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Intracellular pathway of Onconase that enables its delivery to the cytosol

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Summary

Onconase[®] is an RNase with a very specific property because it is selectively toxic to transformed cells. This toxin is thought to recognize cell surface receptors, and the protection conferred by metabolic poisons against Onconase toxicity indicated that this RNase relies on endocytic uptake to kill cells. Nevertheless, its internalization pathway has yet to be unraveled. We show here that Onconase enters cells using AP-2/clathrin-mediated endocytosis. It is then routed, together with transferrin, to the receptor recycling compartment. Increasing the Onconase concentration in this structure using tetanus toxin light chain expression enhanced Onconase toxicity, indicating that recycling endosomes are a key compartment for Onconase cytosolic delivery. This intracellular destination is specific to Onconase because other (and much less toxic) RNases follow the default pathway to late endosomes/lysosomes. Drugs neutralizing

endosomal pH increased Onconase translocation efficiency from purified endosomes during cell-free translocation assays by preventing Onconase dissociation from its receptor at endosomal pH. Consistently, endosome neutralization enhanced Onconase toxicity up to 100-fold. Onconase translocation also required cytosolic ATP hydrolysis. This toxin therefore shows an unusual entry process that relies on clathrin-dependent endocytic uptake and then neutralization of low endosomal pH for efficient translocation from the endosomal lumen to the cytosol.

Supplementary material available online at <http://jcs.biologists.org/cgi/content/full/120/8/1405/DC1>

Key words: Onconase, RNase, Coated pits, Recycling endosomes, Endocytosis, Translocation

Introduction

Onconase[®] (Onco, also termed ranpirnase) is selectively cytotoxic to transformed cells (Darzynkiewicz et al., 1988) and possesses potent in vivo antitumour activity (Mikulski et al., 1990). Onco is currently undergoing phase III clinical trials for the treatment of malignant mesothelioma (Favaretto, 2005).

Onco toxicity essentially results from its ability to degrade tRNAs (Saxena et al., 2002), whereas intracellular routing that enables Onco delivery to the cytosol remains elusive. Pioneer studies showed that Onco binding to cells was saturable and probably involved membrane receptors. Moreover, metabolic poisons were found to protect cells against Onco toxicity, indicating that this toxin does not access the cytosol by direct transport across the plasma membrane, and that endocytosis is required for cytotoxicity (Wu et al., 1993).

Several receptors enter cells using clathrin-mediated endocytosis (CME). This process is dependent on clathrin, adaptors and the GTPase dynamin (Conner and Schmid, 2003). The involvement of dynamin in internalization processes is usually studied using the dynamin-K44A mutant that has a weak affinity for GTP and acts in a dominant-negative manner (Damke et al., 1994). Regarding Onco entry, toxicity was found to be slightly enhanced upon dynamin-K44A expression by stably transfected and inducible HeLa cells, indicating that Onco internalization is dynamin independent (Haigis and Raines, 2003).

Following initial uptake, endocytosed tracers are delivered to sorting endosomes. From there they can be directed to the endosome recycling compartment located in the pericentriolar region of the cell. This is the case for recycling ligands such as transferrin (Tf). Alternatively, endocytosed proteins can be transported to late endosomes/lysosomes (Gruenberg and Maxfield, 1995). Most RNases seem to follow this degradation pathway, because neither RNase A (Haigis and Raines, 2003) nor a human pancreatic RNase variant colocalized with Tf upon uptake. The latter mutant indeed accumulated within late endosomes/lysosomes (Bosch et al., 2004).

Onco intracellular routing has been evaluated using drugs, such as those able to increase acidic endosomal pH, which is in the pH 5.5–6.5 range (Gruenberg and Maxfield, 1995). Among these molecules, ammonium chloride did not show any effect, whereas monensin enhanced Onco toxicity by 10-fold. Brefeldin A, which prevents retrograde transport to the endoplasmic reticulum (ER), either had no effect (Wu et al., 1993) or potentiated Onco toxicity by 10-fold (Haigis and Raines, 2003). Both studies concluded that Onco probably gains access to the cytosol from endosomes and not from the Golgi or the ER (Haigis and Raines, 2003; Wu et al., 1993). Accordingly, Onco could be visualized within unidentified intracellular vesicles (Haigis and Raines, 2003). The role of endosome acidification in Onco toxicity has yet to be identified.

Results and Discussion

To visualize the internalization of Onco by human cells, we introduced a Cys residue into Onco for labeling. We selected position 72, which is located in a solvent-exposed surface loop remote from the active site (see supplementary material Fig. S1). OncoS72C was fluorescently labeled to obtain Onco-Red, which was as toxic as Onco and therefore biologically active.

To assess the Onco initial uptake pathway, we used dynamin-K44A and other well-established CME inhibitors such as a dominant-negative mutant of Eps15 that selectively inhibits clathrin/AP-2-dependent endocytosis (Benmerah et al., 1999), and the intersectin SH3 domain that, when overexpressed, impairs clathrin-dependent uptake (Simpson et al., 1999).

We transiently overexpressed these constructs in Jurkat cells and monitored the capacity of transfected cells, identified by the enhanced green fluorescent protein (EGFP) tag of the construct (or by cotransfection with EGFP in the case of dynamin), to internalize Onco-Red. The control version of Eps15 (Eps15D3Δ2) or dynamin [wild type (WT)] did not affect the internalization of Onco or the early endosome marker Tf, whereas dominant-negative constructions impairing coated vesicle formation (i.e. Eps15Δ95/295, the intersectin SH3A domain and dynamin-K44A) prevented, with almost the same efficiency, Tf and Onco uptake (Fig. 1A). These results indicated that Jurkat cells endocytosed Onco through an Eps15-, intersectin- and dynamin-dependent route, and presumably the well-characterized clathrin/AP-2-mediated endocytic pathway.

We then examined Onco localization at the plasma membrane by electron microscopy (EM). Because RNases are conserved proteins it is difficult to raise good antibodies against them (Beintema and Kleineidam, 1998). We therefore directly labeled Onco with gold and incubated Jurkat cells at 37°C with this conjugate. Onco-gold was found within coated pits (Fig. 1B). A morphometric analysis showed that more than 20% of plasma membrane-associated Onco localized within these structures. Because coated pits occupy ~2% of the cell surface in T cells (Foti et al., 1997), we concluded that Onco is specifically concentrated within coated pits at the plasma membrane.

To examine whether CME was required by Onco to access the cytosol, we tested the ability of the above described effectors to protect cells against this toxin. Because Onco kills cells by inducing apoptosis (Grabarek et al., 2002; Iordanov et al., 2000), we set up a fluorescence-activated cell sorting (FACS) assay to specifically monitor Onco toxicity to transfected cells using fluorescent annexin-V binding to EGFP-positive cells. Although Onco-induced apoptosis was not significantly affected by overexpression of the control constructions (Fig. 1C), all the dominant-negative constructs inhibited Onco toxicity with an efficiency ranging from 32% (Dynamin-K44A) to 54% (Eps15 Δ95/295). Hence, Onco enters cells using clathrin-mediated uptake, and this enables its subsequent delivery to the cytosol.

We then examined Onco routing along the endocytic network. This study was performed on HeLa cells that enable easier visualization and compartment discrimination than lymphocytes. Onco-Red was internalized in the same endosomal structures as Tf-Cy5 (Fig. 1A, Fig. 2). In contrast to other RNases that enter cells slowly and are directed to the

degradative pathway (Bosch et al., 2004; Haigis and Raines, 2003), Onco-Red was quickly internalized (data not shown) and did not significantly colocalize with established late endosome/lysosome markers such as lysosomal-associated membrane proteins (Lamp-1 and Lamp-2), internalized dextran, lysobisphosphatidic acid (Fig. 2) or the mannose 6-phosphate receptor (data not shown). Onco-containing structures were negative for calnexin or TGN46, indicating that this protein is not transported to the ER or the *trans*-Golgi network. Onco intracellular routing was restrained to Tf-positive structures, and the efficiency of Onco colocalization with Tf (75-80%) was the same as that observed when using two Tf bearing different fluorophores (Sabharanjak et al., 2002).

More precisely, Onco seemed to concentrate within a compartment at the center of the cell where fluorescent Tf accumulates upon labeling, i.e. the recycling compartment (Mallard et al., 1998). Recycling endosomes are the sole structure in the endocytic pathway where the small GTPase Rab11 is present (Sonnichsen et al., 2000). Internalized Onco colocalized very efficiently with Rab11-EGFP and Tf in the pericentriolar recycling compartment at the center of the cell (see supplementary material Fig. S2). Together, these data indicated that Onco follows Tf internalization, from coated pits to the recycling compartment.

To further show that Onco endocytosis followed a similar pathway to Tf internalization and that Onco was not, as other RNases, targeted to lysosomes (Bosch et al., 2004), we monitored the effects of overexpressing Rab5 or Rab7 mutants on Onco endocytosis. Rab5 controls traffic to the sorting endosome compartment (Gruenberg and Maxfield, 1995), and a dominant-negative mutant of Rab5 (Rab5S34N) slows down endocytosis of ligands such as Tf (Stenmark et al., 1994). Active Rab7 is required for transport to late endosomes/lysosomes. Indeed, a dominant-negative version of Rab7 (Rab7N125I) prevents tracer delivery to late endosomes, but does not significantly inhibit Tf uptake (Bucci et al., 2000; Vitelli et al., 1997). Neither the activated version of Rab5 (Ceresa et al., 2001) nor of Rab7 (Bucci et al., 2000; Vitelli et al., 1997) were found to influence uptake of Tf or lysosome-targeted molecules. Accordingly, we observed that the WT or the activated version of Rab5 and Rab7 did not significantly affect Onco or Tf internalization (Fig. 3A). Regarding dominant-negative mutants, Onco and Tf uptake was strongly inhibited in cells overexpressing Rab5S34N, but was not affected by Rab7N125I (Fig. 3A).

Similar data were obtained when Onco toxicity to transfected cells was examined, and the sole effector that significantly affected Onco toxicity was dominant-negative Rab5 (Rab5S34N) that partially protected cells against Onco (~25%, Fig. 3B). Together with morphological examination, the Rab5 data showed that Onco internalization and toxicity is regulated by this small GTPase. The result that dominant-negative Rab7 affects neither Onco intracellular routing nor its toxicity confirmed that the Onco intracellular pathway is restricted to early endosomes, and that Onco does not need delivery to late endosomes/lysosomes to reach the cytosol and kill cells.

To examine whether recycling endosomes were implicated in Onco toxicity we transfected cells with tetanus toxin light chain (TeNT-LC), a highly specific protease that cleaves and

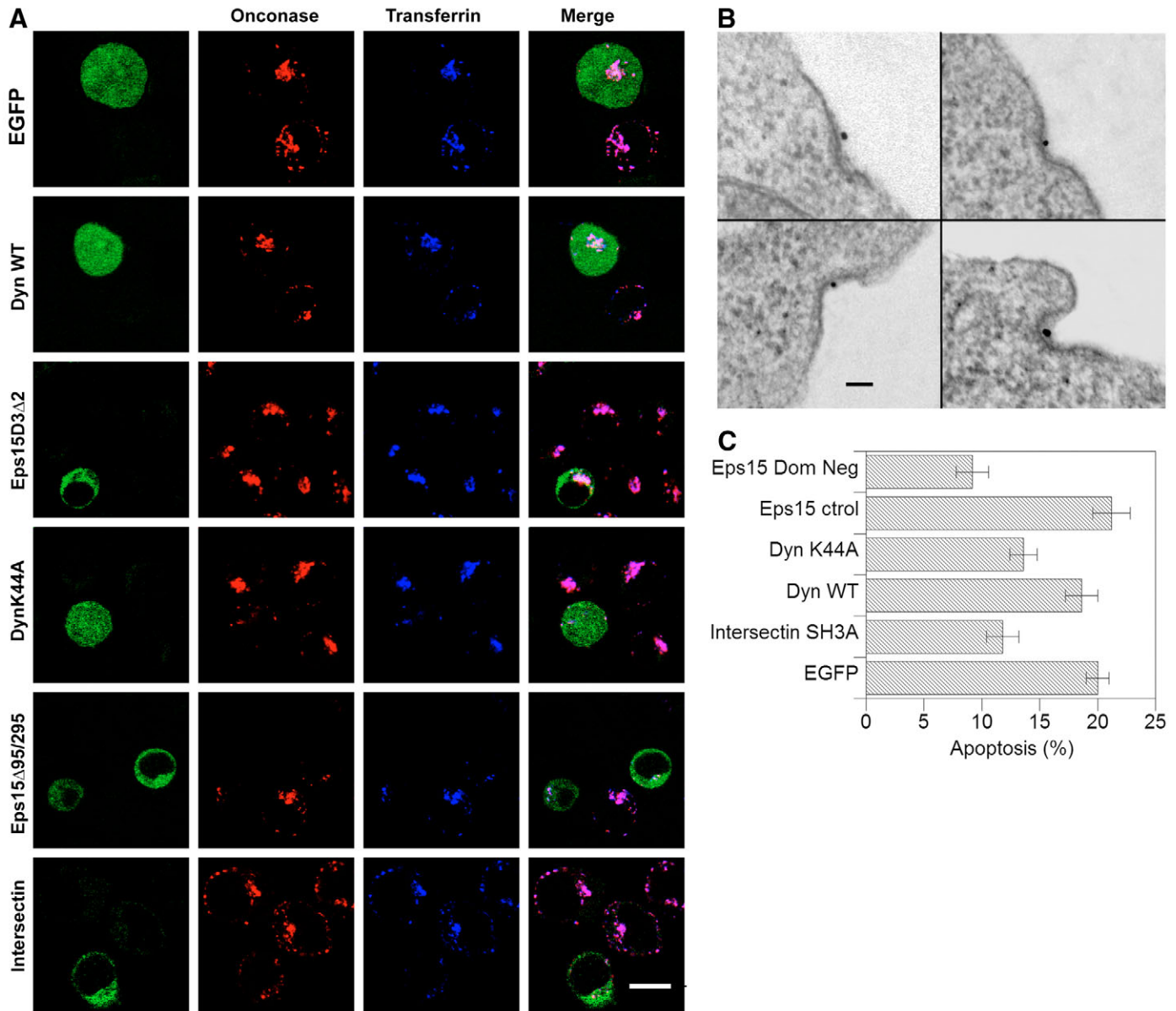


Fig. 1. Onco enters cells using the clathrin-mediated pathway. (A) Inhibition of AP-2-dependent endocytosis prevents Onco internalization. Jurkat cells were transiently transfected with a vector coding for an EGFP-tagged protein (Eps15D3Δ2, Eps15Δ95/295 or intersectin SH3A), or with both an EGFP vector and another coding for dynamin II-WT or dynamin II-K44A. After 24 hours, 250 nM Onco-Red and 100 nM Tf-Cy5 were added for 45 minutes. Cells were then washed, fixed and examined under a confocal microscope. Representative sections through the middle of the cell. Bar, 10 μm. (B) Onco is found within coated pits at the plasma membrane. Jurkat cells were labeled for 15 minutes at 37°C with Onco conjugated to 10-nm diameter colloidal gold. They were then cooled to 4°C, washed, fixed and processed for conventional electronmicroscopic examination. Bar, 50 nm. (C) Inhibition of clathrin-mediated endocytosis protects cells against Onco-induced apoptosis. Jurkat cells were transfected with a vector coding for EGFP alone or EGFP-tagged proteins, as indicated. After 24 hours, 6 μM Onco was added. Apoptosis was quantified 24 hours later by monitoring annexin-V-Cy5 binding to transfected cells by FACS. The results are expressed as the mean ± s.e.m. of three separate experiments.

inactivates cellubrevin, a SNARE protein present on different types of vesicles, but colocalizing with Tf at the recycling endosome level only (Wilcke et al., 2000). Cellubrevin is required for efficient Tf recycling, although 70% of Tf recycling is cellubrevin independent (Galli et al., 1994). In agreement with these findings, TeNT-LC expression enhanced Onco toxicity by 32%, whereas the proteolytically inactive E234Q mutant had no significant effect (Fig. 3B). This result

is consistent with the view that the recycling compartment enables Onco translocation to the cytosol and that a higher Onco concentration at this level favors toxicity. This interpretation is in agreement with the differential effect of Tf coupling to Onco or RNase A (Rybak and Newton, 1999). When RNase A was coupled to Tf its cytotoxicity increased by ~10,000-fold, probably as a result of both more efficient uptake and routing to the recycling compartment. By contrast,

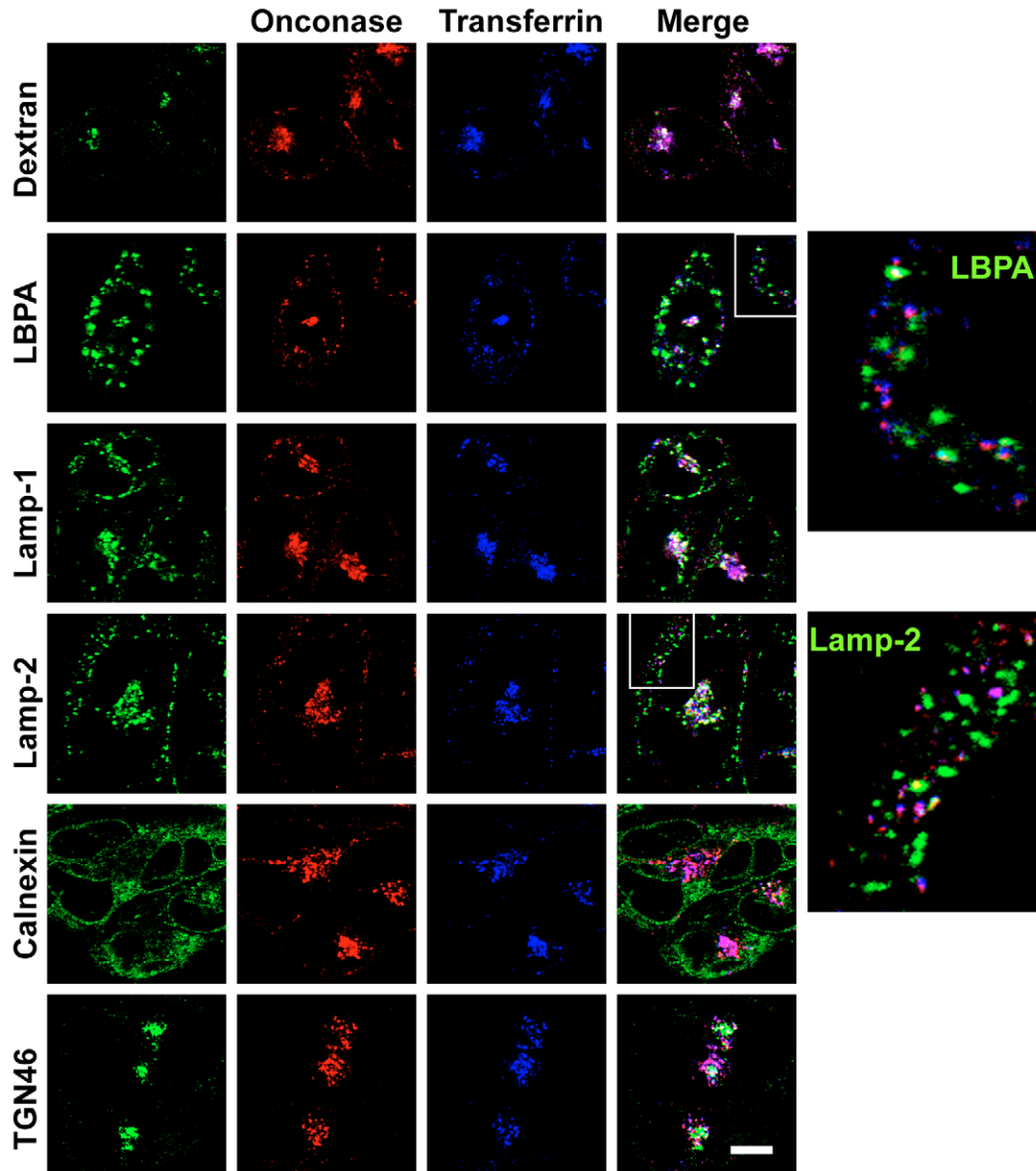


Fig. 2. Onco intracellular pathway. HeLa cells were incubated for 45 minutes with Onco-Red and Tf-Cy5 (to label early endosomes). When indicated, cells were also labeled with dextran-FITC, a tracer that is addressed to lysosomes. Cells were then processed for immunodetection of markers for late endosome/lysosomes [Lamp-1, Lamp-2 or lysobisphosphatidic acid (LBPA)], the ER (calnexin) or the *trans*-Golgi network (TGN46), before mounting and observation under a confocal microscope. Median optical sections; bar, 10 μ m. Boxed areas in LBPA and Lamp-2 images indicate regions enlarged on the right.

conjugation of Onco to Tf only enhanced its cytotoxicity by 10-fold, probably reflecting an increase in internalization efficiency only.

Studies on the role of endosomal low pH in Onco toxicity used 20–30 mM ammonium chloride to neutralize endosomes and failed to reveal any effect of this treatment on Onco toxicity. The less specific drug monensin that also affects Golgi structure nevertheless enhanced toxicity by 10-fold on 9L and K562 cells. It was concluded that Onco toxicity was insensitive to endosome neutralization (Haigis and Raines, 2003; Wu et al., 1993). We re-examined these findings by monitoring endosomal pH and additionally using bafilomycin A1 (Baf), which is a specific inhibitor of the vacuolar ATPase responsible

for endosome acidification (Crider et al., 1994). For these experiments, we used both HeLa and A431 cells, and measured the endosomal pH using double-labeled fluorescent Tf for the latter cell line. We obtained a pH value of approximately pH 5.3 for the Tf compartment in A431 cells, very similar to the pH 5.6 observed in HeLa cells (Baravalle et al., 2005).

In agreement with previous findings (Haigis and Raines, 2003; Wu et al., 1993), we observed that ammonium chloride either did not affect (HeLa) or moderately sensitized (fivefold for A431) cells to Onco (Fig. 4A). Nevertheless, this molecule alone (20 mM) only increased endosomal pH by 0.4 pH units (from pH 5.3 to pH 5.7, Fig. 4B). Only when mixed with methylamine (20 mM) was NH_4Cl able to almost neutralize

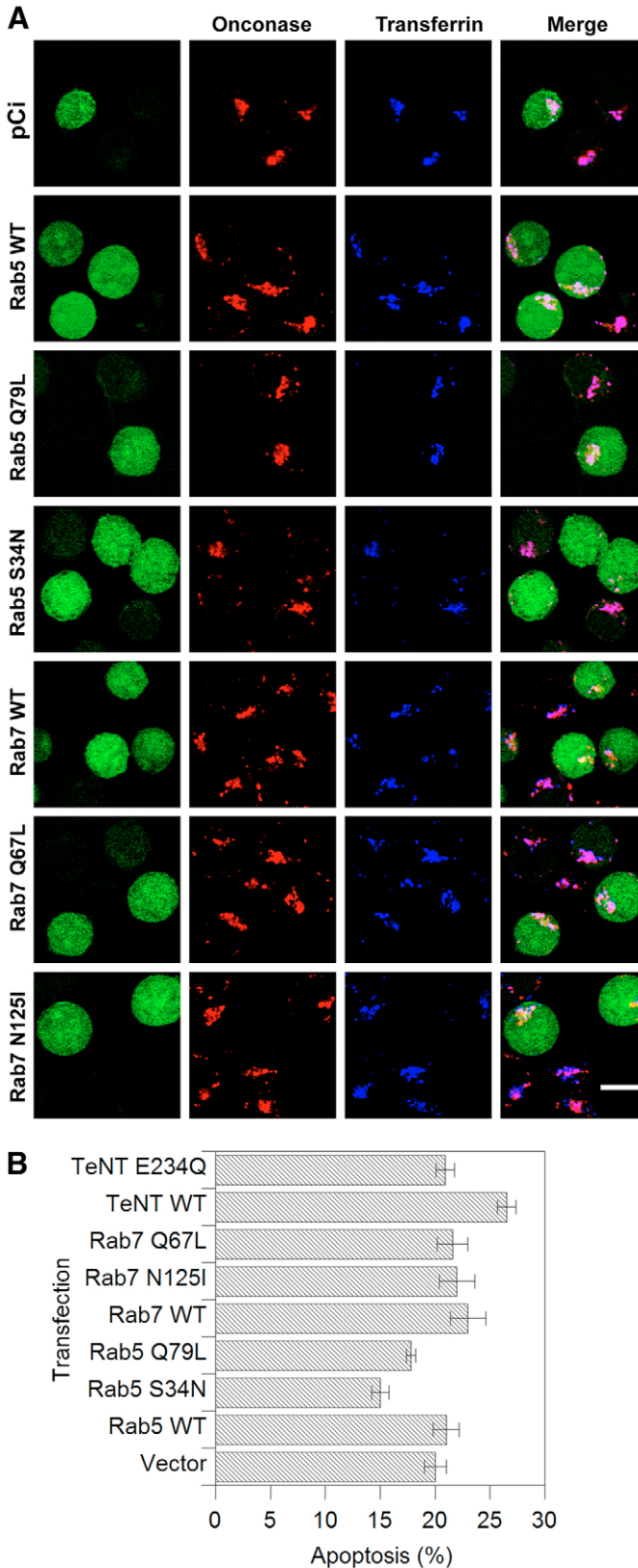


Fig. 3. Onco endocytosis and toxicity are regulated by Rab5. (A) Onco internalization depends on Rab5. Jurkat cells were transfected with the indicated Rab5 or Rab7 expression vectors, together with EGFP. After 24 hours, Onco-Red and Tf-Cy5 were added for 45 minutes before fixation and confocal microscopic examination. Bar, 10 μ m. (B) Cellubrevin and Rab5 regulate Onco delivery to the cytosol. Jurkat cells were transfected with EGFP and either the wild type or a mutated version of Rab5, Rab7 or TeNT-LC, as indicated. After 24 hours, 6 μ M Onco was added. Cytotoxicity was measured after 24 hours, on transfected cells, using annexin-V-Cy5 labeling and FACS analysis.

100-fold and completely neutralized the endosomal pH. We concluded that neutralization of low endosomal pH enhances Onco toxicity by more than one log and that this sensitization effect cannot be obtained using NH_4Cl alone, which weakly increases the endosomal pH. How can endosome neutralization enhance Onco toxicity? Endosome-neutralizing drugs such as Baf do not affect the routing of ligands that are addressed to the recycling compartment, such as Tf (Baravalle et al., 2005; Presley et al., 1997), *Pseudomonas* exotoxin (Alami et al., 1998) or shiga toxin (Mallard et al., 1998). Consistent with these findings, we found that Onco was still efficiently addressed to the recycling compartment in the presence of Baf (see supplementary material Fig. S3), indicating that endosome neutralization did not affect Onco intracellular routing. Hence, these drugs probably affected a later step of the intoxication procedure, most likely transmembrane transport. To examine this issue, we labeled mouse lymphocytes with ^{125}I -Onco (or ^{125}I -Tf as a control), and purified endosomes to directly assay Onco translocation in a cell-free system. We previously used the same approach to characterize the endosomal translocation process of bacterial (Alami et al., 1998; Beaumelle et al., 1992) or viral (Vendeville et al., 2004) toxins.

In the presence of Baf and ATP, Onco translocation reached a plateau after 1 hour, whereas no Tf release was observed under these conditions (see supplementary material Fig. S4). In the absence of endosome neutralization, Onco translocation was still significant, but this process became approximately fivefold more active when endosomal pH was neutralized using Baf or monensin (Fig. 4C). This finding is in agreement with toxicity experiments and indicates that this cell-free assay faithfully reconstitutes the Onco translocation process, and that this transmembrane transport is facilitated by endosome neutralization. Onco translocation required ATP hydrolysis because the nonhydrolysable analog $\text{ATP}\gamma\text{S}$ did not support translocation (Fig. 4D).

The finding that Onco translocates more efficiently from neutralized endosomes was surprising. Indeed, conventional endosome-translocating toxins such as diphtheria and anthrax toxin (Abrami et al., 2005; Beaumelle et al., 1992), *Pseudomonas* exotoxin (Méré et al., 2005) and HIV-1 Tat (Vendeville et al., 2004) usually rely on endosome acidification to trigger membrane insertion and translocation, and drugs increasing endosomal pH prevent translocation. To determine why Onco translocates more efficiently from neutralized endosomes, we tested whether Onco was released from its receptor, and therefore from the membrane at low pH. To this end, we labeled Jurkat cells with ^{125}I -Onco, washed away the excess ligand and incubated labeled cells at different pH values before pelleting them and counting both the cells and

endosomes (20 mM methylamine plus 20 mM NH_4Cl raised endosomal pH to 6.6). Monensin (0.25 μ M) enhanced Onco toxicity by 5-80-fold while efficiently increasing the endosomal pH to pH 6.6. Baf enhanced Onco toxicity by 20-

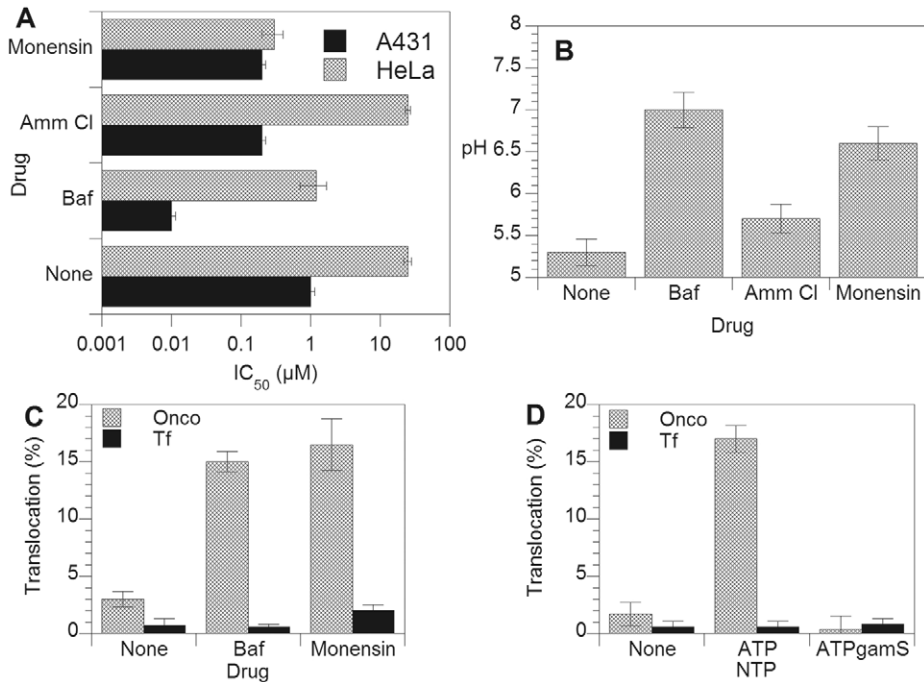


Fig. 4. Onco toxicity and translocation are enhanced by endosome neutralization.

(A) Cytotoxicity to HeLa and A431 cells. Cells were pre-incubated for 30 minutes with 0.25 μ M monensin, 20 mM NH_4Cl (Amm Cl) or 100 nM bafilomycin (Baf), as indicated, before adding various concentrations of Onco. Cell viability was measured after 24 hours (HeLa) or 36 hours (A431). The results are expressed as the Onco concentration generating a growth inhibition of 50% (IC_{50}). Mean \pm s.e.m. of at least three separate experiments. (B) Endosomal pH measurements. A431 cells were labeled for 40 minutes with FI-Tf-Rh before acquiring FI and Rh fluorescence images using a confocal microscope. The FI/Rh intracellular intensity ratio was then used together with a calibration curve to determine the endosomal pH (Dunn et al., 1994; Presley et al., 1997) in living cells treated with the indicated drug. (C) Endosome neutralization enhances Onco translocation. ^{125}I -Onco- or ^{125}I -Tf-loaded lymphocyte endosomes were purified and resuspended in translocation buffer supplemented with 10 mM ATP. Translocation assays (Morlon-Guyot et al., 2003; Vendeville et al., 2004) were performed for 1 hour in the presence or absence of 400 nM Baf or 20 μ M monensin, as indicated. Translocation activity corresponds to the time course increase in the supernatant/(endosome plus supernatant) radioactivity ratio. (D) Onco translocation requires cytosolic ATP hydrolysis. Translocation was assayed in the presence of Baf and 10 mM ATP or $\text{ATP}\gamma\text{S}$, as indicated.

supernatant. The experiment was performed at 4°C to prevent any endocytosis, and ^{125}I -Tf, which is not released from its receptor at low pH (Dautry-Varsat et al., 1983), was used as a control. Although, as expected, ^{125}I -Tf remained bound to its receptor down to pH 4.7, ~60% of ^{125}I -Onco was effectively released from cells below pH 5.5 (see supplementary material Fig. S5). These data showed that endosome-neutralizing drugs potentialize Onco toxicity by preventing Onco release from its receptor(s) at the low pH encountered within the endocytic pathway, and that this receptor probably plays a major role in translocation.

In this work, we found that Onco uses the well-characterized AP-2/CME pathway for initial uptake. This finding seems difficult to reconcile with a previous study that indicated that Onco endocytosis is dynamin-independent (Haigis et al., 2003). Nevertheless, their conclusions were essentially drawn from cytotoxicity experiments using a HeLa cell line stably transfected with inducible dynamin-K44A dominant-negative mutant. Indeed, expression of mutant dynamin in this system did

not protect cells against Onco (Haigis et al., 2003). We obtained the same results using this cell line (data not shown). Nevertheless, mutant dynamin induction leads to an increase in the pH of endocytic compartments by 0.4 to 0.7 pH units, as measured using double-labeled fluorescent dextran (Bayer et al., 2001). In the light of our observations that Onco is taken up by CME (Fig. 1) and that endosomal neutralization enhances Onco toxicity (Fig. 4A), it seems likely that two antagonistic effects are responsible for the absence of protection against Onco observed upon dynamin-K44A expression by these inducible and stably transfected HeLa cells. Indeed, although less toxin was endocytosed upon transgene expression, Onco encountered a higher endosomal pH than in uninduced cells (Bayer et al., 2001), and thus remained tightly bound to its receptor (see supplementary material Fig. S5), thereby accessing the cytosol more easily.

Materials and Methods

Protein expression, purification and labeling

Recombinant Onco was produced from *Escherichia coli* as described (Ribo et al., 2004). Onco was conjugated to 10-nm diameter colloidal gold at pH 9.0 (Beaumelle and Hopkins, 1989). Onco and Tf were labeled with ^{125}I using immobilized lactoperoxidase and Iodogen (Pierce), respectively. Alexa Fluor-594 C5 maleimide (Molecular Probes) was conjugated to OncoS72C (Bosch et al., 2004) to obtain Onco-Red. Tf was labeled with Cy5 (Amersham Biosciences) or with both fluorescein- and rhodamine-succinimidyl esters (Molecular Probes) to obtain FI-Tf-Rh, as described (Presley et al., 1997). Fluorescent labelings were followed by dialysis against PBS at 4°C.

Plasmids and transfections

Plasmids pEGFP (Clontech) containing Eps15 DIII Δ 2, Eps15 Δ 95/295 (Benmerah et al., 1999), intersectin SH3 domain (Simpson et al., 1999) as well as pCMV5-Dynamin II [WT or K44A (Lamaze et al., 2001)] and vectors coding for TeNT-LC [WT or the E234Q inactive mutant (Proux-Gillardeaux et al., 2005)], Rab5a [WT, Q79L or S34N (Mukhopadhyay et al., 1997)] or Rab7 [WT, Q67L, N125I (Vitelli et al., 1997)] have been described. Rab4-EGFP and Rab5-EGFP expression plasmids were provided by Anne Blangy (CRBM, Montpellier, France) and Rab11-EGFP by Jean Salamero (Institut Curie, Paris, France). HeLa and Jurkat cells were transfected using Superfect (Qiagen) or electroporation, respectively.

Immunofluorescence and quantitative fluorescence microscopy

Sample preparations and immunofluorescence observations were performed as described (Bosch et al., 2004; Vendeville et al., 2004). The procedure for endosome-pH measurements has been described in detail (Dunn et al., 1994; Presley et al., 1997). After quantitation of the signal from internalized FI-Tf-Rh using ImageQuant (Amersham Biosciences), the FI/Rh image ratio was plotted against the pH. This calibration curve was then used for endosomal-pH measurements in live cells ($n > 50$).

Cytotoxicity assays on transfected cells

One day after transfection with pEGFP and the indicated effector, cells were treated with 6 μ M Onco for 24 hours, then labeled with annexin V-Cy5 as recommended (BD Biosciences) and analyzed by FACS using a four-color FACScalibur cytometer,

gating on transfected cells using EGFP fluorescence. Apoptosis was monitored using annexin-Cy5 binding to these cells. Approximately 20% of the cells became annexin-V-positive upon Onco treatment. 10,000 events were collected for each assay, which was performed in duplicate. Results are the mean \pm s.e.m. of three separate experiments.

Conventional cytotoxicity and cell-free translocation assays

Cytotoxicity assays with pharmacological agents were performed using HeLa and A431 cells as described (Morlon-Guyot et al., 2003). For translocation assays, mouse BW lymphocytes were labeled with ^{125}I -Onco (or ^{125}I -Tf as control) and purified as described (Vendeville et al., 2004). Endosomes were resuspended in translocation buffer. Translocation was assayed for 0–90 minutes at 37°C, before cooling and ultracentrifugation. Translocation was monitored using the increase in the supernatant/(endosome plus supernatant) radioactivity ratio.

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