Role of endocytosis in the internalization of spermidine-C₂-BODIPY, a highly fluorescent probe of polyamine transport

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The mechanism of transmembrane polyamine internalization in mammalian cells remains unknown. A novel fluorescent spermidine conjugate [Spd-C₂-BODIPY; *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)-*N'*-(*S*-[spermidine-(*N*⁴-ethyl)]thioacetyl)ethylenediamine] was synthesized from *N*⁴-(mercaptoethyl)spermidine by a simple, one-step coupling procedure. In Chinese-hamster ovary (CHO) cells, Spd-C₂-BODIPY accumulation was inhibited by exogenous putrescine, spermidine and spermine, was subject to feedback transport inhibition and was up-regulated by prior polyamine depletion achieved with a biosynthetic inhibitor. Probe internalization was decreased by about 85% in a polyamine-transport-deficient CHO mutant cell line. Using confocal laser scanning fluorescence microscopy, internalized Spd-C₂-BODIPY was concentrated in vesicle-like structures similar to the recycling endosomes observed with fluorescent transferrin, which partly co-localized with the polyamine probe. In yeast, Spd-C₂-BODIPY uptake was stringently dependent on receptor-mediated endocytosis, as determined with a mutant defective in early-

endosome formation. On the other hand, Spd-C₂-BODIPY did not mimic the substrate behaviour of natural polyamines in yeast, as shown by the lack of correlation of its uptake characteristics with the phenotypes of mutants defective in either polyamine transport or biosynthesis. These data suggest that endocytosis might be an integral part of the mechanism of polyamine transport in mammalian cells, and that the mammalian and yeast transport systems use qualitatively different transport mechanisms. However, the current data do not rule out the possibility that sequestration of the probe into vesicular structures might be secondary to its prior uptake via a 'classical' plasma membrane carrier. Spd-C₂-BODIPY, a highly sensitive probe of polyamine transport with biochemical parameters qualitatively similar to those of natural polyamines in mammalian cells, should be very useful for dissecting the pathway responsible for polyamine internalization.

Key words: confocal microscopy, difluoromethylornithine, flow cytometry, membrane transport, yeast.

INTRODUCTION

Polyamines are small ubiquitous molecules involved in various functions, including macromolecular synthesis, ion channel gating and the post-translational modification of eukaryotic initiation factor eIF-5A [1,2]. Intracellular polyamine pools are narrowly controlled by the enzymes involved in their biosynthesis and degradation [1,2], as well as by internalization via specific plasma-membrane carriers [3,4]. High-affinity polyamine transport can be demonstrated in a wide spectrum of tissues and cell lines, and is activated upon entry into the cell cycle, cellular transformation by oncogenes, and various hormonal signals [3,4]. Polyamine-transport activity is negatively regulated by intracellular polyamines, as shown by its up-regulation upon polyamine depletion by agents such as α -difluoromethylornithine (DFMO) [3,5], a suicide substrate of ornithine decarboxylase [1]. Antizymes are major factors involved in the acute inhibition of polyamine transport by newly internalized polyamines [6,7], although the mechanism of their interaction with the polyamine

carrier(s) is currently unknown. Polyamine uptake is membrane-potential-dependent, Na⁺-independent and requires bivalent cations such as Ca²⁺ or Mg²⁺ [8], but its actual contribution to polyamine homeostasis under physiological conditions is still uncertain.

Prokaryotic polyamine carriers have been extensively characterized, and vacuolar polyamine transporters have recently been identified in the yeast *Saccharomyces cerevisiae* [9]. However, little is known about the molecular structure of mammalian polyamine carriers. Genetic evidence indicates that at least two loci control polyamine transport [10], and an as yet unidentified human gene for polyamine transport transfected into a Chinese-hamster ovary (CHO) mutant cell line could restore the polyamine-transport defect present in these cells [11].

Although mammalian polyamine carriers are highly specific for putrescine, spermidine and spermine, the polyamine-binding site of these putative proteins can accommodate substantial modifications of the basic polyamine structure, as shown by the utilization of paraquat (1,1'-dimethyl-4,4'-bipyridinium) and

Abbreviations used: CHO, Chinese hamster ovary; MANT, *N*-methylanthranilic acid; Spd-MANT, *N*-(spermidine-[*N*⁴-(3-aminopropyl)]anthranilamide); Spd-C₂-BODIPY, *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)-*N'*-(*S*-[spermidine-(*N*⁴-ethyl)]thioacetyl)ethylenediamine; BODIPY[®] FL iodoacetamide, *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)-*N'*-iodoacetylthylene-diamine; α -MEM, minimal essential medium with α modification; DFMO, α -difluoromethylornithine; MFI, mean fluorescence intensity.

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methylglyoxal bis(guanyldiazide) as substrates [3]. This property has previously been exploited for the biochemical characterization of the polyamine carrier. For instance, spermidine, spermine and norspermine were derivatized with ^{125}I -labelled 4-azidosalicylic acid to generate photoreactive probes for the labelling of polyamine-binding proteins as potential candidates for the mammalian polyamine carriers [12]. The polyamine-transport system has also been used for the targeting of cytotoxic polyamine analogues [1] or chemotherapeutic agents conjugated to polyamines [13].

Conjugation of aromatic structures such as 4-azidosalicylic acid [12] and chlorambucil [13] to the polyamine core structure does not abrogate its strong binding to the polyamine carrier. This interesting property has recently been exploited for the design of fluorescent probes to study polyamine transport. Mono fluoresceinyl adducts of spermidine and spermine substituted on one of the primary amino groups have been described by Aziz et al. [14], which displayed affinity comparable with the parent polyamine and mostly accumulated in the cytoplasm of mammalian cells. Likewise, amidation of *N*-methylantranylic acid (MANT) to an aminopropyl group of *N*⁴-(3-aminopropyl)-spermidine led to an UV-excitable substrate for polyamine transport in various mammalian cells [13]. Interestingly, *N*-{spermidine-[*N*⁴-(3-aminopropyl)]}anthranilamide (Spd-MANT) mainly accumulated into discrete subcellular structures reminiscent of endocytic vesicles [13].

Polyamines accumulated from exogenous sources exert regulatory effects on polyamine homeostasis at levels representing only a minor fraction of the total polyamine pool [15–17]. Likewise, indirect biochemical evidence suggests that the free (i.e. unbound) pools of spermidine and spermine would be restricted to less than 2 and 7% of total content respectively [18]. Thus a better understanding of the mechanism of polyamine internalization and compartmentalization is required to understand the actual sites of action of these compounds under physiological conditions. In this report we describe the synthesis and characterization of a novel fluorescent probe of polyamine transport, spermidine-*C*₂-BODIPY [Spd-*C*₂-BODIPY; *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)-*N'*-(*S*-[spermidine-(*N*⁴-ethyl)]thioacetyl)ethylenediamine], obtained by grafting a highly sensitive fluorophore to an *N*⁴-mercaptoethyl side arm extending from the spermidine backbone. Like Spd-MANT [13], Spd-*C*₂-BODIPY mainly compartmentalizes into vesicle-like intracellular structures and is excluded from the nucleus. Moreover, the internalization of Spd-*C*₂-BODIPY likely proceeds via endocytosis of a complex formed between the probe and the polyamine transporter(s), rather than carrier-mediated influx.

EXPERIMENTAL

Reagents

Bovine calf serum and Eagle's minimal essential medium (with α modification; α -MEM) were purchased from Wisent (St-Bruno, Quebec, Canada) and Gibco-BRL Life Technologies (Burlington, Ontario, Canada), respectively. D,L- α -Difluoromethylornithine hydrochloride was generously provided by ILEX Oncology (San Antonio, TX, U.S.A.). *o*-Phthaldialdehyde was obtained from MAT Laboratories (Quebec City, Quebec, Canada). BODIPY[®] FL iodoacetamide [*N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)-*N'*-iodoacetyl ethylenediamine] and Texas Red[®]- and BODIPY FL-labelled human serum transferrin as well as FITC were purchased from Molecular Probes (Eugene, OR, U.S.A.). [*terminal-methylene-groups*- ^3H (n)] Spermidine trihydrochloride (3.1×10^5 Ci/mol) was purchased

Figure 1 Structures of Spd-*C*₂-BODIPY and Spd-MANT

from NEN Life Science Products (Lachine, Quebec, Canada). Unless otherwise indicated, other biochemical and tissue culture reagents were from Sigma-Aldrich.

Synthesis of Spd-*C*₂-BODIPY

*N*⁴-(Mercaptoethyl)spermidine was synthesized according to the method of Cohen et al. [19]. Spd-*C*₂-BODIPY (Figure 1) was prepared by alkylation of the thiol group of *N*⁴-(mercaptoethyl)spermidine with BODIPY FL iodoacetamide. Prior to conjugation of the fluorophore, *N*⁴-(mercaptoethyl)spermidine was dissolved in an aqueous solution containing a 5-fold molar excess of dithiothreitol to ensure complete reduction of the thiol group. The mixture was left for 1 h at room temperature, and *N*⁴-(mercaptoethyl)spermidine was re-purified by ion-exchange chromatography as described above, followed by lyophilization. All following operations were carried out under subdued light. To 1 mol of a neutralized solution of *N*⁴-(mercaptoethyl)spermidine in 100 μM Tris/HCl (pH 7.3) was then added 4 mol of BODIPY FL iodoacetamide (extemporaneously prepared as a 20 mM solution in DMSO), and the mixture was stirred overnight in the dark at room temperature. Solvents were removed by lyophilization, the foam was dissolved in deionized water, and the solution was passed through a PVDF syringe membrane filter (Millex-HV4, 0.45 μm pore size; Millipore, Bedford, MA, U.S.A.). The aqueous phase was washed with dichloromethane (HPLC grade) until the organic phase, containing unchanged BODIPY FL iodoacetamide, became colourless. Complete extraction of unchanged BODIPY FL iodoacetamide and formation of a polar BODIPY-spermidine conjugate was confirmed by silica-gel TLC using dichloromethane/methanol (9:1, v/v) as an eluent. The aqueous phase containing Spd-*C*₂-BODIPY was reduced to a small volume by lyophilization. The actual concentration of Spd-*C*₂-BODIPY was then determined on an aliquot of the latter solution by derivatization with an *o*-phthaldialdehyde solution in water [400 mM potassium borate, 0.5 g/l *o*-phthaldialdehyde, 1% methanol (v/v), 0.5% (v/v) β -mercaptoethanol and 0.1% Brij 35 (v/v)] for 20 min in the dark at 37 $^{\circ}\text{C}$ [20], and determination of fluorescence intensity using an SLM-AMINCO-Bowman AB2 spectrofluorimeter (excitation wavelength, 350 nm), using spermidine as standard. Standardization of the solution was con-

firmed by measuring the fluorescence intensity of the BODIPY adduct (excitation wavelength, 493 nm), and the final concentration of Spd-C₂-BODIPY was then adjusted to 1 mM.

Synthesis of Spd-MANT and of monofluoresceinylspermidine conjugates

Spd-MANT was synthesized by *N,N'*-dicyclohexylcarbodiimide-coupled amide formation between anthranilic acid and *N*¹,*N*⁸-di(*t*-butoxycarbonyl)-*N*⁴-(3-aminopropyl)spermidine as described in [19]. A mixture of *N*¹- and *N*⁸-(monofluoresceinyl)-spermidines was synthesized using FITC and purified by TLC on silica-gel plates [14].

Cell lines and cell culture

The parental CHO cell line (CHO-TOR) and a polyamine-transport-deficient subline (CHO-MG) isolated after chronic selection for growth resistance to methylglyoxal bis(guanyldrazone) [10,21] were generously provided by Dr Wayne Flintoff (Department of Microbiology and Immunology, University of Western Ontario, London, Ontario, Canada). Both lines were routinely grown in α -MEM supplemented with 10% CosmicTM calf serum (Hyclone, Logan, UT, U.S.A.) and antibiotics in a water-saturated 5% CO₂ atmosphere at 37 °C.

All yeast strains used in this study were haploid. The wild-type yeast (*S. cerevisiae*) strain RH144-3D (*MATa leu2 his4 ura3 bar1-1*) and its RH266-1D (*MATa leu2 his4 ura3 bar1-1 ts end3*) mutant derivative defective in an early internalization step of endocytosis [22–24] were kindly provided by Dr H. Riezman (Biozentrum, University of Basel, Basel, Switzerland). The DBY747 wild-type strain (*MATa leu2 his3 ura3 trp1*) and its mutant derivative DBY747spe2 Δ (*MATa leu2 his3 ura3 trp1 Δ spe2-5::LEU2*) carrying a chromosomal deletion of the *SPE2* gene encoding S-adenosylmethionine decarboxylase have been described previously [23]. The W303 *ptk2:: Δ* deletion mutant (*MATa ade2 ura3 trp1 his3 leu2 ptk2::TRP1*) was generated by the one-step disruption method [25]. Briefly, the full *PTK2* sequence, including 683 and 262 bp adjacent to the 5' and 3' ends of the coding region, respectively, was isolated as a *Bam*HI fragment (3.4 kb) from a lambda PM-1436 *S. cerevisiae* genomic clone from chromosome X (A.T.C.C. 70345) [26]. The *Bam*HI fragment was subcloned into the same site of the Bluescript II KS(+) plasmid. The resulting plasmid pKSPTK2 was digested with *Hpa*I and *Nde*I restriction enzymes to eliminate a 2631 bp fragment corresponding to the full sequence of the *PTK2* open reading frame. A *Sca*I–*Nde*I fragment (3100 bp) bearing the *TRP1* marker (from the pJG4-5 expression vector) was ligated into the blunt-end *Hpa*I and *Nde*I sites of pKSPTK2. The resulting 3900 bp *Bam*HI fragment, containing the *TRP1* gene flanked with *PTK2* sequences (*ptk2::TRP1*), was isolated and used to transform the wild-type haploid strain W303 (*MATa ade2 ura3 trp1 his3 leu2*) with subsequent selection on tryptophan-free synthetic dextrose (SD) medium [0.17% yeast nitrogen base without amino acids or (NH₄)₂SO₄, with 0.5% (NH₄)₂SO₄ and 2% dextrose added]. The yeast strains used in this study were routinely grown in YPD medium (1% yeast extract, 2% peptone and 2% D-glucose).

Epifluorescence microscopy

Both CHO cell lines were grown on sterile glass coverslips in six-well plates for 24 h. Cells were then incubated in the presence of Spd-C₂-BODIPY (1 μ M) for 2–4 h. Medium was removed, and cell monolayers were washed three times with 1 ml of ice-cold Ca²⁺/Mg²⁺-free PBS containing 1 mM spermidine and then

twice with 1 ml of PBS. Coverslips were then inverted on a droplet of Hanks' balanced salt solution. Intracellular fluorescence was observed using an Axioskop microscope equipped with a Plan-Neofluar 403/0.75 objective (Zeiss) and filter set 487909 (bandpass, 450–490 nm; filter threshold, 510 nm; long pass, 520 nm). Images were obtained with a CCD-300T-RC camera (DAGE-MTI, Michigan City, IN, U.S.A.) coupled to the MetaMorph[®] imaging system (version 3.5; Universal Imaging Corporation, West Chester, PA, U.S.A.).

FACS analysis of fluorescent probe uptake

Both CHO cell lines were seeded at 2.5×10^5 cells/well in 24-well culture plates. Cells were incubated for the indicated time interval with serum-free α -MEM containing 1 μ M Spd-C₂-BODIPY or 1 μ M Spd-MANT. Medium was then removed and cell monolayers were washed three times with 1 ml of PBS containing 1 mM spermidine. Cell cultures were then rinsed twice with 1 ml of PBS, and harvested after a 5-min incubation with pancreatin/EDTA/Hepes solution (2.5 g/l/1 mM/7.25 mM) in Hanks' balanced salt solution. Cells were suspended in ice-cold α -MEM and immediately processed for fluorescence analysis by flow cytometry with an Epics Profile II cytofluorometer (Coulter Corp., Miami, FL, U.S.A.). For Spd-C₂-BODIPY, the argon cytofluorimeter was tuned to 488 nm, using an FL1 photomultiplier (bandpass, 525 ± 30 nm). For Spd-MANT, the He/Cd lamp of the cytofluorimeter was tuned to 325 nm, using an FL2 photomultiplier. The mean value of fluorescence intensity (MFI) distribution was recorded for the analysis of 1×10^4 cells/sample. MFI is the mean channel number recorded for the distribution of fluorescence among the cells counted.

To determine the effect of polyamine depletion and protein synthesis inhibition on Spd-C₂-BODIPY uptake, cells were first grown for 72 h in the presence of 5 mM DFMO or vehicle. Fresh serum-free α -MEM containing 1 μ M Spd-C₂-BODIPY was then added for a 4 h incubation, with or without 200 mM cycloheximide and/or 5 mM DFMO.

Confocal laser scanning fluorescence microscopy

CHO-TOR cells grown on coverslips were first incubated for 4 h with 1 μ M Spd-C₂-BODIPY and Texas Red–transferrin conjugate (3 μ g/ml) in α -MEM at 37 °C. Culture medium was then removed, and cells were washed three times with ice-cold PBS containing 1 mM spermidine, and then twice with ice-cold PBS. Specimens were visualized with a Bio-Rad MRC-1024 confocal imaging system equipped with a Kr/Ar laser and mounted on a Diaphot-TMD inverted microscope (Nikon). A 60 $\times$ oil-immersion objective lens with a 1.4 numerical aperture was used for imaging. The photomultiplier gain was set at maximum, and the confocal aperture was adjusted for maximum resolution. Analysis of fluorophore co-localization was performed using the LaserSharp software (version 3.0; Bio-Rad) with background subtraction.

Competition of spermidine uptake by fluorescent polyamines

The ability of Spd-C₂-BODIPY, Spd-MANT and *N*⁴-(mercaptoethyl)spermidine to compete for [³H]spermidine uptake was determined in CHO-TOR cells by a 20 min uptake assay in the presence of increasing concentrations of competitor, using 5 μ M [³H]spermidine as a substrate, as described in [27]. Data were averaged from three separate experiments with triplicate determinations of the *K*_i value for each experiment. The *K*_m value of spermidine transport was determined by Lineweaver–Burk analysis as described in [27]. *K*_i values for inhibition of spermidine

uptake were determined using the Cheng–Prusoff equation [28] from the IC_{50} value derived by iterative curve fitting of the sigmoidal equation describing the velocity of spermidine uptake in the presence of the respective competitor [5,27].

Uptake of Spd-C₂-BODIPY in yeast

The wild-type DBY747 strain and its *spe2Δ* mutant derivative were incubated at 30 °C for 96 h in H medium, an amine-free minimal medium [29] supplemented with 5 mg/ml tryptophan with or without 5 mg/ml leucine, respectively. Cell growth had completely stopped in the *spe2Δ* mutant at the end of the incubation period, indicating the complete depletion of spermidine and spermine [23,29]. DBY747 cells and the *spe2Δ* mutants were then pre-incubated for 10 min at 30 °C in the appropriately supplemented H medium at 3×10^7 cells/ml. The wild-type W303 strain and its *ptk2Δ* mutant derivative were grown until mid-exponential stage in YPD at 30 °C, and 3×10^7 cells were then transferred into 1 ml of H medium at 30 °C. The four strains were then incubated with 1 μ M Spd-C₂-BODIPY in the dark in a shaking incubator at 250 cycles/min. At the indicated times, cells were processed for FACS analysis as described above.

Wild-type RH144-3D cells and the *end3* mutant were grown to mid-exponential stage in YPD at 25 °C, then rinsed and pre-incubated for 10 min at 37 °C in H medium. Cells were then incubated with either 1 μ M Spd-C₂-BODIPY for the indicated time interval, or 10 μ M [³H]spermidine (50 Ci/mol) for 20 min to determine the rate of high-affinity spermidine uptake [23,26].

Statistical analysis

The statistical significance of differences between means was assessed at the 5% level by unpaired Student's *t* tests or by the multiple-range Duncan–Kramer test [30]. Unless otherwise indicated, results are expressed as the mean $\pm$ S.D. from three separate experiments with duplicate or triplicate determinations for each experiment.

RESULTS

Design and synthesis of the BODIPY-spermidine conjugate

Spermidine analogues derivatized at the *N*⁴ position are better substrates for the mammalian polyamine-transport system than those substituted on primary amino groups [31]. Moreover, spermidine and norspermidine dimers cross-linked through alkylation of the secondary amino group with aliphatic or aromatic linkers display low K_i values for competition against polyamine uptake [32]. In order to generate a sensitive probe of polyamine transport, we thus conjugated the iodoacetamide derivative of the highly fluorescent dye BODIPY FL [33] to the thiol group of *N*⁴-(mercaptoethyl)spermidine [19]. The availability of a thiol as a unique reactive group obviated the need for protecting the amino groups of the spermidine derivative before conjugation to the fluorophore. The resulting conjugate, Spd-C₂-BODIPY (Figure 1) could thus be prepared using a simple, one-step reaction while avoiding the use of extreme pH conditions under which the fluorophore is unstable [34].

The ability of Spd-C₂-BODIPY to interact with the polyamine-transport system was determined by measuring competition by the probe against spermidine uptake in CHO-TOR cells. For comparison, the K_i value against spermidine uptake was also determined for *N*⁴-(mercaptoethyl)spermidine and Spd-MANT (Figure 1), a recently described fluorescent polyamine which bears a smaller hydrophobic fluorophore than Spd-C₂-BODIPY

Figure 2 Heterogeneous cytoplasmic labelling by Spd-C₂-BODIPY in CHO cells

CHO-TOR cells grown on sterile glass coverslips were incubated for 4 h with 1 μ M Spd-C₂-BODIPY. Note the uneven distribution of fluorescence in the cytoplasm in the form of granules or vesicle-like structures.

[13]. The K_i of Spd-C₂-BODIPY (46 ± 13 μ M) was about 30-fold higher than the K_m of spermidine (1.5 ± 0.4 μ M), consistent with the weak inhibitory potency also measured for *N*⁴-(mercaptoethyl)spermidine ($K_i = 25 \pm 1$ μ M). Spd-MANT ($K_i = 0.4 \pm 0.07$ μ M) was a much stronger competitor of spermidine uptake than Spd-C₂-BODIPY, exhibiting an even stronger affinity than spermidine for the polyamine transporter.

Labelling of intact cells with Spd-C₂-BODIPY

As shown in Figure 2, the accumulation of Spd-C₂-BODIPY added in trace amounts (1 μ M) to the growth medium was readily detectable by epifluorescence microscopy in wild-type CHO (CHO-TOR) cells. Intracellular fluorescence was clearly distributed in a discrete fashion, leading to a granular pattern, with a much less intense staining of the cytosol. To evaluate the suitability of Spd-C₂-BODIPY as a probe for polyamine transport, its ability to differentially label CHO-TOR and polyamine-transport-deficient mutant (CHO-MG) cells was next assessed. It had been established previously that CHO-MG cells are completely deficient in diamine and polyamine transport [10,21]. Cells were pre-treated for 72 h with DFMO and were co-incubated with cycloheximide to maximize probe accumulation (see below). Whereas CHO-TOR cells were intensely labelled with Spd-C₂-BODIPY, only a faint green intracellular fluorescence could be detected in the CHO-MG cells (Figure 3). These data indicate that conjugation of the hydrophobic BODIPY moiety to spermidine via a long side chain preserves the specificity of substrate binding to the polyamine carrier, and that Spd-C₂-BODIPY is a reliable probe of high-affinity polyamine uptake.

Polyamine uptake is an active-transport process and is thus strongly dependent on temperature [3,5]. MFI was determined by flow cytometry in CHO-TOR and CHO-MG cells incubated for 1 h at either 4 or 37 °C with Spd-C₂-BODIPY (1 μ M) in serum-free α -MEM, after pre-incubating cells at the respective temperature for 60 min in serum-supplemented growth medium.

Figure 3 Epifluorescence microscopy of Spd-C₂-BODIPY accumulation in CHO-TOR and CHO-MG cells

CHO-TOR (A, B) or CHO-MG (C, D) cells grown on coverslips were incubated for 4 h with 1 μ M Spd-C₂-BODIPY in the presence of 5 mM DFMO and 200 μ M cycloheximide. The same fields were examined by epifluorescence microscopy (A, C) and phase-contrast microscopy (B, D).

The uptake of Spd-C₂-BODIPY was strongly temperature-dependent, being decreased from 2.8 ± 0.2 and 0.6 ± 0.01 channel number units at 37 °C to 0.25 ± 0.01 and 0.05 ± 0.01 channel number units at 4 °C in CHO-TOR and CHO-MG cells, respectively.

We next determined the ability of natural polyamines to compete against intracellular labelling by the fluorescent probe. At a 1000-fold molar excess, putrescine, spermidine and spermine abolished $89 \pm 16\%$, $82 \pm 9\%$ and $82 \pm 9\%$ of the MFI in CHO-TOR cells incubated for 4 h with 1 μ M Spd-C₂-BODIPY. On the other hand, spermidine (1 mM) did not significantly decrease intracellular fluorescence measured in the CHO-MG mutants incubated under the same conditions (14 ± 4 and 17 ± 8 relative fluorescence units for cells labelled in the absence or presence of spermidine respectively; means $\pm$ S.D. from three independent experiments). Thus cell labelling with Spd-C₂-BODIPY largely depends on the binding of the spermidine-like portion of the conjugate to the polyamine carrier.

FACS analysis of intracellular labelling by Spd-C₂-BODIPY was performed over a 6 h period in wild-type and CHO-MG cells. The rate of uptake of the probe was initially linear in CHO-TOR cells for about 2 h and then steadily decreased (Figure 4). In contrast, Spd-C₂-BODIPY accumulated very slowly in CHO-MG cells, and no net uptake could be detected 4 h after addition of the probe. Spd-C₂-BODIPY uptake in the transport mutant represented about 15–20% of that detected in wild-type cells throughout the incubation period, and could not be decreased by co-incubation with a 1000-fold excess of spermidine (results not shown).

The rapid decrease in the rate of Spd-C₂-BODIPY accumulation observed after a 2 h incubation was reminiscent of the

Figure 4 Time course of the uptake of Spd-C₂-BODIPY in wild-type (CHO-TOR) and polyamine-transport-deficient mutant (CHO-MG) cells

Cells were incubated in medium containing 1 μ M Spd-C₂-BODIPY for the indicated interval prior to FACS analysis. Data are means $\pm$ S.D. of mean fluorescence emission values from three separate experiments.

onset of feedback repression of polyamine transport induced by polyamine internalization in mammalian cells [5]. This feedback inhibition results from the rapid induction of antizyme synthesis

Figure 5 Confocal laser scanning microscopy of Spd-C₂-BODIPY and Texas Red–transferrin accumulation in CHO-TOR cells

Cells were incubated for 4 h with either 1 μ M Spd-C₂-BODIPY or 3 μ g/ml Texas Red–transferrin (Texas Red-Tf) and then examined by confocal laser microscopy as described in the Experimental section. Three representative fields (A–C) are shown to illustrate the extent of partial co-localization (arrows) and the pattern of reticular perinuclear staining (pn). For each field shown, Spd-C₂-BODIPY fluorescence is shown in green, Texas Red–transferrin in red and the superimposition of the two images is shown on the right.

by promotion of a +1 frameshifting in the translation of antizyme mRNA by polyamines [6]. We thus determined the effect of protein synthesis inhibition on the accumulation of the polyamine probe. Furthermore, we examined the effect of prior polyamine depletion on Spd-C₂-BODIPY uptake by pre-incubating cells with DFMO, a treatment which up-regulates polyamine-transport activity [3,5,11]. DFMO significantly ($P < 0.05$) increased the apparent uptake of Spd-C₂-BODIPY by about 2-fold (MFI, 108 ± 27 and 45 ± 6 in DFMO-treated and control cells, respectively), as previously observed for the transport of natural polyamines [11]. Moreover, cycloheximide significantly ($P < 0.05$) increased MFI of wild-type CHO cells incubated with Spd-C₂-BODIPY to 149 ± 6 and 196 ± 39 in the absence and

presence of DFMO, respectively, consistent with the relief of feedback inhibition of Spd-C₂-BODIPY uptake by pre-empting stimulation of *de novo* synthesis of antizyme by the internalized polyamine [5,35].

Spectrometric properties of Spd-C₂-BODIPY and Spd-MANT

During the course of this work, the characteristics of two other fluorescent polyamine probes, namely monofluoresceinyl-spermidine [14] and Spd-MANT [13], have been reported. We thus compared the properties of the latter probes with those of Spd-C₂-BODIPY. In our hands, monofluoresceinylspermidine [14] (which is in fact a mixture of the N¹- and N⁸-fluoresceinyl-

Figure 6 Receptor-mediated endocytosis is required for Spd-C₂-BODIPY accumulation in yeast

(A) The time course of high-affinity spermidine uptake activity was determined in wild-type yeast cells as well as their *end3-1* mutant derivative after transfer to the non-permissive temperature (37 °C) using 10 μ M [³H]spermidine as a substrate. (B) The same strains were incubated for the indicated period with 1 μ M Spd-C₂-BODIPY after transfer to the non-permissive temperature (37 °C) prior to FACS analysis. Data are the means $\pm$ S.D. of intracellular radioactivity or MFI values from three separate experiments.

substituted isomers) very faintly labelled CHO cells at 1 μ M, whereas cells were readily stained with an equimolar concentration of Spd-MANT (results not shown) [13]. We next compared the relative sensitivity of Spd-C₂-BODIPY and Spd-MANT to generate a fluorescent signal upon internalization by CHO-TOR cells. For this purpose, CHO-TOR cells were incubated with equimolar amounts of each probe (1 μ M) for 7.5 h, and the intensity of both total fluorescence and autofluorescence was then determined by flow cytometry. At 1 μ M, Spd-C₂-BODIPY exhibited an 18-fold larger specific signal than Spd-MANT, whereas autofluorescence was similar at the respective excitation wavelength used (0.93 and 1.23 for Spd-C₂-BODIPY and Spd-MANT respectively). The difference observed between the intensity of the two probes was consistent with their respective molar absorption coefficients (8.1×10^4 and 5×10^3 cm⁻¹·M⁻¹ for Spd-C₂-BODIPY and Spd-MANT respectively). Thus, despite the approx. 50–100-fold higher apparent affinity of Spd-MANT, a much stronger signal is generated by Spd-C₂-BODIPY at equimolar working concentrations, owing to the favourable optical properties of the fluorophore.

Compartmentalization of Spd-C₂-BODIPY as studied by confocal laser scanning microscopy

The discrete distribution of internalized Spd-C₂-BODIPY into vesicle-like entities noted by epifluorescence microscopy (see Figure 1) was very similar to that observed with Spd-MANT [13]. This pattern was suggested to result from receptor-mediated endocytosis of polyamines upon binding to the membrane-bound polyamine carrier [13]. To better understand the compartmentalization of Spd-C₂-BODIPY, and to assess the hypothesis that the probe is accumulated by receptor-mediated endocytosis, we used confocal laser scanning microscopy to visualize the simultaneous accumulation of Spd-C₂-BODIPY and Texas Red–human transferrin conjugate. Internalization of labelled transferrin has been widely used as a marker of vesicle recycling through receptor-mediated endocytosis, and to visualize the early steps of endosome formation in mammalian cells [36]. Thus extensive co-localization of Spd-C₂-BODIPY and Texas

Red–transferrin would indicate that Spd-C₂-BODIPY is co-internalized with transferrin–transferrin-receptor complexes via receptor-mediated endocytosis.

As shown in Figure 5, fluorescence of both Spd-C₂-BODIPY and Texas Red–transferrin was virtually excluded from the nucleus, and was similarly concentrated mainly as vesicles of various shapes and diameters, often displaying a perinuclear staining pattern. Faint cytoplasmic staining was observed for Spd-C₂-BODIPY, but not Texas Red–transferrin, suggesting that free Spd-C₂-BODIPY may reach the cytosol to a detectable extent. However, co-localization of Spd-C₂-BODIPY and Texas Red–transferrin was only partial, as determined by computer image analysis, and was mostly limited to large vesicle-like structures. These data strongly suggest that Spd-C₂-BODIPY is internalized via a pathway morphologically similar to receptor-mediated endocytosis, but that partly diverges from that used by the transferrin–transferrin-receptor complexes.

To rule out the possibility that the defect in Spd-C₂-BODIPY accumulation found in CHO-MG mutants might be due to general alteration of receptor-mediated endocytosis in the latter cell line, we determined the accumulation of a BODIPY FL–transferrin conjugate (5 μ g/ml) by FACS analysis. MFI associated with BODIPY FL–transferrin accumulation was 1.36 ± 0.08 and 1.67 ± 0.11 for the parental and CHO-MG cells respectively. Thus receptor-mediated endocytosis is normal in CHO-MG cells, and defective internalization of Spd-C₂-BODIPY in polyamine-transport-deficient cells probably cannot be attributed to a defect in the early steps of endocytosis.

Mechanism of Spd-C₂-BODIPY internalization in yeast

The evidence presented above points to a major role of receptor-mediated endocytosis for the internalization of Spd-C₂-BODIPY in mammalian cells. We had previously determined that receptor-mediated endocytosis is also involved in the regulation of high-affinity polyamine transport in the yeast *S. cerevisiae*, and contributes to the acute down-regulation of polyamine carrier activity by newly internalized spermidine [23].

Figure 7 Spd-C₂-BODIPY accumulation does not respond to known phenotypic alterations of polyamine transport in yeast

Yeast mutants deleted for the *PTK2* (A) or *SPE2* (B) genes, as well as their respective parental strains, were incubated for the indicated time intervals with 1 μ M Spd-C₂-BODIPY prior to FACS analysis, as described in the Experimental section. Prior to the experiment shown in (B), cells were grown for 48 h in amine-free medium to achieve total polyamine depletion in the *spe2Δ* cells. Data are the means $\pm$ S.D. of MFI values from three separate experiments.

To assess the involvement of receptor-mediated endocytosis in the uptake of Spd-C₂-BODIPY in yeast, we determined the effect of an *end3* mutation on probe internalization. The product of the *END3* gene is involved in actin cytoskeleton organization and at an early step of receptor-mediated endocytosis, and the temperature-sensitive *end3-1* mutant exhibits a defect in the endocytic internalization of various plasma membrane receptors and permeases [23,37]. As expected [23], high-affinity [³H]spermidine uptake was increased markedly by the presence of the *end3* mutation at the non-permissive temperature (37 °C; Figure 6A). This effect has been attributed to an increased number of polyamine carriers due to their decreased recruitment by recycling endosomes [23]. However, defective *END3* function strongly inhibited the intracellular accumulation of Spd-C₂-BODIPY (Figure 6B).

We next determined Spd-C₂-BODIPY uptake in mutants deleted for the *PTK2* gene, encoding a serine/threonine protein kinase that positively regulates polyamine transport [4,26]. As shown in Figure 7(A), *ptk2Δ* mutants accumulated Spd-C₂-BODIPY at the same rate as wild-type cells, whereas [³H]spermidine uptake was decreased by 70–80% in these mutants (results not shown), as previously reported [26]. We also assessed the effect of polyamine depletion on the uptake of the fluorescent polyamine derivative in mutants (*spe2Δ*) deleted for the gene encoding *S*-adenosylmethionine decarboxylase. Upon incubation in amine-free medium, *spe2Δ* cells exhibit a several-fold up-regulation of polyamine transport due to the relief from feedback inhibition of the high-affinity polyamine carrier by endogenous polyamines [23]. Indeed, pre-incubation of *spe2Δ* cells under polyamine-free conditions led to a significant ($P < 0.01$) increase in the apparent rate of Spd-C₂-BODIPY accumulation as compared with wild-type cells (Figure 7B). However, maximal probe accumulation observed in *spe2Δ* cells was only 40% greater than that found in wild-type cells after 3 h, and differential accumulation per cell mass in the mutant strain was actually less than the observed uptake, since a substantial population (25–30%) of polyamine-depleted *spe2Δ* cells exhibited a significant increase in cell volume (results not shown), as previously reported [29]. Taken together, these data strongly

suggest that Spd-C₂-BODIPY accumulation in yeast proceeds via a pathway that is limited by endocytosis, unlike the natural polyamines, leading to divergent dynamic behaviour as a substrate of the high-affinity polyamine-transport system.

DISCUSSION

FACS analysis based on the differential uptake or display of specific fluorescent probes has been successfully used for the expression cloning of various membrane proteins [38,39]. Moreover, microscopic analysis of intracellular trafficking of transport substrates such as organic cations [40] or folate conjugates [41] has helped to elucidate their mode of delivery and/or compartmentalization. We have thus designed Spd-C₂-BODIPY to obtain insight into the fate of internalized polyamines, and as a tool for monitoring the expression of functional polyamine transporters in our current effort to identify DNA sequences encoding these proteins. Spd-C₂-BODIPY accumulation reliably detects polyamine-transport activity in mammalian cells since (i) it is suppressed by co-incubation with natural polyamines, (ii) it is 5–6-fold lower in polyamine-transport-deficient cells and (iii) it is up-regulated upon prior polyamine depletion or concomitant suppression of feedback transport inhibition by the internalized polyamine.

In our fluorescence-microscopy assays with monofluoresceinated spermidines, the weak labelling intensity did not allow clear visualization of the internalized polyamine, suggesting poor uptake of the probe. The reason for the discrepancy with results by Aziz et al. [14] is not clear. A potential disadvantage of fluoresceinated spermidine derivatives is the fact that the published synthetic procedure involves using a mixture of the *N*¹- and *N*⁸-(monofluoresceinyl)spermidine isoforms, as well as an unknown proportion of the 5- and 6-FITC isomers [14]. Thus lack of stereochemical definition of the mixture used might lead to variable results with the use of fluoresceinated spermidine conjugates. On the other hand, we confirmed the results obtained by Cullis et al. [13] for Spd-MANT, and found that it could also be used as a probe for polyamine transport. Thus, despite the considerably longer and bulkier side arm of Spd-C₂-BODIPY,

Figure 8 Two models for the intravesicular accumulation of N^4 -substituted polyamines

In model A (left), the polyamine (sphere) enters into the cytosol via a *bona fide* plasma-membrane transporter, and a fraction of it can then be sequestered into pre-formed intracellular vesicles (a subpopulation of which mixed with the recycling endosomes) possibly via a putative H^+ /polyamine (PA) antiporter, perhaps similar to the vesicular monoamine transporters of synaptic vesicles and chromaffin granules [45]. However, direct insertion into these vesicles by passive diffusion of the hydrophobic moiety of the probe (broken arrow) cannot be ruled out completely. In model B (right), the polyamine first binds to a putative plasma-membrane receptor that undergoes endocytosis as a polyamine–receptor complex. Acidification of the endosome by insertion of vacuolar (V-) ATPases (shown as truncated cones) might favour dissociation of the latter complex, and promote export of the polyamine towards the cytosol, perhaps via the action of a H^+ –polyamine symporter similar to the NRAMP metal transporters [44].

both probes differentially labelled wild-type and polyamine-transport-deficient CHO cells with the same relative fluorescence intensity (see Figure 4) [13]. Unlike radiolabelled polyamines, either probe generated a non-specific signal (i.e. not suppressed by an excess of exogenous polyamines) in CHO-MG cells that increased linearly with time and which represented about 15–20 % of the fluorescence intensity measured in their wild-type counterparts. Such a fraction of non-specific labelling does not limit the usefulness of Spd- C_2 -BODIPY, since parental COS-7 cells, which exhibit a considerably lower velocity of polyamine uptake than CHO cells, were labelled with the probe with the same relative efficiency over their polyamine-transport-deficient mutants isolated in our laboratory (D. Soulet, M. Kaouass, R. Charest-Gaudreault, M. Audette and R. Poulin, unpublished work).

Spd- C_2 -BODIPY provided a much stronger fluorescence signal than Spd-MANT at equimolar concentrations, using standard argon laser equipment for flow cytometry. This property was expected from the ≈ 16 -fold higher molar absorption coefficient of Spd- C_2 -BODIPY, and from the relatively weak excitation energy available in the multiline UV region of the argon-ion-laser

spectrum for optimal excitation of the Spd-MANT fluorophore [33]. Thus the higher sensitivity of Spd- C_2 -BODIPY as a probe allows its use in trace amounts for FACS analysis, whereas concentrations of $50 \mu M$ were typically used for the cyto-fluorimetric detection of Spd-MANT [13]. The lower affinity of Spd- C_2 -BODIPY for the polyamine carrier was not solely due to the larger size of the fluorophore, since the precursor used for its synthesis, i.e. N^4 -(mercaptoethyl)spermidine, displayed a 60-fold higher K_i value than did Spd-MANT. On the other hand, a derivative closely similar to Spd-MANT, but with a side arm shorter by one ethylene group, N^4 -(azidosalicylamidoethyl)-spermidine, exhibited a 50-fold lower affinity than spermidine for the polyamine transporter in L1210 mouse leukaemia cells [12]. Therefore the thioethyl chain of Spd- C_2 -BODIPY might be less optimal than the aminopropyl group of the polyamine linker present in Spd-MANT, although the structural basis for these differences is unclear.

Our data suggest that, in CHO cells, Spd- C_2 -BODIPY is largely compartmentalized into vesicle-like structures with a distribution morphologically similar to that observed for

fluorescently labelled transferrin, a marker of recycling endosomes [36]. However, the only partial co-localization of Spd-C₂-BODIPY and Texas Red-transferrin indicates that divergent pathways of endocytic internalization might exist for the two ligands. A first possibility is that Spd-C₂-BODIPY bound to the polyamine carrier is sorted from transferrin receptors at the level of sorting endosomes [36], corresponding to the larger vesicles where both probes were found to co-localize. A second hypothesis is that two similar populations of recycling endosomes containing polyamine carriers and transferrin receptors diverge due to the existence of specialized lipid microdomains according to the 'raft' hypothesis [42]. Alternatively, polyamine-transporter-enriched vesicles might exist as specialized endosomes recycling between the plasma membrane and an intracellular compartment, similar to the latent pool of GLUT4 hexose transporters present in insulin-responsive cells [43]. These hypotheses are currently being assessed biochemically using specific protein markers of the various endosomal compartments and detailed kinetics of Spd-C₂-BODIPY internalization.

Our data demonstrate that, in yeast, endocytosis is a rate-limiting step for the internalization of Spd-C₂-BODIPY, but not for spermidine transport. Thus Spd-C₂-BODIPY cannot be considered as a mimic of the natural substrates of the yeast polyamine permease, as further supported by the fact that its accumulation was not affected by disruption of the *PTK2* gene that is required for polyamine transport [4,26], and was only marginally responsive to polyamine depletion. These marked qualitative differences between the yeast and mammalian polyamine plasma-membrane transporters remain to be elucidated, since molecular information is lacking for either carrier(s).

The fact that Spd-C₂-BODIPY is a good surrogate of the natural polyamines for the biochemical parameters of polyamine transport in mammalian cells, but not in yeast, might suggest that receptor-mediated endocytosis is an integral part of the mechanism of polyamine transport in higher eukaryotes. Only a few substrates are known to rely mainly on an endocytic pathway for intracellular delivery, e.g. the Fe(II)/transferrin complex [44] and folates [41,42]. Such pathways involve binding of the substrate to a membrane receptor, followed by endocytosis of the substrate-receptor complex, and transfer of the sequestered substrate from the acidic endosome to the cytosol. Spd-C₂-BODIPY indeed reaches the cytosolic compartment, as shown by the induction of feedback repression of its own accumulation and by the presence of diffuse intracellular staining by the probe. Thus, if polyamine internalization initially proceeds via receptor-mediated endocytosis, polyamine uptake would require two steps, i.e. initial binding to a receptor-like membrane protein, followed by endocytosis and release of the polyamine via a putative endosomal polyamine exporter (see Figure 8, model B). The efficiency of polyamine delivery through such a mechanism would clearly be limited by the rate of recycling of the putative polyamine 'receptor' to the cell surface, since the substrate and the polyamine transporter would most likely be internalized with a 1:1 stoichiometry.

The present data do not rule out the possibility that the accumulation of Spd-C₂-BODIPY or Spd-MANT into vesicle-like structures may not be representative of the fate of natural polyamines. For instance, a substantial fraction of Spd-C₂-BODIPY might be endocytosed as a probe/transporter complex, but remain sequestered into endosomes without an actual translocation of the probe into the cytosol because of steric constraints due to the bulky fluorophore moiety. However, the fact that Spd-MANT, which has a pattern of uptake similar to that of Spd-C₂-BODIPY, is intracellularly accumulated at a rate comparable with that of spermidine import [13], supports the notion that

both probes are taken up by mechanisms basically similar to those of natural polyamines. On the other hand, the current results still allow for the possibility that Spd-C₂-BODIPY or Spd-MANT might be initially taken up by a 'classical' polyamine transporter, and then be sequestered into the observed vesicle-like structures (Figure 8, model A). Such a secondary sequestration might occur via active carriers similar to H⁺-dependent vesicular monoamine transporters [45]. Alternatively, the free base form of the N⁴-substituted spermidine probes could passively diffuse into small acidic compartments, as in the case of other amphipathic amines such as chloroquine [46] or quinacrine [40], although such a mechanism should be limited by the fact that the probes are much stronger bases than the latter amines.

Notwithstanding the pathway of polyamine internalization, a fluorescent probe with highly favourable spectral and biochemical properties such as Spd-C₂-BODIPY should be very useful in delineating the various steps in polyamine transport and to identify proteins associated with the latter process(es).

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