under control conditions (without bafilomycin) was observed after 35-min and 55-min chases.

In contrast, after the blocking of the V-ATPase with bafilomycin, FITC-dextran remained in peripheral early endosomes after the 5-min pulse and after various chase

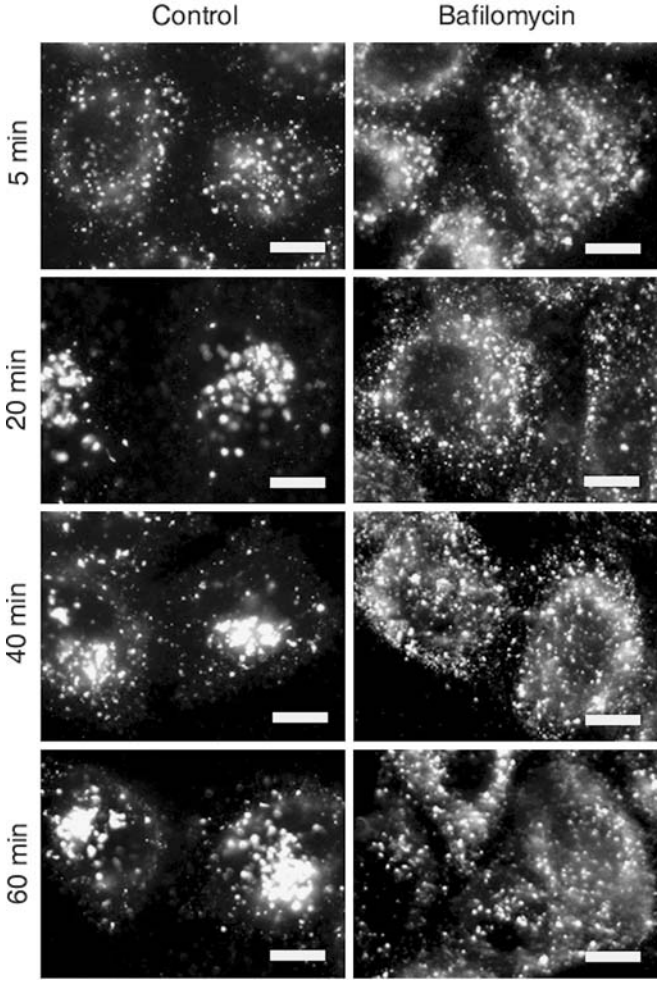


Fig. 3 Effect of bafilomycin on continuous dextran internalization. Cells were pre-treated as described in Fig. 3 and then continuously incubated with 10 mg/ml FITC-dextran for 5, 20, 40, and 60 min in the absence or presence of 200 nM bafilomycin. Afterwards, cells were processed for fluorescence microscopy. Approximately 100 cells were inspected; one experiment out of four is shown. *Bars* 10 μm

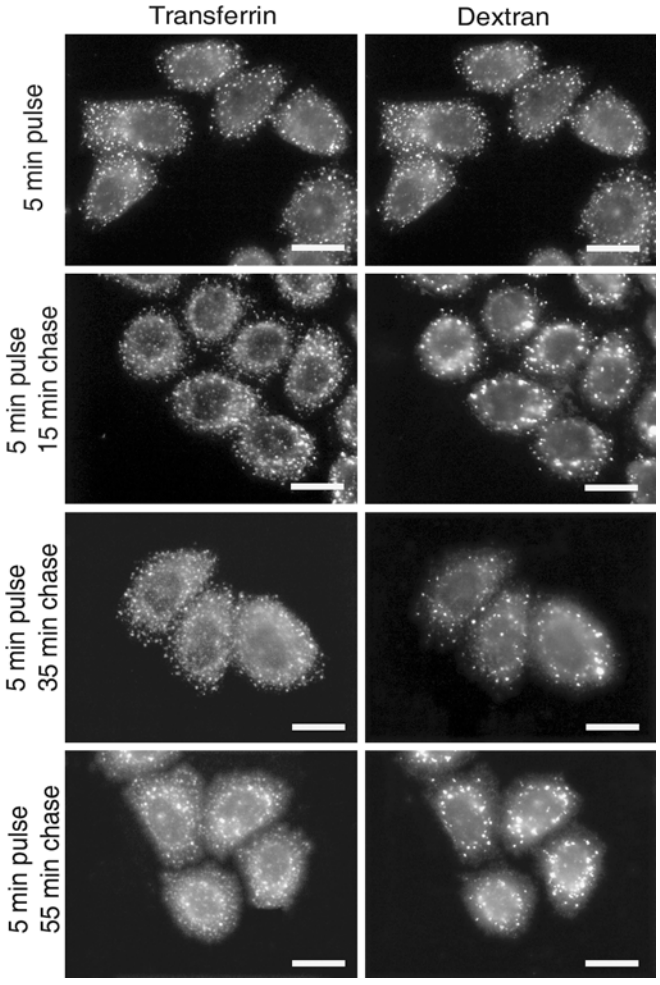


Fig. 4 Effect of nocodazole on transferrin and dextran internalization. Cells were incubated with Alexa568-transferrin and FITC-dextran and processed for fluorescence microscopy as in Fig. 1; nocodazole (20 μM) was present in the medium throughout the experiments. To allow for comparison with the respective control (Fig. 1), images were taken at the same exposure time and processed identically in AxioVision and Adobe Photoshop. Under each experimental condition, approximately 100 cells were inspected; one typical experiment out of 10 is shown. *Bars* 20 μm

periods (Fig. 2). No significant differences in the fluorescence pattern from the control experiments could be observed after the pulse, although after different chase periods, the FITC-staining was greatly reduced in the presence of bafilomycin (Fig. 2) compared with the control (Fig. 1). Therefore, to improve our analysis of the effect of bafilomycin on the lysosomal pathway, FITC-dextran was continuously internalized into Hela cells in the absence or in the presence of bafilomycin. Without bafilomycin, FITC-dextran accumulated in the perinuclear region of the cells after 20, 40, and 60 min (Fig. 3), whereas FITC-dextran remained distributed in small peripheral vesicles in the presence of bafilomycin, even after prolonged incubation times. Thus, bafilomycin inhibits the transport of cargo from peripheral early to (perinuclear) late endosomal compartments but does not prevent the delivery of transferrin to the PNRC in HeLa cells.

Effect of nocodazole on transferrin and dextran endocytosis

To investigate the influence of the microtubule-disrupting agent nocodazole on the endocytic pathways, the same pulse chase experiment (see above) was carried out in the absence or presence of nocodazole. The disruption of microtubules after a 30-min pretreatment with 20 μM nocodazole was verified in each experiment by indirect immunofluorescence microscopy with anti-tubulin antibody (data not shown). Our fluorescence microscopy experiments revealed that, after the 5-min pulse, peripheral vesicles were stained with Alexa568-transferrin and FITC-dextran under control conditions (see Fig. 1) and in the presence of nocodazole (Fig. 4) demonstrating that the transport from the plasma

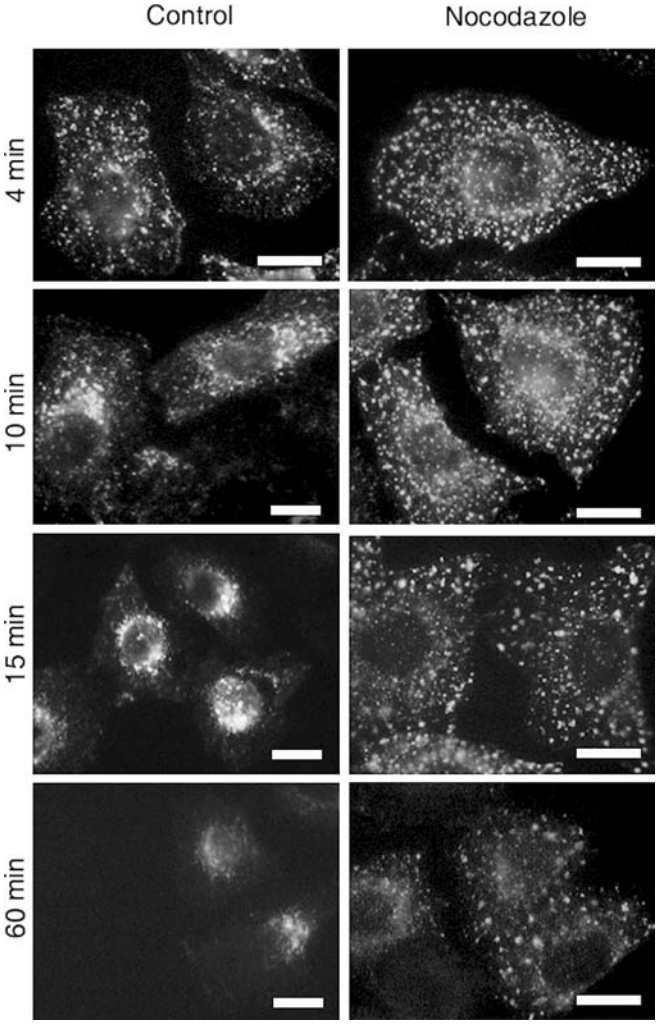


Fig. 5 Effect of nocodazole on the transferrin recycling pathway. HeLa cells were pre-incubated for 30 min in MEM -/+ 20 μM nocodazole. Alexa568-transferrin (25 μg/ml) was bound to the plasma membrane of HeLa cells for 1 h at 4°C in the presence or absence of 20 μM nocodazole. After washes, pre-warmed medium (37°C) with or without nocodazole was added, and at the stated times, endocytosis was stopped by cooling cells to 4°C. Transferrin still present at the plasma membrane was removed, and the cells were fixed and further prepared for fluorescence microscopy. One typical experiment out of five is shown. *Bars* 10 μm

membrane to early endosomes was unaffected by this drug and was therefore microtubule-independent. After a 15-min chase and up to a 55-min chase, Alexa568-transferrin still remained in small peripheral vesicles, presumably early endosomes (Fig. 4), whereas FITC-dextran accumulated in large peripheral vesicles, presumably ECV (Fig. 4). Thus, disruption of the microtubule-network in HeLa cells with nocodazole blocks the transport from early endosomes to PNRC and from ECV to late endosomes.

To analyze kinetically the effect of nocodazole on the recycling pathway, experiments were carried out in which Alexa568-transferrin was bound to the plasma membrane for 1 h at 4°C, and internalization was then induced by warming to 37°C. Cells were then cooled, residual transferrin remaining at the plasma membrane was removed, and the cells were further processed for fluorescence microscopy. Under control conditions, transferrin was rapidly transported (within 10 min) via early endosomes to the PNRC (Fig. 5), whereas in the presence of nocodazole, transferrin accumulated in large peripheral vesicles irrespective of the time after the temperature shift.

Effect of bafilomycin and nocodazole on the kinetics of transferrin acidification

Endosomes were labeled with a mixture of transferrin conjugated either to a pH-dependent (FITC) fluorophore or to a pH-independent (Cy5) fluorophore, and the fluorescence values were then determined by flow cytometry (Murphy et al. 1984). By this method, the ratio of fluorescence values of FITC/Cy5 could be converted to the endosomal pH-value with the help of a calibration curve. HeLa cells were incubated with FITC- and Cy5-transferrin for 1 h at 4°C. After extensive washes, cells were resuspended in pre-warmed medium at 37°C, and at the indicated time points, an aliquot of the cells was analyzed by flow cytometry. The amount of transferrin either remaining at the plasma membrane or being recycled to the membrane was taken into consideration and therefore did not contribute to the calculated pH values. Thus, the average pH of all labeled endocytic compartments was depicted at each time. As shown in Fig. 6, within 2 min after transfer to 37°C, a rapid acidification to pH 6.0 was seen followed by a slower pH decrease to 5.6 within 14–20 min before slow alkalinization to pH 6.5 after 60 min occurred. Thus, the kinetics of transferrin acidification seemed to be biphasic in HeLa cells, with an initial rapid and a delayed slower acidification.

As expected, the inhibition of the V-ATPase by bafilomycin increased the pH of the compartments labeled for 30 min with FITC/Cy5-transferrin to neutrality (data not shown). Therefore, the effect of bafilomycin on the kinetics of transferrin acidification was not further investigated.

Next, we determined the influence of 20 μM nocodazole on the kinetics of transferrin acidification in HeLa cells by flow cytometry. HeLa cells were pre-treated with 20 μM nocodazole for 30 min at 37°C, and the drug was present during FITC/Cy5-transferrin-binding (1 h at 4°C) and subsequent warming to 37°C. In the presence of nocodazole, the amount of bound transferrin was reduced by 10% compared with the absence of the drug (data not shown). Within 4 min, transferrin-labeled endosomes rapidly acidified to a significantly higher pH (pH 6.2) in nocodazole-treated cells than under control conditions (pH 5.8; see Fig. 6), followed by fast alkalinization to pH 6.7 after 12 min. However, prolonged incubation (60–105 min) resulted in a slight pH decrease to pH 6.1 followed by alkalinization at 120 min. These data indicate that nocodazole blocks transferrin transport from mildly acidic early endosomes to more acidic PNRC.

Effect of bafilomycin on the kinetics of acidification of dextran-labeled compartments

Next, we investigated the acidification of endosomes labeled with the fluid-phase marker FITC/Cy5-dextran in HeLa cells at 37°C. Endosomes were labeled with FITC-

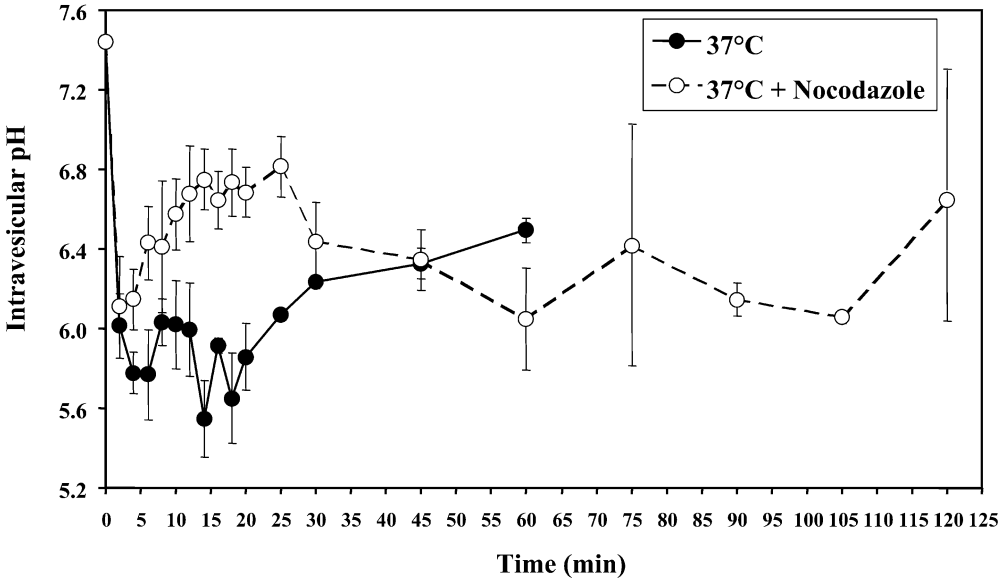
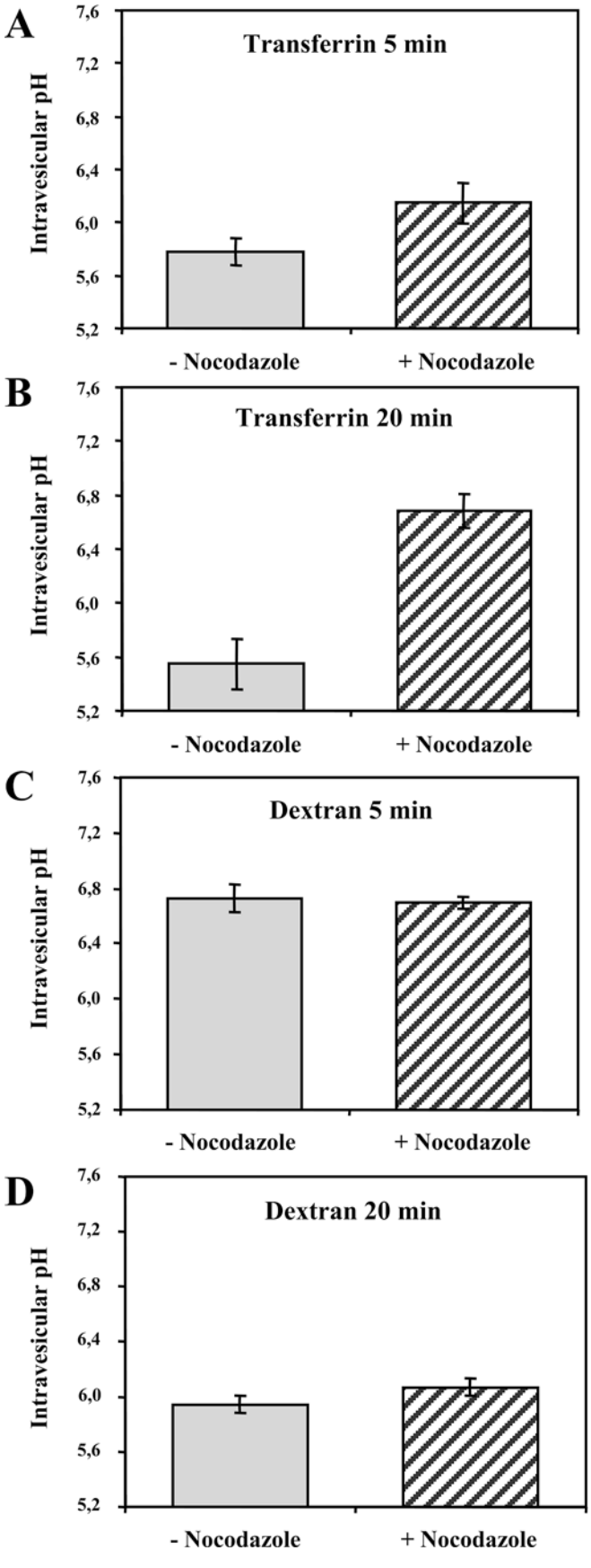


Fig. 6 Effect of nocodazole on the kinetics of transferrin acidification. Cells were pre-treated with MEM -/+ 20 μM nocodazole for 30 min at 37°C. FITC-transferrin (3 μg/ml) and Cy5-transferrin (0.5 μg/ml) were then bound to HeLa cells (1 h, 4°C), and cells were subsequently warmed to 37°C in the absence or presence of 20 μM nocodazole. The mean pH of the labeled compartments was measured at the times stated. Data shown are the mean ± SEM of four to nine different experiments



◀ **Fig. 7** Comparison of the effect of nocodazole on the pH of transferrin-labeled and dextran-labeled compartments. HeLa cells were preincubated in medium with or without 20 μM nocodazole for 30 min at 37°C. Endosomes were labeled with FITC/Cy5-transferrin or with FITC/Cy5-dextran in medium (± nocodazole) for 5 min and then chased for 15 min in marker-free medium (± nocodazole) at 37°C. The fluorescence intensities of the internalized markers were determined by flow cytometry, and the average pH of labeled compartments was calculated. Data shown are the mean ± SEM of three to nine different experiments

and Cy5-dextran for 5 min at 37°C and then chased in marker-free medium for the times indicated. Internalization was halted by the addition of ice-cold PBS (pH 7.4), and cells were immediately analyzed by flow cytometry to determine the pH of all labeled compartments. After the pulse, an endosomal pH of 6.8 was obtained (see Fig. 10;

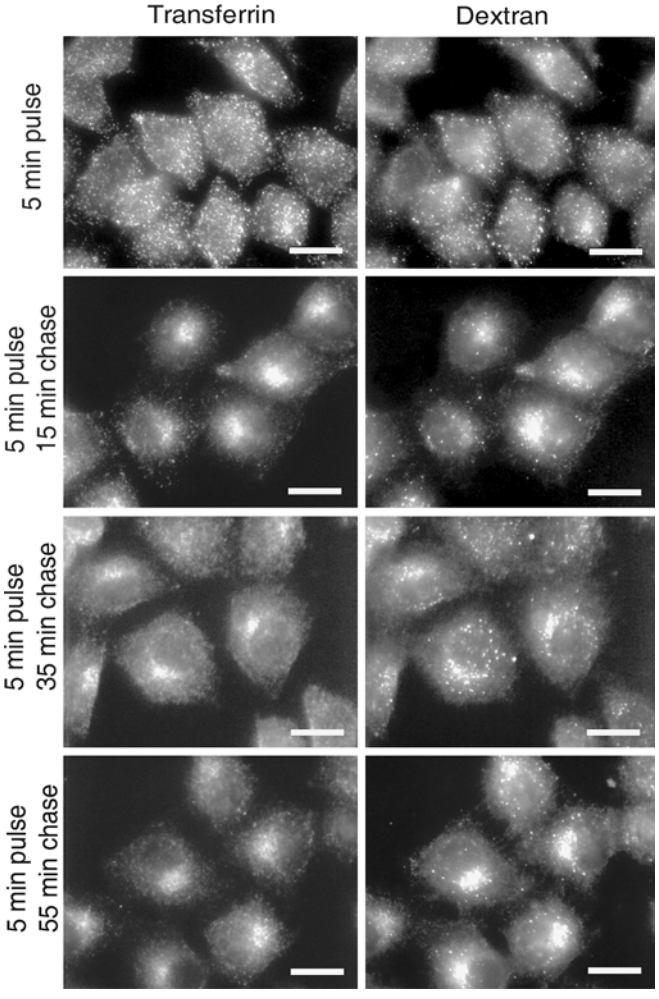


Fig. 8 Transferrin and dextran co-internalization at 20°C. Alexa568-transferrin 25 μg/ml and 10 mg/ml FITC-dextran were co-internalized into HeLa cells at 20°C by applying the same pulse-chase protocol as that for Fig. 1. Transferrin remaining at the plasma membrane was removed, and cells were fixed and processed as described in Fig. 1. To allow for comparisons with the respective control at 37°C (Fig. 1), images were taken at the same exposure time and processed identically in AxioVision and Adobe Photoshop. Under each experimental condition, approximately 100 cells were inspected; one typical experiment out of five is shown. *Bars* 20 μm

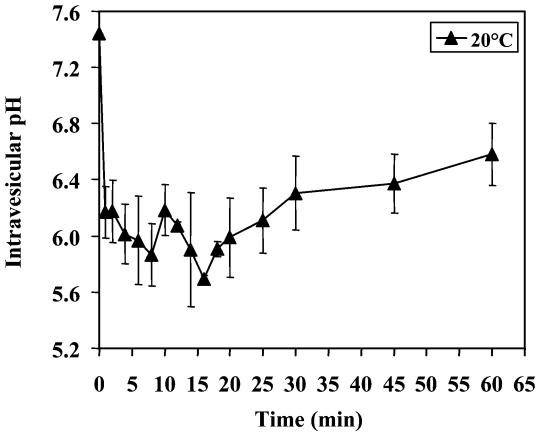


Fig. 9 Effect of low temperature on the kinetics of transferrin acidification. The kinetics of transferrin acidification was determined as described in Fig. 6; however, internalization was carried out at 20°C. Data shown are the mean ± SEM of four to six different experiments.

control 37°C - filled circles) that continuously decreased to 5.6 after 60 min and to 5.3 after 120 min. This is in good agreement with published data of decreasing pH values of compartments en route to lysosomes (Roederer et al. 1987). In our previous studies, we had shown that the addition of 200 nM bafilomycin to HeLa cells in which the endosomes had been labeled with FITC/Cy5-dextran for 25 min was sufficient to raise the pH to neutrality within 30 min (Bayer et al. 1998). Therefore, all our experiments were carried out after a 30-min pre-incubation with this drug.

Comparison of the effect of nocodazole on the endosomal pH of transferrin-labeled and dextran-labeled compartments

To compare the effect of nocodazole on the pH of early and perinuclear endosomes labeled with transferrin and dextran, respectively, endosomes were labeled either with FITC/Cy5-transferrin or with FITC/Cy5 dextran for 5 min (pulse) in the absence or presence of 20 μM nocodazole and then chased for 15 min in marker-free medium (with or without nocodazole). As shown in Fig. 7a, b, the mean pH of all transferrin-labeled endosomes was 5.8 after 5 min and further decreased to 5.5 after 20 min under control conditions, whereas in the presence of nocodazole, the average pH of labeled compartments was elevated immediately after the pulse (pH 6.2) and after a 15-min chase (pH 6.7).

Using FITC/Cy5-dextran as a fluid-phase marker, we obtained an average endosomal pH of 6.7 after a 5-min pulse and a pH of 6.0 after the chase under control conditions. However, disrupting the microtubule network with nocodazole had no significant influence on the pH of endosomes labeled with dextran for 5 min or after a 15-min chase (Fig. 7c, d). These data are in agreement with the influence of nocodazole on the kinetics of transferrin acidification shown before (Fig. 6), where transport to the PNRC was completely blocked by the drug, whereas the pH of compartments labeled with dextran within 20 min was unaffected.

Effect of low temperature on transferrin and dextran endocytosis

To determine the influence of low temperature on the transferrin recycling pathway and on the lysosomal pathway in HeLa cells, we first applied fluorescence microscopy. HeLa cells were depleted of endogenous transferrin, and then, Alexa568-transferrin and FITC-dextran were co-internalized either at 37°C (control) or at 20°C (5 min pulse followed by a chase in marker-free medium up to 55 min). As shown in Figure 8, internalization at 20°C did not significantly affect the uptake and transport of Alexa568-transferrin and FITC-dextran from early to perinuclear compartments (compare with Fig. 1, control). However, transferrin and dextran recycling appeared to be delayed, since the fluorescence staining of either marker was brighter after 40 and 60 min at 20°C compared with that seen in the control experiment at 37°C (Fig. 1).

Effect of low temperature on the kinetics of transferrin and dextran acidification

To investigate the influence of reduced temperature on the kinetics of transferrin acidification, FITC- and Cy5-transferrin were bound to the plasma membrane (1 h at 4°C), and the cells were subsequently warmed to 20°C. The pH of FITC/Cy5-transferrin labeled endosomes was determined by flow cytometry as described before. As depicted in Fig. 9, transferrin rapidly acidified to pH 6.2 within 2 min at 20°C and declined to pH 5.7 after 16 min. Thereafter, slow alkalinization occurred. Compared with the kinetics of acidification at 37°C (Fig. 6), acidification at 20°C is

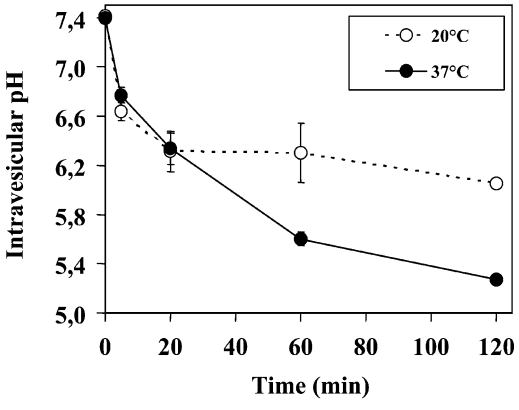


Fig. 10 Effect of reduced temperature on the pH of dextran-labeled endosomes. HeLa cells were preincubated in serum-free medium for 30 min at 20°C or 37°C. Endosomes were labeled with FITC (6 mg/ml)- and Cy5 (1 mg/ml)–dextran in medium for 5 min and then chased for the time indicated in marker-free medium. The fluorescence intensity of the internalized markers was determined by flow cytometry. The average pH of endocytic compartments labeled at 20°C (*open circles*) and 37°C (*filled circles*) was calculated. Data shown are the mean ± SEM of three to nine different experiments

slightly delayed but is otherwise not significantly affected by reduction of the incubation temperature.

Finally, we investigated the acidification of endosomes labeled with the fluid-phase marker dextran at 20°C. The cells were pulse-labeled with FITC/Cy5-dextran and chased in the absence of marker as described before, and the mean pH of all labeled compartments was determined by flow cytometry. After a 5-min incubation at 20°C, the pH of the labeled endosomes was 6.6 (Fig. 10; open circles) compared with pH 6.8 at 37°C. After a 15-min chase, a pH of 6.3 was observed that only slightly decreased to pH 6.2 after a 115-min chase, in contrast to the pH drop to 5.3 at 37°C. Consequently, incubation at low temperature did not affect acidification and transport into late endosomal compartments but blocked transport to the most acidic compartments, i.e. from late endosomes to lysosomes.

Discussion

To investigate the influence of the V-ATP-ase inhibitor bafilomycin, the microtubule-disruptiong agent nocodazole, and low temperature (20°C) on recycling and the lysosomal pathway, respectively, we have applied fluorescence microscopy to determine the morphology of endocytic compartments labeled with Alexa568-transferrin and FITC-dextran in a time-dependent manner. It should be noted that the fluorescence microscopy experiments are not quantitative kinetic studies and thus minor changes in the transport rates will not be observed. In addition to microscopy, flow cytometry has been used to analyze the kinetics of transferrin and dextran acidification, respectively. In the acidification experiments that involved transferrin, the pH values shown are indeed vesicular pH values, since transferrin that remains at or that has been recycled to the plasma membrane is taken into consideration when calculating the endosomal pH ("external transferrin", see above).

Effect of bafilomycin, nocodazole, and low temperature on the transferrin pathway

Based on studies of the kinetics of transferrin acidification, Murphy and co-workers (Rybak and Murphy 1998; Sipe et al. 1991) have proposed that there are at least two different classes of cell lines and primary cell cultures based on their early endosomal pH. In the first group (class H), transferrin acidification is limited to a mild pH of 6.0–6.2 followed by alkalinization during recycling (Cain et al. 1989; Sipe and Murphy 1987). Cells of the second group (class L) acidify transferrin to pH 5.4 but do not appear to alkalinize it during recycling (Killisch et al. 1992; Sipe et al. 1991). In HeLa cells, we have now shown that transferrin acidification is clearly biphasic, with both an initial rapid (pH 6.0–5.8) and a delayed (pH 5.6) slower acidification, indicative of fast and slow recycling pathways. Thereafter, the pH slowly increases to a pH of 6.5 within 60 min attributable to transferrin alkalinization within recycling compartments derived from the PNRC. The kinetics of transferrin acidification for HeLa cells is thus similar to those of class L cells ,such as K562 (Cain et al. 1989; Sipe and Murphy 1987; Zen et al. 1992). However, previous experiments with K562 cells have only been carried out for 20 min, and whether transferrin is alkalinized thereafter is unknown.

In contrast to previous publications by Maxfield and co-workers (for a review, see Maxfield and McGraw 2004), we here demonstrate that early endosomes are less acidic than the PNRC in HeLa cells. This could be attributable to differences in the cell type investigated or the methodology applied. The same technique, i.e., a confocal microscopic pH assay, has shown that transferrin encounters a progressively lower pH in Hep G2 cells (from a median of pH 6.4 at 2 min to pH 5.8 at 5 min), whereas corresponding values in CHO cells amount to pH 6.1 and 6.5, respectively (Presley et al. 1997; van Weert et al. 1995). In addition, the PNRC in lymphocytes is also considerably more acidic (pH 5.5) then early endosomes (Alami et al. 1998). Taken together, the PNRC involved in transferrin recycling can either be less or more acidic than early endosomes dependent on the cell type, whereas a progressively lower pH is encountered in endocytic compartments en route to lysosomes in all cell types investigated thus far.

The biphasic kinetics of acidification, together with pulse (5 min)/chase (15–55 min) fluorescence microscopy experiments, demonstrate that transferrin passes through both peripheral early endosomes and the PNRC during its recycling in HeLa cells (see scheme in Fig. 11). Extended chase periods (up to 55 min) result in less intense staining of transferrin in the PNRC (Fig. 1) correlated with the time-dependence of transferrin alkalinization (Fig. 6), which must occur in compartments derived from the PNRC. Raising the endosomal pH with bafilomycin (Fig. 2) does not prevent the entry of transferrin into the PNRC. However, at prolonged chase times (55 min), a brighter and more intense staining of Alexa568-transferrin in the PNRC can be observed. This could be attributable to a bafilomycin-induced delay in recycling, as has been reported in CHO and in TRVb-1 cells (Johnson et al. 1993; Presley et al. 1997).

Disruption of microtubules does not affect the initial (within 2 min) rapid acidification to pH 6.1 in early endosomes but leads to an increased endosomal pH (6.2) of compartments labeled with transferrin after 4 min and completely prevents the second phase of transferrin acidification (Fig. 6). The kinetics of transferrin acidification in HeLa cells in the presence of nocodazole is therefore similar to those in class H cells such as 3T3 and A549 (Cain et al. 1989; Sipe and Murphy 1987). The acidfication data, together with the morphological results (Fig. 5), demonstrate that the rapid transport of transferrin from early endosomes to the more acidic (pH 5.6) PNRC is inhibited by nocodazole and is thus microtubule-dependent, as in, for example, CHO cells (Daro et al. 1996). In contrast to hepatocytes in which microtubule disassembly results in the reduction in initial levels of transferrin accumulation (Runnegar et al. 1997), nocodazole does not

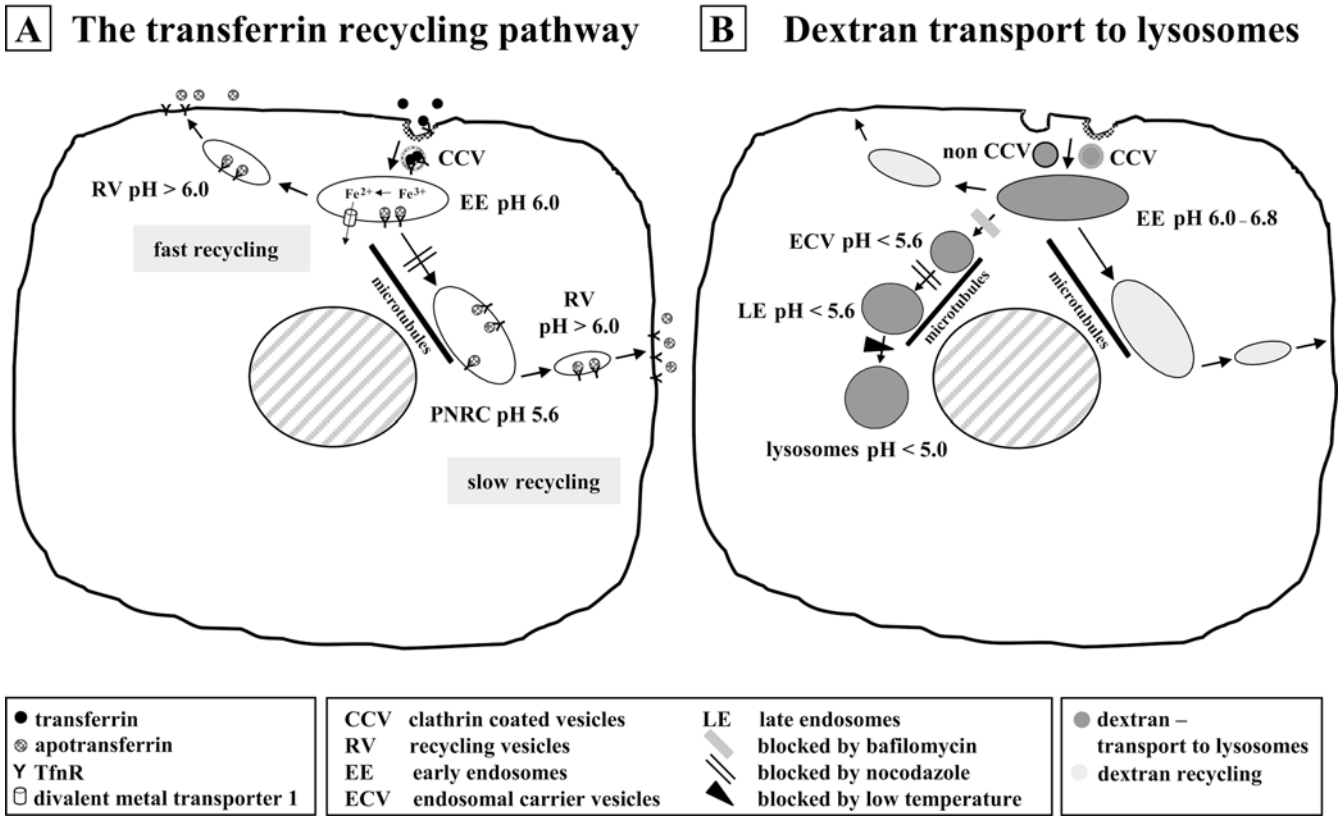


Fig. 11 Schematic representation of the effect of bafilomycin, nocodazole, and reduced temperature on the transferrin recycling pathway and on dextran transport to lysosomes. The endocytic subcompartments and their intravesicular pH of the transferrin recycling pathway (**a**) and of dextran transport to lysosomes (**b**) is summarized (*dark gray* main route of the fluid-phase marker dextran to lysosomes, *light gray* entry of dextran into recycling compartments). Intravesicular pH values are deduced from acidification experiments shown in Figs. 6, 9, 10, and from our previous investigations with HRV2 as a selective "pH meter" to determine the pH of late endocytic compartments (Bayer et al. 1998). Based on the kinetics of acidification, we differentiate early endosomes labeled by transferrrin after 2 min from the PNRC where the ligand is visualized after 1 min. **a** Transferrin transport to and therefore recycling via the PNRC is blocked by nocodazole, whereas this pathway is delayed but not inhibited by bafilomycin and reduced temperature (20°C). **b** Budding of ECV from early endosomes is prevented by bafilomycin and results in marker accumulation in early endosomes, whereas nocodazole inhibits transport of cargo from ECV to late endosomes. Finally, incubation at 20°C leads to the accumulation of dextran in late endosomes by blocking delivery to lysosomes.

affect transport into early endosomes in HeLa cells. Furthermore, fast alkalinization in the presence of nocodazole indicates that microtubules are not required for vesicle movement from early endosomes to the plasma membrane in HeLa cells (McGraw et al. 1993). Moreover, transport from early endosomes to the plasma membrane must occur through compartments of higher or neutral pH. This is in agreement with data in CHO cells in which the overexpression of wild-type Rab4 results in transferrin accumulation in non-acidic (recycling) compartments (van der Sluijs et al. 1992). Consequently, transferrin recycling to the plasma membrane via either the fast (from early endosomes) or the slow (from the PNRC) pathway involves compartments of higher or neutral pH.

Reduction of the internalization temperature to 20°C does not drastically affect transferrin transport to the PNRC, although transferrin recycling appears to be delayed (Fig. 1, Fig. 8). Similarly, the kinetics of transferrin acidification are slightly delayed at 20°C compared with 37°C but are otherwise unaffected. This is in contrast with 3T3 (class L) and K562 (class H) cells in which temperatures below 20°C completely block transferrin recycling and thus its alkalinization, as shown in 3T3 cells (Sipe et al. 1991; Sipe and Murphy 1987).

Effect of bafilomycin, nocodazole, and low temperature on dextran transport to lysosomes

Fluid-phase markers such as dextran or HRP preferentially label endocytic compartments en route to lysosomes (Ellinger et al. 1998; Griffiths et al. 1989; Leung et al. 2000). Nevertheless, the markers also access recycling compartments (Cupers et al. 1994; Klapper et al. 1992). Furthermore, fluid-phase markers are internalized not only by clathrin-dependent endocytosis, but also via all endocytic pathways (Conner and Schmid 2003). The kinetics of dextran acidification have revealed a time-dependent pH decrease from pH 6.8 after 5 min to pH 5.3 after 120 min. Thus, the pH in early compartments is significantly higher than the pH of transferrin-labeled early endosomes (6.0–5.8) because an average pH of all labeled compartments and

Table 1 Factors differently affecting the transferrin recycling pathway and transport of fluid-phase marker to lysosomes

Process	Inhibited by bafilomycin	Inhibited by nocodazole	Inhibited at 20°C
Early endosome formation	No	No	No
Early endosome—PNRC traffic	No	Yes	No
Transferrin recycling from early endosomes	No, but delayed?	No	No, but delayed?
Transferrin recycling from PNRC	No, but delayed?	Yes	No, but delayed?
Early endosome—endosomal carrier vesicle transfer	Yes	No	No
Endosomal carrier vesicle—lysosome transfer	Yes	Yes	Yes

pathways is obtained by using the fluid-phase marker. Fluorescence microscopy studies (Fig. 1) and our previous subcellular fractionation experiments demonstrate that the decrease in pH corresponds to transport of dextran from peripheral early endsomes (5 min), through ECV and late endosomes (15 min), to lysosomes (35–120 min; Bayer et al. 1998). Bafilomycin does not seem to affect dextran internalization into early endosomes or recycling from early endosomes but prevented transfer to late endocytic compartments. In agreement with studies in BHK and CHO cells (Aniento et al. 1996; Daro et al. 1997), low pH in early endosomes is thus required for the budding of ECV in HeLa cells.

Disruption of the microtubules with nocodazole does not affect the acidification of endosomes labeled with dextran up to 15 min after a 5-min pulse. However, a further drop in pH thereafter is prevented. Under this condition, dextran accumulates in large peripheral vesicles (Fig. 4) identified as so-called ECV (Bayer et al. 1998; Gruenberg et al. 1989). Furthermore, ECV have a similar low pH (pH≤5.6) as late endosomes in HeLa cells when a virus (HRV2) is used as a "pH meter". This is shown by the complete conversion of HRV2 into modified viral particles that occurrs below a threshold pH 5.6 (Bayer et al. 1998; Prchla et al. 1994). Thus, disruption of the microtubule network in HeLa cells with nocodazole blocks the transport from ECV to late endosomes. ECV and late endosomes, which are also located in the perinuclear area, are nevertheless clearly dinstinct from the transferrin-recycling PNRC (Daro et al. 1996; Miaczynska and Zerial 2002).

Using fluorescence microscopy, we show here that dextran is internalized into perinuclear late endosomes at 20°C, but this staining appears brighter after prolonged incubation times as compared with internalization at 37°C (compare Figs. 8, 1) indicative of delayed dextran recycling. In HeLa cells, we have previously shown that the conformational change of HRV2 takes place at reduced temperature (20°C), but HRV2 degradation is blocked. Consequently, transport from late endosomes (pH≤5.6) to lysosomes is prevented at 20°C (Prchla et al. 1994). This is in accordance with various other cell types (hepatocytes, BHK, MDCK cells) in which lysosomal degradation does not occur when ligands are internalized at low temperature (Barroso and Sztul 1994; Dunn et al. 1980; Ellinger et al. 2002). Incubation at low temperature does not affect dextran acidification up to a 15-min chase (Fig. 10) or transport into late endosomal compartments but blocks transport from late endosomes to lysosomes as revealed by the lack of a further pH decrease and absence of lysosomal degradation of HRV2 (Prchla et al. 1994).

Conclusions

Taken together, our data clearly demonstrate, in the same cell type, that inhibition of the V-ATP-ase by bafilomycin A1, disruption of microtubules by nocodazole, and reduction of the internalization temperature to 20°C differently affect the transferrin recycling pathway and transport of fluid-phase marker to lysosomes (Table 1, Fig. 11). Consequently, these treatments can be applied to differentiate whether a ligand follows a recycling pathway via mildly acidic early endosomes and more acidic PNRC to the plasma membrane or is transported via early endosomes to more acidic ECV that maintain a similar pH as late endosomes and is finally degraded in the even more acidic lysosomes. Differences in transferrin acidification kinetics between cell types may be attributable to differences in the balance between slow and fast recycling, with fast recycling dominating in class H cells.

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