

Chemistry-based protein modification strategy for endocytic pathway analysis

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Background information. The integrated analysis of intracellular trafficking pathways is one of the current challenges in the field of cell biology, and functional proteomics has become a powerful technique for the large-scale identification of proteins or lipids and the elucidation of biological processes in their natural contexts. For this, new dynamic strategies must be devised to trace proteins that follow a specific pathway such that their initial and final destinations can be detected by automated means.

Results. Here, we report a novel vectorial strategy for trafficking pathway analysis. This strategy is based on a chemical modification of plasma membrane proteins with a bSuPeR (biotinylated sulfation site peptide reagent) and metabolic labelling in the Golgi apparatus, such that plasma membrane proteins that traffic via the retrograde route become detectable in complex mixtures. Efficient synthesis schemes are presented for tailor-made chemical tools that are then applied to the step-by-step validation of the strategy, using a known retrograde cargo protein: the STxB (Shiga toxin B-subunit). bSuPeR modification at the plasma membrane does not affect STxB transport to the Golgi apparatus, where the protein is metabolically labelled, allowing its detection in cell lysates.

Conclusions. Our vectorial concept proposes a new chemical approach for traffic-based profiling of proteins that may prove to be applicable to the analysis of diverse endocytic pathways.

Introduction

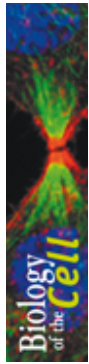
Compartment and pathway identity is given by a specific complement of proteins and lipids. The emerging fields of proteomics and lipidomics aim at developing high-throughput methods for large-scale protein and lipid identification. A number of descriptive or quantitative proteomics approaches have been presented for the identification and the localization of proteins at steady state (Taylor et al., 2003;

Gilchrist et al., 2006), but they fail to provide an integrated view of dynamic processes such as intracellular transport. Thus the current challenge resides in the functional annotation of proteomics data by dynamic information on pathways that allow proteins to reach defined intracellular localizations, and the development of traffic-based methodologies marks a milestone for protein profiling of pathway proteomes.

The retrograde route permits some proteins and lipids to leave the endocytic pathway and to reach the Golgi apparatus, the ER (endoplasmic reticulum), and in some cases the cytosol (Johannes and Popoff, 2008). Recent studies demonstrate the importance of retrograde transport in pathological situations such as toxin entry, viral replication (Crump et al., 2004; Brass et al., 2008), and Alzheimer’s disease (He et al., 2005; Wahle et al., 2005), and in cellular functions

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Key words: autoradiography, intracellular trafficking, post-translational modification, proteomics, retrograde transport, Shiga toxin B-subunit (STxB).
Abbreviations used: BFA, brefeldin A; bSuPeR, biotinylated sulfation site peptide reagent; ER, endoplasmic reticulum; HRP, horseradish peroxidase; MALDI, matrix-assisted laser-desorption ionization; NHS, *N*-hydroxy-succinimidyl; RP-HPLC, reverse-phase HPLC; STxB, Shiga toxin B-subunit; Tf, tagged transferrin; TGN, *trans*-Golgi network.



such as antigen presentation (Ackerman et al., 2005), glucose and copper transport (Voskoboinik and Camakaris, 2002; Shewan et al., 2003) and cell signalling (Liao and Carpenter, 2007). Yet, the full scope of cellular retrograde transport functions remains to be elucidated.

Shiga toxin has become one of the best-studied model cargo proteins of the retrograde route (Johannes and Römer, 2010). This protein toxin is produced by *Shigella dysenteriae* and enterohaemorrhagic strains of *Escherichia coli*. Notably the so-called verotoxins (or Shiga-like toxins) from *E. coli* are responsible for pathological manifestations that can lead to haemolytic-uraemic syndrome, the leading cause for pediatric renal failure in the world. Shiga toxin binds via its homopentameric B-subunit [STxB (Shiga toxin B-subunit)] to the glycosphingolipid Gb3 (globotriaosylceramide) at the cell surface. After its endocytosis, Shiga toxin is targeted to the Golgi apparatus and then to the ER, via the retrograde transport route. The molecular determinants of the different transport steps between plasma membrane and ER have been analysed in quite some detail (Johannes and Decaudin, 2005; Sandvig and van Deurs, 2005), with a special focus on retrograde trafficking at the interface between endosomes and the Golgi apparatus (Bonifacino and Rojas, 2006; Johannes and Popoff, 2008). From the lumen of the ER, the catalytic A-subunit is translocated by ERAD (endoplasmic-reticulum-associated degradation) to the cytosol, where it catalyses the removal of a specific adenine residue from position 4324 (rat ribosomes) in the sarcin-ricin loop of 28S rRNA in the 60S ribosomal subunit.

In this report, a novel chemistry-based approach was devised that may allow capturing cargos of the retrograde route. This vectorial approach is based on the premise that only retrograde cargos have the characteristic of trafficking from one position in the cell, the plasma membrane, to another position, the Golgi apparatus. We modify plasma membrane proteins with a biotinylated capture reagent, and, of all cell surface-tagged proteins, only those that undergo retrograde transport to the Golgi apparatus will be modified by sulfotransferase, allowing their detection in complex mixtures. We present proof of concept that a model retrograde cargo protein, the STxB, can be cell surface-tagged and subsequently metabolically labelled within the cells without perturbing its intra-

cellular transport. This demonstration creates a new paradigm for the chemical biology community. The underlying concept is not limited to the retrograde route, but may also be applicable to other endocytic pathways that lead to cellular compartments harbouring known post-translational modification enzymes.

Results

Outline of the approach

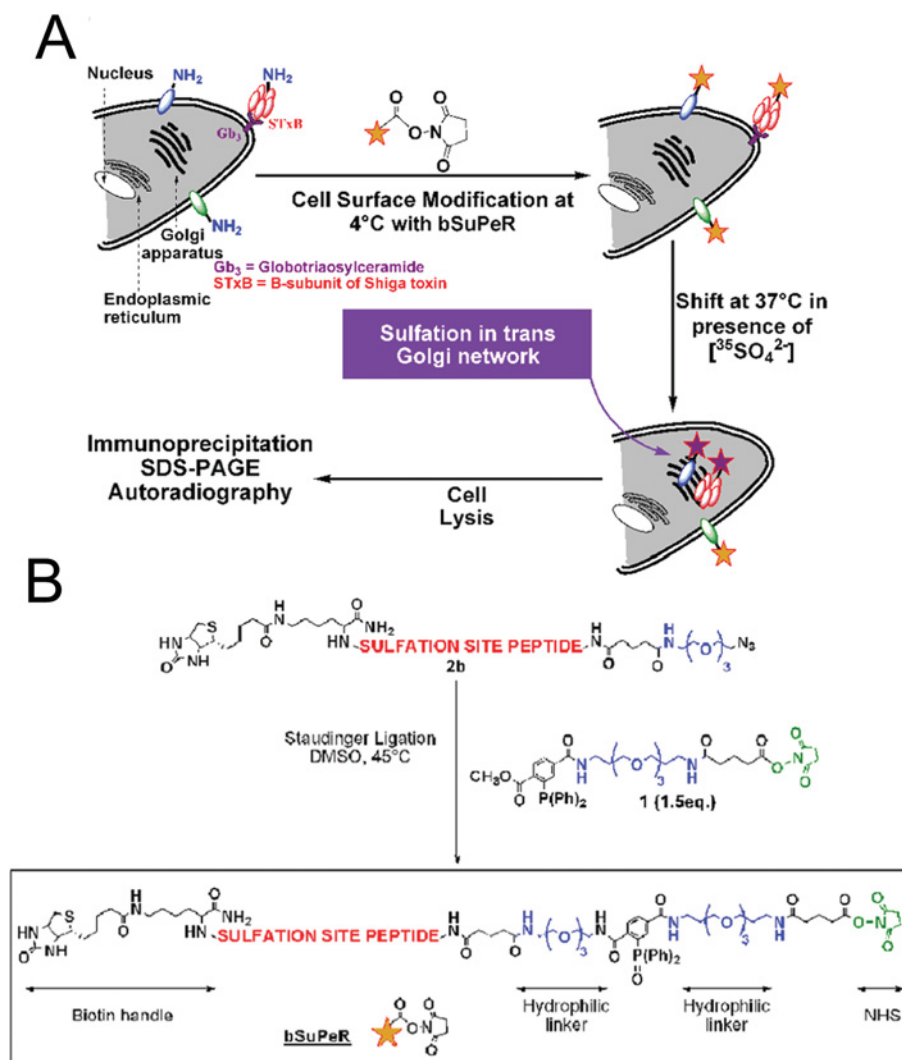
The experimental strategy for the vectorial analysis of the retrograde route is outlined in Figure 1(A). Cell surface proteins are modified at 4°C (endocytosis inhibited) by a bSuPeR (biotinylated sulfation site peptide reagent). bSuPeR bears three essential functionalities: a NHS (*N*-hydroxysuccinimidyl) ester group as an amino-reactive moiety for cell surface labelling, a sulfation site peptide, and a biotin handle for protein capture. The choice of NHS-based reactivity is inspired by previous cell surface biotinylation studies (Scheurer et al., 2005; Elia, 2008). The EEPEYGE sulfation peptide sequence of bSuPeR is recognized by a TGN (*trans*-Golgi network)-localized sulfotransferase that catalyses the transfer of inorganic sulfate from the medium to its tyrosine residue. Therefore, of all cell surface bSuPeR-tagged proteins, only those that undergo retrograde transport to the TGN are modified by radioactive sulfate that can be added to the culture medium. After cell lysis, bSuPeR-tagged proteins are pulled down via their specific biotin anchor and separated by electrophoresis (one-dimensional and/or two-dimensional) followed by autoradiography. In principle, this approach could ultimately be transposed to a proteomics format, in the case of which sulfated species would be isolated from gels and identified by MS. Here, we have aimed for proof-of-concept validation using STxB as a model cargo.

Preparation of bSuPeR

bSuPeR and a model version without sulfation site peptide were obtained exploiting the orthogonal and chemoselective Staudinger ligation (Saxon and Bertozzi, 2000) (Figure 1B). This reaction generates an amide bond from an azide and a triarylphosphine. The reaction is compatible with a large number of functional groups and therefore has various uses in either organic (Nilsson et al., 2001) or biological chemistry (Lemieux et al., 2003; van Swieten et al.,

Figure 1 | Presentation of the strategy and bSuPeR preparation

(A) Strategy for vectorial proteomics of the retrograde route: plasma membrane proteins are modified at 4°C (no endocytosis) with bSuPeR. On shift to 37°C, bSuPeR-tagged proteins that undergo retrograde transport are modified by [$^{35}\text{SO}_4^{2-}$] on arrival in the TGN (star indicates sulfation site, and change from yellow to purple indicates sulfation). (B) Staudinger ligation for assembling phosphine block 1 with the biotinylated azido blocks 2b, giving bSuPeR.

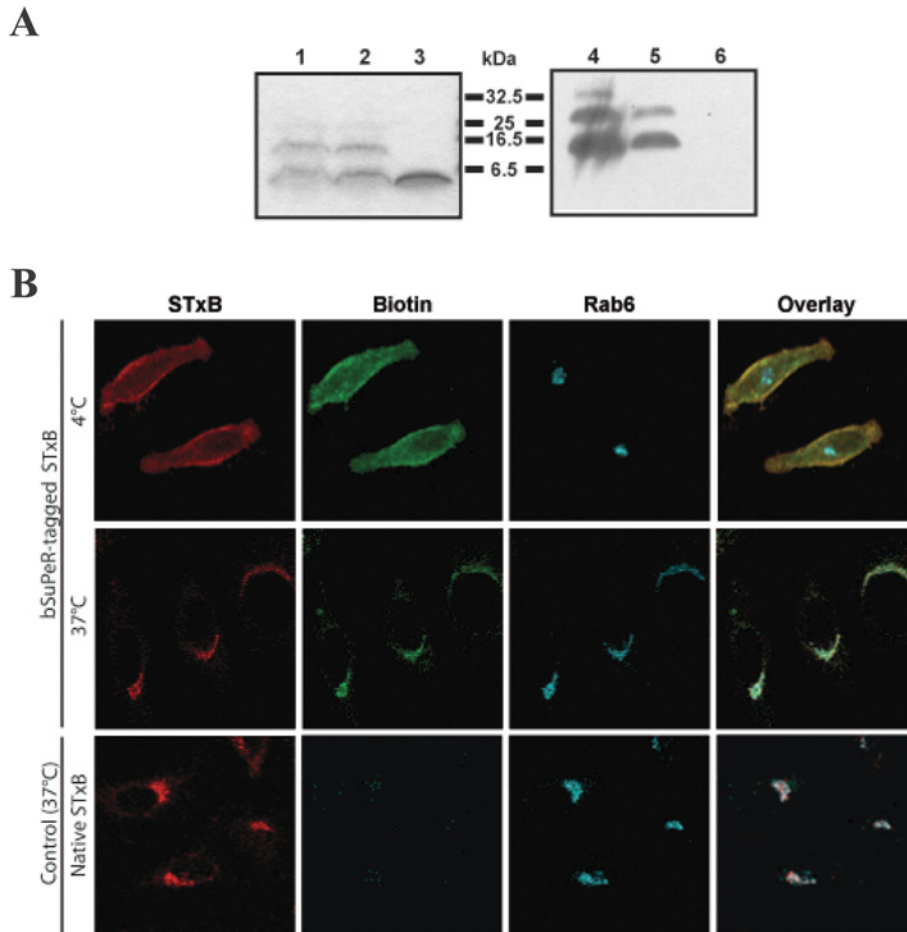


2005; Azoulay et al., 2006). Thus the NHS moiety is easily linked to the azido sulfation site peptide using the NHS-containing triarylphosphine linker 1, without any side-chain protections of 2b (Figure 1B). The azido-biotinylated sulfation site peptide (REEPEYGE) 2b was prepared using Fmoc (fluoren-9-ylmethoxycarbonyl) solid-phase peptide synthesis. For preparing 2a and 2b in a convergent manner, the same starting molecule is used to insert the essential azido group for Staudinger reaction.

We performed Staudinger ligation under smooth conditions, at 45°C in DMSO, between phosphine 1 and the unprotected azido building blocks 2a or 2b to generate the amino-reactive biotinylated capture reagents 3a and bSuPeR, respectively. Progress of the reactions was monitored by HPLC, and considering that a conversion of >99% was achieved, 3a was used without further purification. After completion of the reaction, bSuPeR was directly purified by RP-HPLC (reverse-phase HPLC). After

Figure 2 | *In vitro* modification STxB with bSuPeR and co-localization studies

(A) *In vitro* labelling of STxB with bSuPeR. Purified native STxB (70 μ M) was incubated with bSuPeR (210 μ M, 3 equiv.) for 120 min (lanes 1 and 4), 30 min (lanes 2 and 5), or with the buffer alone (lanes 3 and 6). Lanes 1–3: Coomassie Brilliant Blue; lanes 4–6: Western blot with streptavidin–HRP (1/1500). (B) Fluorescence microscopy. HeLa cells were incubated for 45 min at 37°C with native (control) or bSuPeR-tagged STxB, or at 4°C with bSuPeR-tagged STxB. The cells were then fixed and STxB was immunolabelled with a mouse monoclonal anti-STxB antibody (13C4) and a cyanin 3-conjugated secondary goat anti-mouse antibody (red), biotin was labelled using FITC-conjugated streptavidin (green) and the Golgi marker Rab6 was immunolabelled using a rabbit monoclonal anti-Rab6 antibody and a cyanin 5-conjugated secondary donkey anti-rabbit antibody (blue).



lyophilization, the purity of bSuPeR (94.5%) was determined by analytical HPLC, and the mass of the product was confirmed by MALDI (matrix-assisted laser-desorption ionization)–TOF (time-of-flight)–MS. Chemical synthesis of 1–3 is further described in the Supplementary online data (see Supplementary Schemes S1–S3 at <http://www.biocell.org/boc/102/boc1020351add.htm>). This methodology has the potential to be exploited as a generic means to quickly generate various enzyme recognition site-carrying

peptide tools for the study of other endocytic pathways.

bSuPeR can modify STxB *in vitro* without perturbing its retrograde transport

To validate the strategy, we used STxB as a model. In a first step, the reactivity of bSuPeR was shown by modifying STxB *in vitro*. Western blot analysis and Coomassie Brilliant Blue staining (Figure 2A) proved that the desired modification of the protein

was obtained (>50% of modified B-fragments, based on Coomassie Brilliant Blue). Considering the homopentameric structure of STxB in solution, this level of modification ensures that, statistically, the vast majority of STxB pentamers bears one or more bSuPeR molecules. On incubation of bSuPeR-modified STxB (1 μ M) with cells for 30 min at 4°C, STxB (Figure 2B, red; top panel) and biotin (green) labelling was detected at the cell surface, but not in the Golgi apparatus (Rab6, blue). The functionality of bSuPeR-modified STxB was shown after temperature shift for 45 min to 37°C, where STxB and biotin perfectly co-localized with the Golgi marker (Figure 2B, middle panel). As a control for peptide delivery, we showed that incubation at 37°C of HeLa cells with non-bSuPeR modified STxB led to the absence of signals in the biotin channel (Figure 2B, bottom panel).

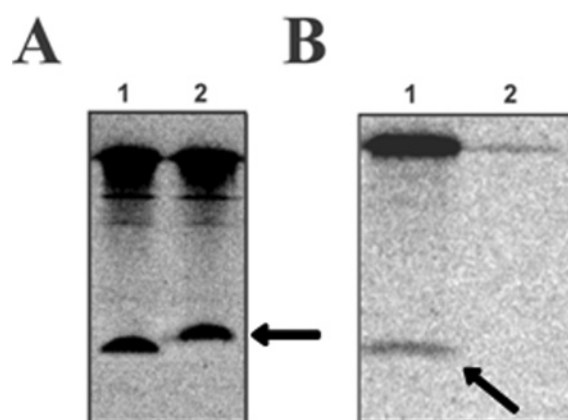
In vitro bSuPeR-tagged STxB is specifically sulfated in Golgi apparatus

The functionality of bSuPeR-modified STxB was further confirmed on incubation with cells for 1 h at 37°C in the presence of radioactive sulfate [$^{35}\text{SO}_4^{2-}$]. A sulfation product could be obtained under these conditions (indicated by an arrow in Figure 3A, lane 2; note that sulfation bands at the level of the load originate from contaminating endogenous proteins), whose apparent electrophoretic mobility was slightly lower than that of the positive control STxB-Sulf2 (Figure 3A, lane 1). STxB-Sulf2 is a genetic fusion that bears at its C-terminus the same sulfation site peptide as found in bSuPeR (Mallard and Johannes, 2003). As further controls, we first showed that no sulfation signal was obtained on incubation of cells with bSuPeR-modified STxB at 4°C, a condition under which endocytosis is inhibited (Figure 3B, lane 2; lane 1: 37°C incubation, the arrow indicates the sulfation product).

To address specificity of our vectorial strategy, we investigated bSuPeR-Tf (tagged transferrin) transport. The transferrin receptor is a marker of early and recycling endosomes, and only small amounts are mis-sorted to the Golgi apparatus after long times of incubation (Snider and Rogers, 1985). As shown by Western blot and MS, Tf could be successfully modified with bSuPeR (see Supplementary Figure S1A at <http://www.biocell.org/boc/102/boc1020351add.htm>). bSuPeR-tagged Tf was internalized with similar efficiency as non-modified

Figure 3 | Metabolic sulfate labelling of *in vitro* bSuPeR-tagged STxB

(A) Sulfation analysis. HeLa cells were incubated for 30 min at 4°C with native (control) or bSuPeR-tagged STxB (1 μ M), and then incubated for 20 min at 37°C in the presence of [$^{35}\text{SO}_4^{2-}$] (0.8 mCi/ml), followed by cell lysis and immunoprecipitation with STxB-specific antibodies. Lane 1: STxB-Sulf2; lane 2: bSuPeR-tagged STxB. Labelling in the upper part of the gel originates from contaminating endogenous proteins. (B) Comparative sulfation analysis of bSuPeR-tagged STxB at 37°C (lane 1) or at 4°C (lane 2). Note that no sulfation was observed on incubation at 4°C (endocytosis is inhibited). The arrows indicate bSuPeR-tagged STxB sulfation product (left).



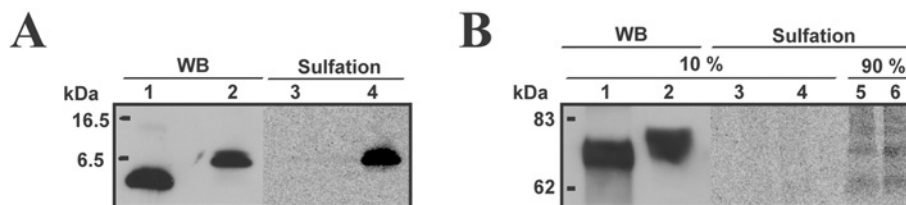
Tf (Supplementary Figures S1B and S1C), demonstrating that the protein retained activity. We then applied our vectorial method to monitor arrival in Golgi membranes.

As a positive control, we again used STxB and sulfation site-tagged STxB-Sulf2 that were incubated with HeLa cells for 20 min in the presence of radioactive sulfate (Figure 4A). After cell lysis and immunoprecipitation, wild-type STxB (lanes 1) and STxB-Sulf2 (lane 2) can be revealed by Western blotting with the expected difference in electrophoretic mobility. We then adjusted our conditions such that radioactive signal could be detected on the same membrane. As expected, an autoradiography band was observed for STxB-Sulf2 (lane 4), but not for sulfation wild-type STxB (lane 3).

These conditions were then transposed to Tf (Figure 4B). Again, wild-type (lane 1) and bSuPeR-tagged Tf (lane 2) could be detected by Western blotting after their incubation with cells. In this case, however, no sulfation signal could be obtained, even after 4 h incubation with cells in the presence of

Figure 4 | Sulfation analysis of bSuPeR-tagged STxB and Tf

(A) Non-modified STxB (lanes 1 and 3) or STxB-Sulf2 (lanes 2 and 4) were incubated with HeLa cells for 20 min at 37°C in the presence of [$^{35}\text{SO}_4^{2-}$] (0.8 mCi/ml), followed by cell lysis and immunoprecipitation with STxB-specific antibodies. Proteins were transferred on to nitrocellulose filters after SDS/PAGE. On the same filters, the presence of STxB was revealed by Western blotting using specific antibodies (WB, lanes 1 and 2), and the presence of the radioactive protein by autoradiography (sulfation, lanes 3 and 4). Note that only STxB-Sulf2 was sulfated (lane 4). (B) Non-modified Tf (lanes 1, 3 and 5) or bSuPeR-modified Tf (lanes 2, 4 and 6) were incubated for 4 h at 37°C with cells in a serum-free growth medium supplemented with 0.8 mCi/ml of [^{35}S]sulfate. Cells were lysed, Tf was immunoprecipitated, and proteins were then analysed by Western blotting (WB, lanes 1 and 2) and autoradiography (lanes 3–6) on the same membrane, as described above. Portions [10% (lanes 3 and 4) and 90% (lanes 5 and 6)] of the immunoprecipitate were loaded for sulfation analysis, and Western blotting was performed on the 10% samples (lanes 1 and 2). Note that Tf could be detected under all conditions by Western blotting, while no sulfation signal was revealed.



radioactive sulfate and the loading of the totality of the immunoprecipitation samples (lanes 3–6). These results confirmed the specificity of our read-out and strongly suggested that this strategy allows focusing on true retrograde cargoes.

Validation of vectorial strategy by cell surface modification

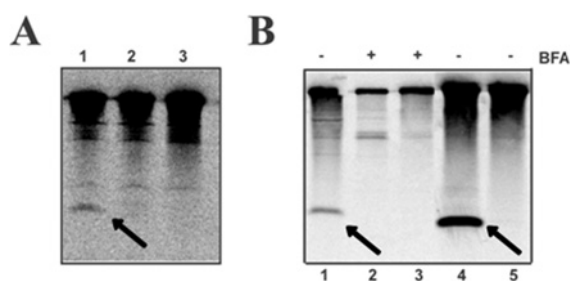
We then turned to cell surface modification using the validated bSuPeR tool. In a first step, the procedure was optimized with the model compound 3a, lacking the sulfation site peptide. On incubation of the cells for 2 h at 4°C, strong labelling of a wide range of plasma membrane proteins could be observed, including STxB (see Supplementary Figures S2A and S2B at <http://www.biocell.org/boc/102/boc1020351add.htm>). Cell surface proteins could also be efficiently modified with bSuPeR (Supplementary Figure S2C). Importantly, we could show that no modification of intracellular proteins was observed on treatment of cells with bSuPeR (see Supplementary Figure S3 at <http://www.biocell.org/boc/102/boc1020351add.htm>), demonstrating that, due to the two hydrophilic spacer arms associated and the charge of bSuPeR (net negative charges –8), the probe did not diffuse across the plasma membrane.

Based on these optimizations, we sought to obtain proof-of-principle demonstration of our vectorial strategy. STxB was bound to cells, which were then surface-modified for 2 h at 4°C by bSuPeR (1.5 mM), and incubated at 37°C in the presence of [$^{35}\text{SO}_4^{2-}$]. On immunoprecipitation using anti-STxB antibody, a sulfation band of the expected size was observed (Figure 5A, lane 1), demonstrating that the cell surface modification had occurred and that STxB subsequently underwent retrograde transport. As controls, no sulfation bands of the size of STxB were observed in the absence of bSuPeR (Figure 5A, lane 2) or STxB (Figure 5A, lane 3).

Sulfated STxB could also be isolated from cell lysates in experiments such as those described in Figure 5(A), using streptavidin–agarose (Figure 5B, lane 1), demonstrating that the biotin moiety on bSuPeR allows selecting plasma membrane proteins from complex protein mixtures. On incubation with BFA (brefeldin A), a fungal metabolite that disrupts Golgi integrity and inhibit retrograde transport (Mallard et al., 1998), this signal was lost (Figure 5B, lane 2), demonstrating again the retrograde transport specificity of the approach. STxB-Sulf2 was used as a positive control in these experiments (Figure 5B, lanes 3 and 4). In lane 5, no STxB was added.

Figure 5 | Metabolic sulfate labelling of STxB-bound cells that were cell-surface-tagged with bSuPeR

(A) Following the general procedure for metabolic sulfation of bSuPeR-tagged proteins, all STxB entities were collected using specific anti-STxB antibodies. The arrows indicate sulfated STxB. Lane 1: bSuPeR-tagged STxB; lanes 2 and 3: as lane 1, except for omission of bSuPeR or STxB, respectively. (B) Biotinylated proteins were collected using streptavidin-agarose and STxB-Sulf2 using specific STxB antibodies. The arrows indicate sulfated STxB. Lane 1: standard condition with 1.5 mM bSuPeR; lane 2: as lane 1, in the presence of 5 μ g/ml BFA during chase period; lane 3: STxB-Sulf2 with BFA; lane 4: STxB-Sulf2; lane 5: no STxB. Note that sulfation signals in the upper part of the gel originates from contaminating endogenous proteins at the level of the load.



Discussion

Intracellular trafficking plays a critical role in many cell biological processes ranging from membrane homeostasis regulation to nutritional uptake and signalling. High-throughput profiling of intracellular pathways should reveal the complete picture of their biological importance and their functions in normal or pathological situations. This is especially true for poorly explored pathways, such as the retrograde route. Yet, the characterization of intracellular pathways is notoriously challenging because of their highly dynamic nature. We report here proof-of-concept validation of a novel approach that, in principle, should allow for the static labelling of the plasma membrane proteome with a dynamic probe to discriminate among the cell surface proteins those that follow the retrograde route.

By combining solid-phase peptide synthesis and chemical ligation, we efficiently obtained a biotinylated peptide probe (bSuPeR) that carries an amino reactive functionality for random covalent conjugation with proteins. Our results show that bSuPeR is adequate for modification of proteins, either

in vitro or on the plasma membrane of cells in culture. bSuPeR is not membrane-permeant, and intracellular proteins are not modified. One major concern with approaches that rely on covalent modification is the possibility of perturbation of protein functions. Our results here show that bSuPeR-tagged STxB is transported as efficiently as wild-type STxB from the plasma membrane to the TGN. In addition, sulfation analysis revealed that bSuPeR-tagged STxB is metabolically sulfated with the same efficiency as the STxB-Sulf2 genetic fusion protein. The validity of our approach is further demonstrated by the finding that even after cell surface modification, STxB still reaches the Golgi apparatus, is metabolically sulfated, and can then be selectively pulled down with streptavidin beads. The selective retrieval of biotinylated proteins among which some are radioactive is the key feature to discriminate retrograde proteins from the pool of other proteins that are only sulfated (i.e. TGN localized) or biotinylated (i.e. plasma-membrane-localized).

Owing to the covalent link between the bSuPeR capture reagent and target proteins and the high affinity between biotin and streptavidin, stringent washing conditions should permit the removal of all contaminating proteins. In addition, the bSuPeR sequence starts with an arginine tryptic cleavage site for the efficient release of retrograde cargo proteins from streptavidin beads and the removal of the tag before MS analysis.

The relatively weak signal of sulfated STxB obtained after cell surface modification, when compared with *in vitro* bSuPeR-modified STxB, indicates that the process is suboptimal at this stage. A likely explanation is that amino groups on STxB are hidden on the membrane-oriented face of the protein. Furthermore, the accessibility of the capture reagent to the plasma membrane may also be a limiting factor. We are currently optimizing the experimental conditions of cell surface modification and are designing second-generation peptides with higher reactivity to proteins.

Our current results are a proof-of-concept advance towards the development of a traffic-based profiling approach for the retrograde proteome. This strategy will be applied to diverse biological contexts, including different cell types and pathological situations. The vectorial profiling concept that we have established here using TGN-localized sulfotransferase

activity could also be applied to other endocytic trafficking routes, exploiting for this post-translational modification enzymes of diverse intracellular compartments. Owing to this generic nature, the vectorial concept may find a wide range of application for the traffic-based profiling of several intracellular pathways, including retrograde transport to the ER.

Materials and Methods

Materials

STxB-Sulf2 and the monoclonal antibody 13C4 were prepared as described previously (Johannes et al., 1997; Mallard et al., 1998). HRP (horseradish peroxidase)-coupled secondary antibodies, ECL detection kits and films were purchased from GE Healthcare. Primary antibodies against GFP (green fluorescent protein; Roche), Hsc70 (heat-shock cognate 70), calnexin, mannosidase (Abcam) and biotin (Rockland) were purchased from the indicated suppliers. The mouse anti-transferrin antibody was purchased from SAPU (Law Hospital, Carlisle, Lanarkshire, Scotland, U.K.). Fluorophore-coupled secondary antibodies and Alexa633-tagged Tf were purchased from Invitrogen.

Cell culture

HeLa cells were cultured in complete DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) fetal calf serum, 5 mM L-glutamine, 100 units/ml of penicillin and 100 µg/ml of streptomycin in 5% CO₂ at 37°C.

Procedure for Staudinger ligation

Unprotected azido building blocks **2a** or **2b** (300 mM) were resuspended with a solution of the *N*-hydroxysuccinimide phosphine 1 (1 equiv. or 1.5 equiv. for **3a** and bSuPeR respectively) containing dry and oxygen-free DMSO. The mixture was stirred at 45°C until completion of the reaction as followed by analytical HPLC. The conversion yield of **3a** (>99%) was determined by analytical HPLC and **3a** was used without further purification. The product was analysed by ESI-MS (electrospray ionization MS): *m/z* 1179.29 was calculated for compound **3a**; 1180.7 Da was the molecular mass of the [M+H]⁺ peak product. The bSuPeR crude mixture was directly purified by RP-HPLC on a 100 Å/5 µm C18 Nucleosil column (1 Å = 0.1 nm) using a linear water/acetonitrile gradient containing 0.05% (v/v) TFA (trifluoroacetic acid) (6 ml/min, detection 215 nm). The fraction were collected and freeze-dried to give bSuPeR (94.5% purity). Peptide bSuPeR was stored as a fluffy powder at -20°C. Spectral data: MS (MALDI) calculated *m/z* 3356.8 for bSuPeR and found 3357.4 Da as the [M+H]⁺ peak product. Prior to use, peptide was solubilized in DMSO (300 mM) and integrity was validated by analytical HPLC.

Procedures for indirect immunofluorescence on HeLa cells

HeLa cells (0.75×10^5) on glass coverslips were placed on ice, incubated with STxB solutions at 1 µM in PBS++ for 30 min. The cells were then washed three times with an ice-cold culture medium and shifted for 45 min to 37°C in a pre-heated culture medium. Cells were fixed using 4% (w/v) paraformaldehyde,

quenched with ammonium chloride (50 mM) and permeabilized with 0.05% saponin. STxB was visualized by labelling with the mouse monoclonal anti-STxB antibody 13C4 and the cyanin 3-conjugated secondary goat anti-mouse antibody. Biotin was visualized by labelling with streptavidin conjugated to FITC. Golgi membranes were labelled with the rabbit polyclonal anti-Rab6 antibody and the cyanine 5-conjugated anti-rabbit secondary antibody. Coverslips were mounted with Mowiol and analysed by confocal microscopy.

Procedure for in vitro STxB or Tf labelling

STxB or Tf in PBS (pH 7.4) (20 µl, 70 µM) were added to a solution of bSuPeR in DMSO (2 µl, 210 µM, 3 equiv.). The mixtures were incubated for 2 h at 4°C, and then dialysed against PBS at 4°C in 10 kDa cut-off Slide-A-Lyzer mini dialysis units (Pierce). Conversion yields were monitored using Coomassie Brilliant Blue and Western blotting. Samples were prepared in a loading buffer without DTT (dithiothreitol), and electrophoresis was performed on Tris-Tricine gels (Figure 2A). For Western blotting, the samples were transferred to nitrocellulose filters, which were blocked for 2 h at room temperature (20°C) using milk powder, and then incubated for 1 h at room temperature with HRP-coupled streptavidin (1/1500) in blocking solution. After washing, HRP was visualized by chemiluminescence.

Procedures for cell surface modification with biotinylated probes **3a** and bSuPeR

For experiments on HeLa cells in suspension, 2×10^6 cells were incubated for 30 min on ice with STxB (1 µM) in PBS. To remove excess of STxB, cells were washed with PBS by three 4°C centrifugations (5 min at 288 g). The cells were then resuspended in PBS (1% DMSO) containing the indicated concentrations of probes **3a** (Supplementary Figures S2A and S2B) or bSuPeR (Figure S2C) and incubated for 2 h on ice. Cells were subsequently washed, as described above, and lysed in PBS containing 1% Triton X-100 and a protease-inhibitor cocktail. Nuclei were removed by centrifugation for 10 min at 1800 g. Biotinylated proteins were precipitated with streptavidin-agarose, and STxB with the monoclonal antibody 13C4 and Protein G-Sepharose. Immunoprecipitates were washed three times with lysis buffer and once with 50 mM Tris/HCl (pH 7.5). Beads were boiled in the sample buffer (15 min, 90°C), and analysed by SDS/PAGE on polyacrylamide gels (Supplementary Figures S2A and S2C) or Tris-Tricine gels for STxB (Supplementary Figure S2B).

Procedure for metabolic sulfation of in vivo bSuPeR-tagged proteins

HeLa cells (2×10^6) were incubated for 1.5 h in a minimal sulfate-free growth medium. STxB was bound to the cells on ice (4°C, 30 min), and cell surface modification was performed as described above. After washing, the cells were incubated at 37°C for the indicated times in a minimal sulfate-free growth medium containing 0.8 mCi/ml of [³⁵S]sulfate (1000 Ci/mmol, Amersham). The cells were lysed with 1% Triton X-100 in PBS in the presence of protease-inhibitor cocktail. Nuclei were removed by centrifugation for 10 min at 3000 rev./min. STxB was immunoprecipitated using the 13C4 antibody and Protein G-Sepharose, or streptavidin-agarose beads (for bSuPeR-tagged proteins). Immunoprecipitates were washed three times in lysis

buffer, and once with 50 mM Tris/HCl (pH 7.5). Beads were boiled in the sample buffer (15 min, 90°C), proteins were separated by SDS/PAGE on Tris-Tricine gels, and results were analysed using a PhosphorImager. BFA was used at 5 µg/ml.

Chemical synthesis, bSuPeR permeability analysis and pattern of cell surface modification

Experimental procedures for chemical synthesis, bSuPeR permeability analysis and pattern of cell surface modification are available as Supplementary online data at <http://www.biocell.org/boc/102/boc1020351add.htm>.

Acknowledgements

We acknowledge Jean-Marc Clavel, Wolfgang Faigle and Damarys Loew (Mass Spectrometry Laboratory of the Curie Institute) for their help.

Funding

This work was supported by the Centre National de la Recherche Scientifique (CNRS) [grant number PGP04-048]; and the Association pour la Recherche contre le Cancer (ARC).

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Received 15 January 2010; accepted 25 January 2010

Published as Immediate Publication 25 January 2010, doi:10.1042/BC20100008

Supplementary online data

Chemistry-based protein modification strategy for endocytic pathway analysis

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Supplementary experimental

General methods for chemical synthesis

All reaction conditions dealing with air- and moisture-sensitive compounds were carried out in a dry reaction vessel under an argon atmosphere. All reagents were obtained from commercial suppliers and were used as received unless otherwise noted. Solvents used in synthesis were used as received [anhydrous DMF (dimethylformamide)] or distilled (CH₂Cl₂ or CH₃CN from P₂O₅, THF from Na/benzophenone and TEA from KOH). The ¹H, ¹³C and ³¹P NMR spectra were recorded on a 300 MHz Bruker AC spectrometer. Chemical shifts are reported in δ values relative to tetramethylsilane or phosphoric acid (³¹P spectra). Chemical ionization (CI-MS; C₄H₁₀, positive ion mode), FAB [positive ion mode, either with a matrix as MB (magic bullet) or NBA (3-nitrobenzyl alcohol)] and mass spectra were recorded on a Nermag R 10-10C spectrometer. Flash chromatography was conducted over silica gel (230–400 Mesh). Analytical TLC was performed on a plastic-plate-supported Silica Gel 60 F254 (E. Merck, layer thickness 0.2 mm). The detection was achieved by treatment with a solution of ninyhydrin (0.2% in ethanol) and/or UV light. Electrospray ionization mass spectra were acquired with a quadrupole instrument with a Waters ZQ 2000.

N-(*t*-Butoxycarbonyl)-*N'*-(1'-methyl-2'-iodoterephthalyl)-4,7,10-trioxatridecane-1,13-diamine [6]

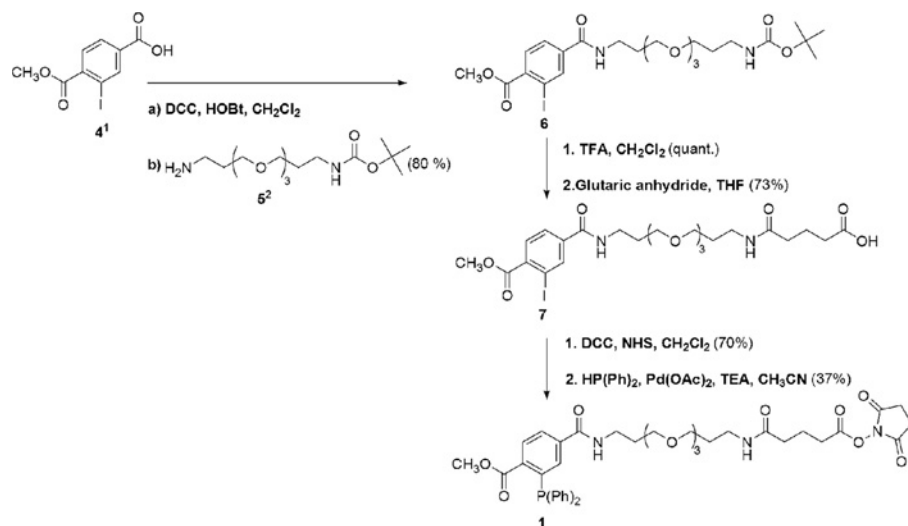
The syntheses of 4 (Saxon and Bertozzi, 2000) and 5 (Trester-Zedlitz et al., 2003) were carried out according to procedures reported in the literature. To a solution of compound 4 (0.2 g, 0.65 mmol) in dry CH₂Cl₂ (20 ml), 715 μl of 1 M DCC solution in CH₂Cl₂ and 0.96 g (0.715 mmol) of HOBT were added under anhydrous conditions. The solution was stirred for 20 min at 0°C, and allowed to reach room temperature during 1 h. After filtration of DCU, the crude solution was mixed with a solution of compound 5 (0.4 g, 1.25 mmol) in CH₂Cl₂ (10 ml) and 100 μl of TEA. The reaction was stirred overnight at room temperature. The solvent was then removed and the residue was flash chromatographed on silica gel (CH₂Cl₂/CH₃OH, 9.3:0.3) to give compound 6 (0.642 g, 83%). Rf: 0.267; ¹H NMR (CDCl₃): δ = 8.37 (s, 1H), 7.8 (m, 2H), 7.40 (s, 1H, NH), 4.90 (bs; 1H, NH), 3.93 (s, 3H), 3.66–3.55 (m, 12H), 3.41 (m, 2H), 3.16–3.14 (m, 2H), 1.90–1.87 (m, 2H), 1.70–1.66 (m, 2H), 1.41 (s, 9H); and CI-MS (C₄H₁₀) for C₂₄H₃₇IN₂O₈: *m/z* 609 as the [M+H]⁺ peak product.

N-(Glutaryl)-*N'*-(1'-methyl-2'-iodoterephthalyl)-4,7,10-trioxatridecane-1,13-diamine [7]

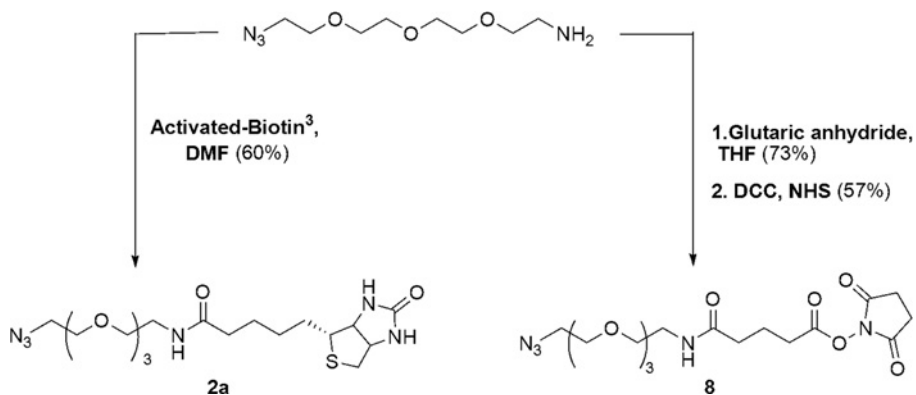
Compound 6 (0.33 g, 0.54 mmol) was dissolved in a CH₂Cl₂/TFA (3:2) mixture at 0°C; this solution was stirred at the same temperature for 1 h and then for 3 h at room temperature. The removal of the protective group was followed by TLC, and the corresponding amine was purified by flash chromatography

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Scheme S1 | Synthesis of the NHS-phosphine reagent [1]



Scheme S2 | Synthesis of modified azido-reagents [2a] and [8]



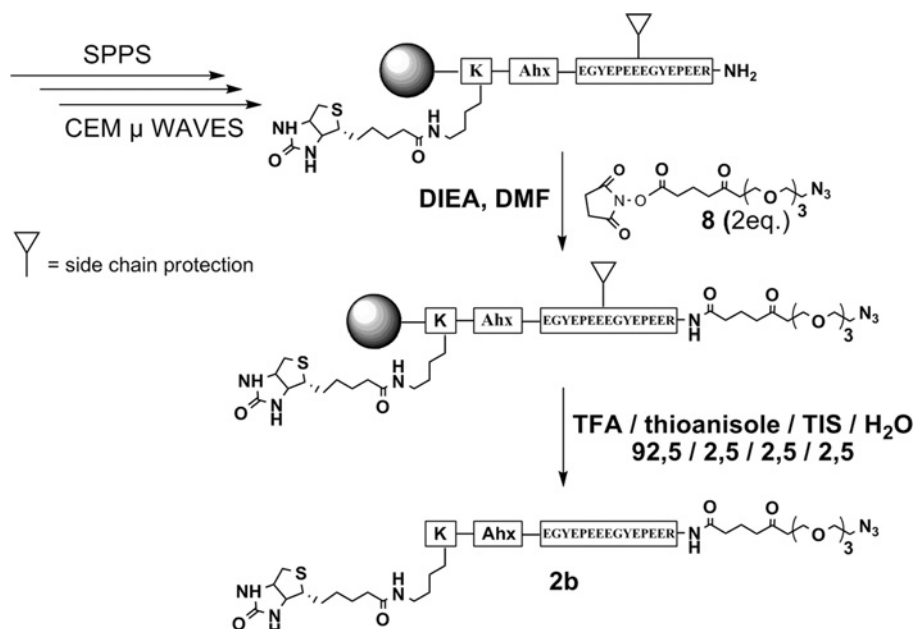
(CH₂Cl₂/MeOH/Et₃N, 0.9:0.5:0.5). To a solution of the preceding compound (0.72 g, 1.15 mmol) in anhydrous THF (71 ml) was added 230 μ l (1.56 mmol) of TEA and 0.206 g (1.7 mmol) of glutaric anhydride. The reaction was allowed to proceed for 3 h at room temperature. The reaction solution was concentrated and the crude product was purified by flash chromatography (CH₂Cl₂/CH₃OH/CH₃COOH, 0.9:0.5:0.5) to give compound 7 (0.44 g, 62%) as an oil. ¹H NMR (CDCl₃): δ = 8.34 (s, 1H), 7.81–7.70 (m, 2H), 6.74 (bs, 1H, NH), 3.87 (s, 3H), 3.65–3.38 (m, 14H), 3.22 (m, 2H), 2.29 (m, 2H), 2.18 (m, 2H), 1.84 (m, 4H), 1.64 (m, 2H); and CI-MS (C₂₄H₃₅IN₂O₉): *m/z* 623 as the [M+H]⁺ peak product.

***N*-(Succinimido-glutaryl)-*N'*-(1'-methyl-2'-diphenylphosphinoterephthalyl)-4,7,10-trioxatridecane-1,13-diamine [1]**

A solution of compound 7 (0.17 g, 0.27 mmol) in CH₂Cl₂ (10 ml) was treated with 300 μ l of 1 M DCC solution in CH₂Cl₂ and 34 mg (0.3 mmol) of *N*-hydroxysuccinimide at 0°C for 1 h. The reaction solution was then stirred for 4 h at room temperature. DCU was removed by filtration and the solvent was evaporated in a vacuum. The crude product was purified by flash chromatography (CH₂Cl₂/MeOH, 0.95:0.05) to give the corresponding activated acid (0.363 g, 77%). The previously activated compound was dissolved in 8 ml of dry and degassed CH₃CN with 195 μ l (0.76 mmol) of TEA and palladium

Endocytic pathway analysis

Scheme S3 | Synthesis of the sulfation site peptide [2b]



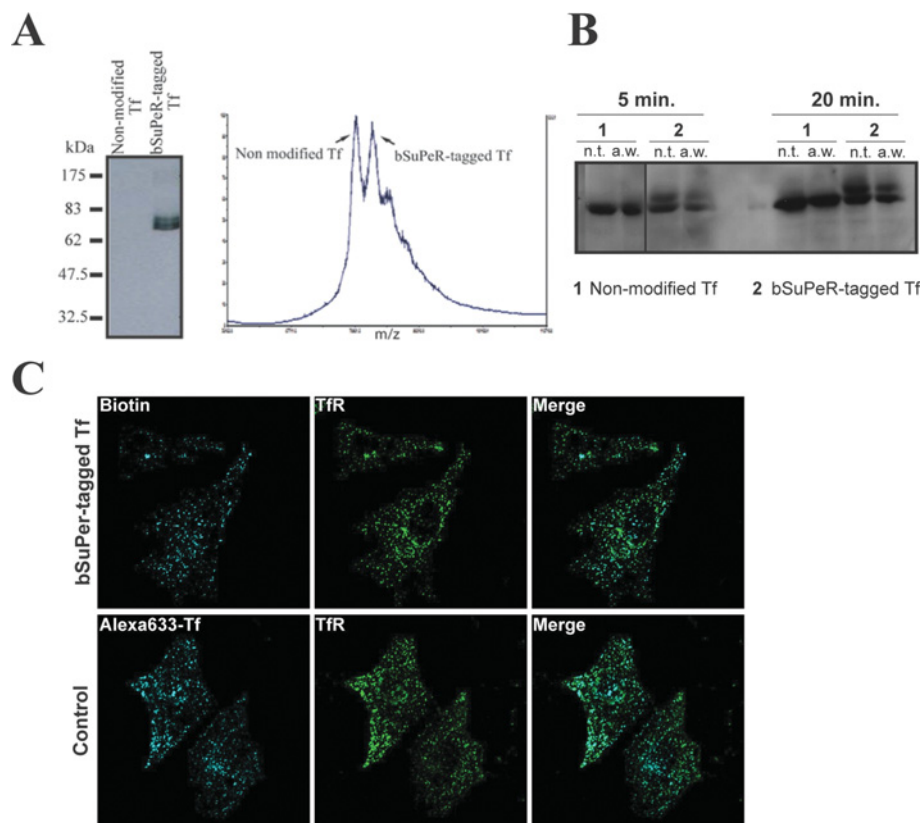
acetate (594 mg). While the mixture was being stirred under an atmosphere of argon, 114 μ l (0.57 mmol) of diphenylphosphine was injected into the flask by means of a syringe. The resulting solution was heated at reflux overnight, allowed to cool at room temperature before being concentrated and purified under argon pressure by flash chromatography ($\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}/\text{CH}_3\text{COOH}$, 0.94:0.03:0.03). The expected compound 1 (0.53 g, 37%) was obtained as a yellowish oil. Rf: 0.375; ^1H NMR (CDCl_3): δ = 8.04 (dd, J = 8.1, 3.6 Hz, 1H), 7.76 (dd, J = 8.1, 1.5 Hz, 1H), 7.38–7.24 (m, 11H), 6.85 (bs, 1H, NH), 6.39 (bs, 1H, NH), 3.72 (s, 3H), 3.61–3.41 (m, 14H), 3.29 (m, 2H), 2.8 (s, 4H), 2.65 (m, 2H), 2.25 (m, 2H), 2.05 (m, 2H), 1.77 (m, 2H), 1.7 (m, 2H); ^{13}C NMR (CDCl_3): δ = 171.6, 169.3, 168.4, 166.8, 166.3, 141.6, 137.6, 137.2, 137.1, 136.6, 136.3, 134.0, 133.8, 133.1, 130.1, 129.0, 126.4, 70.6, 70.4, 70.2, 70.1, 70.0, 69.6, 52.3, 38.6, 37.5, 34.6, 30.0, 29.1, 28.8, 25.6, 20.8; ^{31}P NMR (CDCl_3) δ = –3.3(s, 1, P) (reduced form); and HRMS (FAB+): calculated for $\text{C}_{40}\text{H}_{48}\text{N}_3\text{O}_{11}\text{P}$ [$M+\text{H}$] $^+$ 778.3105, found 778.3120. The product was kept as aliquots of a stock solution (300 mM) in degassed DMSO at -20°C .

N-(11-Azido-3,6,9-trioxatridecanyl)biotinamide [2a]

Commercially available biotin was activated as previously described (Booth et al., 2001). First of all, 0.112 g (0.46 mmol) of biotin, 0.97 g (0.38 mmol) of DSC and 264 μ l (1.90 mmol) of TEA in DMF (15 ml) were stirred at room temperature for 6 h. The commercial available amine 11-azido-3,6,9-trioxatridecan-1-amine (100 μ l, 0.46 mmol), was added and the reaction mixture was stirred overnight. Solvents were removed under vacuum and the crude product was purified with two successive flash chromatography steps: the first using only ethyl acetate and the second with a mixture of dichloromethane/methanol (9:1) as an eluent to provide the coupling product 2a (124 mg, 60%). Rf: 0.417; ^1H NMR (CDCl_3): δ = 6.76 (bs, 1H, NH), 6.64 (bs, 1H, NH), 5.73 (bs, 1H, NH), 4.48 (m, 1H), 4.31 (m, 1H), 3.67–3.53 (m, 12H), 3.41–3.36 (m, 4H), 3.12 (m, 1H), 2.87–2.85 (m, 1H), 2.75–2.71 (m, 1H), 2.21 (m, 2H), 1.67–1.65 (m, 4H), 1.40 (m, 2H); ^{13}C NMR (CDCl_3): δ = 173.4, 164.1, 70.7, 70.6, 70.5, 70.2, 70.0, 69.7, 61.7, 60.2, 55.6, 50.7, 40.5, 39.1, 36.0, 28.2, 28.1, 25.6; and HRMS (CI+): calculated for $\text{C}_{18}\text{H}_{32}\text{N}_6\text{O}_5\text{S}$ [$M+\text{H}$] $^+$ 444.2233, found 444.2215.

Figure S1 | *In vitro* modification of Tf with bSuPeR and endocytosis analysis

(A) *In vitro* labelling of Tf with bSuPeR following the general procedure for *in vitro* protein modification. Commercially available holotransferrin (Sigma, 120 μ M) was incubated for 2 h at room temperature with bSuPeR (1.08 mM, 9 equiv.) during (bSuPeR-tagged Tf), or with PBS buffer alone (Non-modified Tf). After dialysis and SDS-/PAGE, bSuPeR modification of Tf was revealed by Western blotting with streptavidin-HRP (1:1500; left panel) or by MALDI-TOF (right panel). (B) Comparative endocytosis of non-modified Tf (1) and bSuPeR-tagged Tf (2). Cells in suspension (2.5×10^6) were first pre-incubated at 37°C in a serum-free growth medium and then incubated with 1 or 2 for the indicated times. Cells were washed twice in all conditions with ice-cold PBS, and cell surface-bound Tf was removed by incubation for 5 min in ice-cold acid wash buffer pH 2.5 (a.w.). Under control conditions (n.t.), the cells were incubated with the buffer at neutral pH. After pH neutralization, cells were washed twice with ice-cold PBS and lysed. 10% of the lysate was loaded on to polyacrylamide gel (8%) and transferred to nitrocellulose filters. Western blotting was performed using a mouse anti-Tf antibody (1:1000) and an HRP-conjugated goat anti-mouse secondary antibody (1:5000). Note that non-modified Tf and bSuPeR-modified Tf are both internalized with similar efficiencies. (C) Fluorescence microscopy. Serum-starved HeLa cells were incubated for 30 min at 37°C with commercially available Alexa Fluor® 633-tagged Tf (control) or bSuPeR-tagged Tf. The cells were then fixed and biotin was immunolabelled with a rabbit monoclonal anti-biotin antibody and a cyanin 5-conjugated secondary donkey anti-rabbit antibody (cyan), and the Tf receptor was immunolabelled using a mouse monoclonal anti-Tf receptor antibody and an Alexa Fluor® 488-conjugated secondary goat anti-mouse antibody (green).



***N*-(11-Azido-3,6,9-trioxatridecanyl)-succinimidoglutarylamide [8]**

To a solution of the commercial available amine 11-azido-3,6,9-trioxatridecan-1-amine (0.550 g, 2.5

mmol) in anhydrous THF (40 ml) was added 430 μ l (3 mmol) of TEA and 0.350 g (3 mmol) of glutaric anhydride. The reaction was allowed to proceed for 24 h at room temperature. Purification by flash

Endocytic pathway analysis

Figure S2 | Visualization of biotinylated proteins following the general procedure for cell surface modification

(A) Analysis of general plasma membrane protein biotinylation patterns. Cells were incubated for 2 h on ice or at 12°C with two concentrations of **3a**, **2a** (1.5 mM and 0.15 mM) or PBS buffer. The cells were lysed, and lysates were incubated with streptavidin–agarose. Proteins that were retained on the beads were separated by SDS/PAGE, and after transfer to nitrocellulose filters, biotinylated proteins were detected with streptavidin linked to HRP. (B) On the same lysates as in (A), STxB was immunoprecipitated using specific antibodies. A star (*) indicates STxB-specific labelling. Labelling in the upper part of the gel originates from contaminating endogenous proteins. (C) Following the same procedure described in (A), cells were incubated for 2 h on ice with (bSuPeR) or without (PBS) bSuPeR.

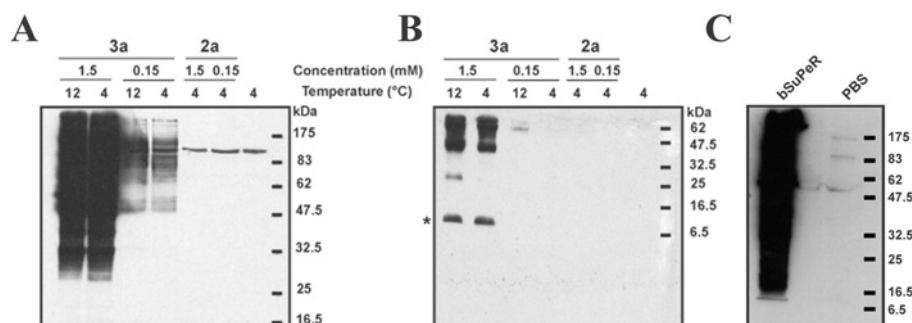
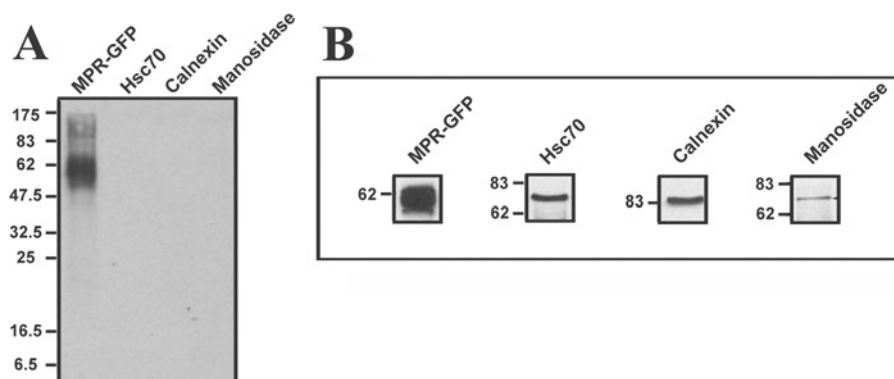


Figure S3 | Analysis of biotinylation of organelle markers in the conditions of cell surface modification

The cells were incubated for 2 h on ice with 1.5 mM of bSuPeR, washed and lysed and lysates were incubated with specific antibodies to the following organelle markers: MPR–GFP (Waguri et al., 2003) (plasma membrane), Hsc70 (cytosol), mannosidase (Golgi apparatus) and calnexin (endoplasmic reticulum). Western blotting was performed with streptavidin–HRP (A) or antibodies to the proteins (B). Note that all proteins were recovered by immunoprecipitation, but only the plasma membrane marker, MPR–GFP, was biotinylated. These findings confirm that bSuPeR does not diffuse spontaneously across the plasma membrane.



chromatography (cyclohexane/acetone/acetic acid, 6:4:1) of the crude product led to the corresponding acid (0.610 g, 73%). A solution of the previous acid compound (0.610 g, 1.8 mmol) in CH₂Cl₂ (10 ml) was treated with 2.2 ml of 1 M DCC solution in CH₂Cl₂ and 0.253 g (2.2 mmol) of *N*-hydroxysuccinimide at 0°C for 1 h and then the reaction solution was stirred for 4 h at room temperature. DCU was filtered, and the crude solution was pur-

ified by flash chromatography (cyclohexane/acetone, 1:1). The expected activated acid **8** (0.610 g, 57%) was obtained as a colourless oil. Rf: 0.27; ¹H NMR (CDCl₃): δ = 6.35 (bs, 1H, NH), 3.61 (m, 10H), 3.51 (m, 2H), 3.41 (m, 2H), 3.35 (m, 2H), 2.81 (s, 4H), 2.66 (m, 2H), 2.28 (m, 2H), 2.05 (m, 2H); ¹³C NMR (CDCl₃): δ = 171.7, 169.3, 169.3, 168.4, 70.7, 70.6, 70.5, 70.2, 70.0, 69.7, 50.6, 39.2, 34.4, 30.0, 25.6, 25.4, 20.7. HRMS (CI⁺):

calculated for $C_{17}H_{27}N_5O_8$ $[M+H]^+$ 430.1938, found 430.1940.

Synthesis of the sulfation site peptide [2b]

Synthesis of the biotinylated sulfation site peptide Lys(Biot)-Ahx-(EGYEPEE)2-NH₂ was carried out by solid-phase synthesis using the standard Fmoc (fluoren-9-ylmethoxycarbonyl)-based strategy on a Novasyn TGR resin (0.2 mmol scale) in a microwave peptide synthesizer (CEM μ WAVES, Saclay, France). Couplings were performed using 5-fold molar excess of each Fmoc L- or D-amino acid, 4.5-fold molar excess of HBTU and 10-fold molar excess of DIEA. Reaction with NHS (*N*-hydroxysuccinimidyl) compound **8** allowed attachment of the azido group. **8** (429 mg, 1 mmol, 5 equiv.) dissolved in DMF (1 ml) and DIEA (147 μ l, 1 mmol, 5 equiv.) was added to a peptide synthesis vessel containing the biotinylated sulfation site peptide on the solid support. The coupling reaction was allowed to proceed under argon at room temperature. Completion of the reaction was monitored with the Kaiser test. After 1 h, the reaction mixture was removed and the resin was washed with DMF followed by CH₂Cl₂ and ether. After drying for 2 h under vacuum, the peptide was removed from

the resin by treatment with TFA/thioanisol/TIS/H₂O (92.5:2.5:2.5:2.5, by vol.). The crude peptide was precipitated in diethyl ether/heptane (1:1, v/v), dissolved in water and freeze-dried. The crude peptide was purified by RP-HPLC on a 100 Å 5 μ m C18 Nucleosil column (1 Å = 0.1 nm) using a linear water/acetonitrile gradient containing 0.05% TFA by volume (6 ml/min, detection 215 nm). The fractions were collected and freeze-dried to give **2b** (67% yield). The purity (>95%) was determined by analytical RP-HPLC and MS. Spectral data: MS (MALDI) calculated m/z 2621.4 for **2b** and found 2622.3 as the $[M+H]^+$ peak product.

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Received 15 January 2010; accepted 25 January 2010

Published as Immediate Publication 25 January 2010, doi:10.1042/BC20100008