SUPPLEMENT FOR EMBO 48044
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Material and Methods

Scrape wounding and healing

HeLa monolayers, grown to confluency on glass coverslips, were transferred to HMEM supplemented with 2.5 mg/ml lysine fixable, 10 kDa Alexa594-dextran (Molecular Probes) (to follow membrane injury and opening to the media) during wounding by scratching 20 times in a grid pattern with the tip of a single-edge surgical blade (Feather industries LTD) at room temperature (Swanson and McNeil, 1987). Cells were rapidly washed (<1 min) three times with PBS, and transferred to warm (37°C) HMEM medium under 5% CO_2. After 5 min, the media was exchanged with HMEM containing 2.5 mg/ml lysine fixable 10 kDa Alexa-647-dextran (Molecular Probes) and incubation was pursued for 2 min at 37°C also under CO_2. Exclusion of Alexa-647 dextran from the cytosol reflects successful healing after wounding. Coverslips were washed three times with ice-cold PBS and then incubated at 0° for 30 min with antibodies specific for Lamp-1 or the enlargeosome marker dissolved in HMEM. The cells were then fixed, immunolabeled and imaged.

Flow Analysis of Resealing

Sub-confluent cultures of HeLa cells were trypsinized and re-suspended in Dulbecco's PBS with or without 1 mM $CaCl_2$. Vacuolin (10 μM in DMSO) or DMSO were added to paired samples of each group, followed by incubation for 2 hr at 37°C. Cells were wounded by 6 uptakes and expulsions through a 30 gauge needle in an automated device at 30 psi (Clarke and McNeil, 1994). After 5 min at 37°C, 1μM FM1-43 dye was added to the cells, and then placed on ice until analysis. Controls, for the evaluation of dye staining of the cell surface and endocytic compartments, received the FM1-43 dye but were not wounded. Staining with FM1-43, gated to eliminate dead cells and debris, was evaluated by flow cytofluorometry (FACS Calibur, Becton, Dickinson).

Uptake of transferrin, LDL and EGF

BSC-1 cells were incubated for 2 hrs in DMEM supplemented with 10% FBS and 0.2% DMSO with or without 1 μM Vacuolin-1 which was also present during the uptake of cargo. The cells were washed once with HMEM (MEM supplemented with 20 mM Hepes and 1% BSA) followed by a 30 min labeling period with fresh media containing 25μg/ml Alexa594-conjugated human transferrin (Molecular Probes). For the simultaneous uptake of transferrin and LDL, the labeling experiments were carried for 20 min using media containing 25μg/ml Alexa488-conjugated human transferrin and 10 μg/ml 1,1' dioctadecyl-3,3,3',3'-tetrametylindocarbocyanine perchlorate (Dil)-labeled LDL (Gu et al., 1998) (provided by M. Krieger). EGF uptake was monitored using A431 cells incubated with media containing 25μg/ml tetramethylrodamine-EGF for 30 min followed by a 1h chase period in HMEM. All incubations were done at 37°C in the presence of 5% CO_2 and cells imaged after fixation.

RESULTS

Identification of vacuolins using an image-based phenotypic screen and chemical structure of vacuolins

The screen was performed at 40°C with a collection of 10,240 compounds (part of the Diverset E, Chembridge library) tested at a nominal concentration in the range of 10-30 μM. We used BSC-1 monkey-derived fibroblast cells transiently expressing the temperature-sensitive mutant of the vesicular stomatitis virus glycoprotein, ts045, fused to green fluorescent protein (VSVG^{ts}-EGFP). Circular structures of varying size completely devoid of EGFP fluorescence were seen with some compounds administered to cells maintained at 40°C (Fig. 1A), the condition at which VSVG^{ts}-EGFP is retained in the endoplasmic reticulum (ER), or after shifting the cells from 40 to 32°C (Fig. 1B), the condition leading to synchronous export of VSVG^{ts}-EGFP first to the Golgi apparatus and then to the plasma membrane. Because these empty spaces are vacuole-like structures, we have called the compounds vacuolins (Fig. 2). Vacuolins-1 to -13 have related triazine-based structures, while Vacuolins-14, -15 and -16 are distinct from the triazine-based vacuolins and are unrelated to each other. Additional compounds with closely related structures, although present in the library, had no effect in the assay (Fig. 2). A limited structure-activity relationship analysis of the triazine-based compounds suggests that the triazine and morpholine moieties are required for activity. The tightness of the structure-activity relationship suggests that these compounds have a specific protein target. The vacuolins were not selected as high-priority hits in any of the other ~150 screens performed with the same compounds at the Institute of Chemistry and Cell Biology (ICCB), Harvard Medical School. Based on the highest potency of Vacuolin-1, we have selected it for characterizing the biological effects of this group of compounds. All control experiments were done in the presence of the carrier DMSO (0.5-1%).

General effects of Vacuolin-1

We asked whether the Vacuolins interfered with constitutive exocytic traffic using BSC-1 cells to follow the movement of VSVG^{ts}-EGFP from the ER to the plasma membrane. None of the compounds had any effects, even when tested at concentrations at least 10 times higher than the IC_{50} required to induce vacuolation (100 times for Vacuolin-1, as illustrated in Fig. 1D, main text; data not shown for the remaining vacuolins). The activity of Vacuolin-1 does not require the expression of VSVG^{ts}-EGFP since non-transduced BSC-1 cells also show the formation of vacuoles with the same appearance and distribution (Fig. 1F, main text). Vacuolin-1 did not change the appearance of the actin and the tubulin cytoskeleton (Fig. 3) and had no discernable effect on the overall uptake of transferrin and LDL mediated by clathrin coated vesicles (see below).

The appearance of vacuoles induced by Vacuolin-1 and the potency of the compound were similar when tested in different cell types isolated from humans (HeLa, A431 and *in vitro* differentiated macrophages from peripheral blood), mice (*in vitro* differentiated dendritic cells and peritoneal macrophages), hamsters (CHO) and monkeys (CVC-1, and COS). The activity of Vacuolin-1 in BSC-1 or HeLa cells is long lasting, as vacuoles remained when cells were kept for 7 days in the same medium containing 1 μM Vacuolin-1. Longer incubations with Vacuolin-1 (of up to 21 days) did not increase the number of vacuoles. The vacuolation effect was reversible, as no swollen structures were apparent 13 hrs after removal of Vacuolin-1 from the medium (Fig 4).

Vacuolin-1 induces the fusion of the inner and the limiting membranes in endosomes/lysosomes

In one approach we loaded the endosomal compartment including multi vesicular bodies by incubation of HeLa cells for a brief period with bovine serum albumin (BSA) coupled to colloidal gold (5 nm) followed by a 4 hr chase (Fig. 2A. main text). We then compared the appearance of endosomal/lysosomal structures in such cells to those in cells incubated with Vacuolin-1 for 20 min or 2 hrs prior to the end of the chase period. The images show the generation of electron lucent vacuoles often containing gold particles. In cells treated for 20 min with Vacuolin-1 we observed that some of the vacuoles contained remnants of the inner membranes (Fig. 2B, main text) whereas all vacuoles were completely clear in cells treated for 2 hrs (Fig. 2C, main text). In the second approach, we pulsed HeLa cells with BSA coupled to colloidal gold (5 nm). After a 180 min chase period, the cells were processed for cryo-immuno staining with an antibody specific for CD63, which was identified using a secondary antibody labeled with colloidal gold particles (10 nm). As expected, in the untreated cells, only the inner membranes of multi vesicular bodies contain CD63 (Fig. 2D, E, main text). In contrast, the BSA-gold containing vacuoles in cells treated with Vacuolin-1 during the last hr of the chase period completely lack inner membranes, and their surrounding membranes contain CD63 (Fig. 2F, main text).

In the presence of Vacuolin-1, vacuoles can participate in endocytic traffic

Fluorescence microscopy was used to evaluate the effects of Vacuolin-1 on the uptake of Alexa594-transferrin and DiI-LDL in HeLa cells and of tetramethylrodamine-EGF in A431 cells. In control cells Alexa594-transferrin internalized for 20 min and reached the early endosomal compartment, as identified by their labeling with EEA1 (Fig. 5A). In cells pre-treated with Vacuolin-1, the internalized transferrin reached enlarged vacuoles also labeled with EEA1 (Fig. 5B). As only vacuoles labeled with EEA1 were competent to receive transferrin, it seems that sorting of newly internalized transferrin is preserved in the presence of Vacuolin-1. Likewise, not only does Vacuolin-1 not prevent the uptake of DiI-LDL (Fig. 5 D), it also does not affect the sorting of these two cargos into vacuoles derived from different endosomal compartments. The preservation of endocytic traffic was further demonstrated by the separation of the signals of internalized transferrin and DiI-LDL observed upon the simultaneous incubation of HeLa cells with Alexa594-transferrin and DiI-LDL for 20 min, a time that in the absence of Vacuolin-1 is sufficient to allow for the sorting of these cargos into different endosomal compartments (compare Fig. 5 C and 5 D). These data suggest that the intrinsic molecular and functional complexity of the endosomal compartment has not been disrupted by vacuolization.

The internalization and delivery of EGF to the vacuoles is not affected by Vacuolin-1. We incubated A431 cells expressing the EGF receptor with tetramethylrodamine-EGF for 20 min, a condition that promotes activation of the receptor followed by internalization and accumulation in endosomes (Fig. 5E), and found accumulation of EGF in a subset of vacuoles in cells pre-treated with 1 μM Vacuolin-1 (Fig. 5F). From these results we conclude that the swollen structures generated by Vacuolin-1 are not only competent to receive different cargo internalized by receptor-mediated endocytosis, but that they also maintain their ability to sort internalized receptor-ligand complexes.

Vacuolin-1 impairs lysosomal exocytosis in cells that reseal

Attached monolayers of HeLa cells were injured by scratching, and the extent of injury and repair was monitored qualitatively by the incorporation of Alexa594-dextran (red) added before the injury (room temperature) and the exclusion of Alexa647-dextran (black) added 5 min later (37°C). In agreement with the inhibition of lysosome exocytosis in response to ionomycin, pretreatment with Vacuolin-1 abolished the surface appearance of Lamp-1 in cells induced by scratching (Fig. 6 B). In striking contrast, the process of wound healing apparently was not affected in Vacuolin-1-treated cells, as all injured cells recovered and excluded Alexa647-dextran (Fig. 6 B, D). Under the conditions used for scratching we found that appearance of the fluorescence signal corresponding to the enlargeosome marker, AHNAK, at the cell surface was too weak for reliable determination.

Vacuolin-1 does not block resealing

FM 1-43 is a normally membrane impermeant dye that rapidly partitions between water and lipid, and is ~ 50-fold more fluorescent in this latter environment. Thus, if a cell fails to reseal, FM 1-43 present in the medium will enter through the disruption and rapidly stain its cytoplasmic membrane, greatly increasing its total fluorescence as determined by flow fluorocytometry (Fig. 7). As a positive control used to validate the capacity of the method to detect failure to reseal, we wounded cells in the absence of extracellular Ca^{2+}. Ca^{2+} is well documented to be required for resealing by acting as the trigger for requisite exocytotic and homotypic fusion events (reviewed in McNeil and Steinhardt, 2003); in its absence, cells fail to reseal.

Comparison between the effects of Vacuolin-1 and other vacuolating agents

The identity of the molecular target(s) of Vacuolin-1 and the detailed mode of action of the compound remain to be determined but its observed organellar specificity is clear. We can therefore draw useful parallels and differences by comparing its effects with those of other agents or conditions known to elicit swelling of the endosomal and/or lysosomal compartments. The fungal toxin Wortmannin, an inhibitor of phosphoinositide-3-kinase, induces marked vacuolation, particularly of early endosomes, due to acute depletion of phosphorylated phosphoinositides and concomitant loss of EEA1 from the endosomal membranes (Patki et al., 1997; Simonsen et al., 1998). The vacuoles generated in the presence of Wortmannin, although large, appear rugged rather than round and smooth as the vacuoles generated with Vacuolin-1 (Spiro et al., 1996). Unlike Vacuolin-1, Wortmannin does not block Ca^{2+} mediated exocytosis of lysosomes (Cerny and Kirchhausen, unpublished). Another way to enlarge the endosomal compartment is to express VacA protein from *Helycobacter pylori.* In this case, the enlargement is mostly of the late endosomal compartment (Morbiato et al., 2001). It requires the presence of weak bases in the media, suggesting that VacA acts as a chloride channel (Morbiato et al., 2001). Probably Vacuolin-1 does not act as a chloride channel, since unlike the effects of VacA, the swelling effects of Vacuolin-1 are manifested regardless of the media composition. It is unlikely that class E VPS products such as Hrs, STAM, ESCRT, ALIX/AIP1 are the target of Vacuolin-1 as the functions of these proteins are organelle-specific whereas the effects of Vacuolin-1 are multi-compartmental.

FIGURE LEGENDS

Figure 1. **Identification of vacuolins.** VSVG^{ts}-EGFP was expressed in BSC-1 fibroblast cells by transduction and used to probe the effect of 10,240 compounds along different membrane traffic steps from the ER to the plasma membrane (Feng et al., 2003). After expression of VSVG^{ts}-EGFP for 18 hrs at 40°C, each well of a 384-well plate received a different compound by pin-transfer. Following an additional 2 hr incubation with the compounds at either 40° C **(A, B)** or 32°C (**C, B**) the cells were fixed and imaged (Yarrow et al., 2003). Images shown correspond to actual views obtained during the primary screen for chemical inhibitors. **A, C** are control cells (1% DMSO used as carrier); **B, D** are cells treated with 10 μM Vacuolin-1. At 40°, the non-permissive temperature for membrane traffic, control cells (**a**) show retention of VSVG^{ts}-EGFP in the ER. At 32°, the permissive temperature, they show synchronous traffic to the Golgi apparatus and the cell surface (**C**). In the presence of Vacuolin-1 (**B,D**) cells show extensive vacuolation which however has no effect on the retention of VSVG^{ts}-EGFP in the ER (**B**) nor on its transport to the cell surface (**D**). Visualization by phase contrast of BSC-1 cells not transfected with VSVG^{ts}-EGFP in the absence (**e**) or presence of 10 μM Vacuolin-1 (**F**) shows that the vacuolated phenotype is induced by vacuolin independently on the expression of the viral protein.

Figure 2. **Structure of compounds eliciting a vacuolated phenotype.** The compounds are listed according to their potency to elicit the vacuolated phenotype in BSC-1 cells upon 1 hr incubation. The data was gathered by visual inspection of fluorescence images obtained from cells expressing VSVG^{ts}-EGFP and represent a limited degree of structure activity relationship for Vacuolin-1 to 13.

Figure 3. **Vacuolin-1 does not affect the actin and tubulin-based cytoskeleton.** Bsc-1 cells were treated with 0.2 mM Vacuolin-1 for 2 hr or with 1 mM Vacuolin -1 for 1 hr followed by fixation and staining for actin or tubulin, using phalloidin - TRITC or antibody labeling, respectively.

Figure 4. **The effects of Vacuolin-1 are reversible.** BSC-1 cells were imaged using phase contrast microscopy, upon treatment with 1 mM Vacuolin-1 for 1h (top panel), with Vacuolin-1 for 1 h followed by a 12 h incubation (middle panel) or without Vacuolin –1 (bottom panel).

Figure 5. **Vacuoles generated by Vacuolin-1 accept ligands internalized by receptor-mediated endocytosis.** (**A, B**) Hela cells were incubated with Alexa594-transferrin (red) for 20 min in the absence or presence of 1 μM Vacuolin-1 added to the cells for 120 min before initiation of the endocytic assay. The fluorescent images show that Vacuolin-1 does not prevent receptor-mediated internalization of transferrin and permits its accumulation in vacuoles containing EEA1 (green). (**C, D**) Hela cells were incubated with Alexa488-transferrin (green) and DiI-LDL (red) for 20 min in the absence or presence of 1 μM Vacuolin-1 added to the cells for 120 min prior to the uptake assay. The control cells (**C**) show the expected accumulation of both ligands in distinct endocytic compartments; in the presence of Vacuolin-1, transferrin and Di-LDL also accumulate in distinct vacuoles. (**E,**

F) A431 cells were incubated with tetramethylrodamine-EGF for 20 min to allow delivery of EGF to the lysosomal/endosomal compartment in the absence of Vacuolin-1 (**E**) and to a subset of vacuoles in cells pretreated for 2 hr with 1 μM Vacuolin-1 (**F**). Nuclear staining with DAPI (blue) and phase contrast data (gray) is included. Bar, 10 μM.

Figure 6. **Vacuolin-1 does not prevent healing following wounding by scratching.** Mechanical wounding of cells was performed by gentle scratching a monolayer of BSC-1 cells in the absence (**A**) or presence (**B**) of 1 μM Vacuolin-1. Plasma membrane breakage was documented by the uptake of Alexa594-Dextran (red) present in the media during the wounding step. Cell healing was monitored by the cellular exclusion of Alexa 647-dextran (black) present in the media after 5 min of recovery at 37°C. Upon wounding and in the absence of Vacuolin-1, the marker for lysosomes (Lamp-1, green) appears at the cell surface. In contrast, pretreatment with Vacuolin-1 prevents lysosome fusion with the plasma. Bar, 100 μM.

Figure 7. **Vacuolin does not inhibit shear-induced resealing.** Flow cytometric analysis (20,000 events each record) of FM 1-43 fluorescence intensity of HeLa cell populations exposed to FM 1-43 dye 5 min after the imposition of wound-inducing shear stress. Cells with open disruptions become heavily stained with the dye. (**A**) Cells were treated (1 hr) with DMSO only prior to wounding in resealing permissive medium containing 1. 5 mM Ca^{2+}. The percentage of the population heavily labeled with FM 1-43 ('M2' gate) was 5.47%. (**B**) Cells were treated with Vacuolin-1 (10 μM) 1hr prior to wounding in medium containing Ca^{2+}. The percentage of the population heavily labeled with FM 1-43 was 6.72% %, nearly identical to that of the control cells wounded in Ca^{2+}, indicating the lack of a resealing defect. (**C**) Cells were treated with DMSO only prior to wounding in medium containing no added Ca^{2+}, a condition that inhibits resealing. The percentage of the population heavily labeled with FM 1-43 was 39.7%, indicating that, at a minimum, the shear treatment wounded this proportion of cells. (**D**) Cells were treated with Vacuolin-1 prior to wounding in medium containing no added Ca^{2+}. The percentage of the population heavily labeled with FM 1-43 was 58.0 %, indicating that the Vacuolin-1 treatment did not reduce the level of shear induced wounding.

Figure 8 (Movie). **Live-cell imaging of vacuoles induced by the presence of Vacuolin-1**. BSC1 cells transiently expressing rab7-EGFP were imaged at 37°C using a spinning disk confocal microscope. The cells were pre-incubated with 1 mM Vacuolin-1 for 1 hr and then incubated for 20 min with DiI-LDL before the acquisition of the time-lapse series for ~22 min). Rab7-EGFP labels the outline of the membranes, and some but not all of the vacuoles contain internalized DiI-LDL (arrow head). An example of fusion between two vacuoles is shown (arrow).

REFERENCES

Borgonovo, B., Cocucci, E., Racchetti, G., Podini, P., Bachi, A. and Meldolesi, J. (2002) Regulated exocytosis: a novel, widely expressed system. *Nat Cell Biol*, **4**, 955-962.

Clarke, M.S. and McNeil, P.L. (1994) Syringe loading: a method for inserting macromolecules into cells in suspension. *Cell Biology: A Laboratory Handbook*, Academic Press, San Diego, CA, 30-36.

Feng, Y., Yu, S., Lasell, T.K., Jadhav, A.P., Macia, E., Chardin, P., Melancon, P., Roth, M., Mitchison, T. and Kirchhausen, T. (2003) Exo1: A new chemical inhibitor of the exocytic pathway. *Proc Natl Acad Sci U S A*, **100**, 6469-6474.

Gu, X., Trigatti, B., Xu, S., Acton, S., Babitt, J. and Krieger, M. (1998) The efficient cellular uptake of high density lipoprotein lipids via scavenger receptor class B type I requires not only receptor-mediated surface binding but also receptor-specific lipid transfer mediated by its extracellular domain. *J Biol Chem*, **273**, 26338-26348.

Morbiato, L., Tombola, F., Campello, S., Del Giudice, G., Rappuoli, R., Zoratti, M. and Papini, E. (2001) Vacuolation induced by VacA toxin of Helicobacter pylori requires the intracellular accumulation of membrane permeant bases, Cl(-) and water. *FEBS Lett*, **508**, 479-483.

Patki, V., Virbasius, J., Lane, W.S., Toh, B.H., Shpetner, H.S. and Corvera, S. (1997) Identification of an early endosomal protein regulated by phosphatidylinositol 3-kinase. *Proc Natl Acad Sci U S A*, **94**, 7326-7330.

Simonsen, A., Lippe, R., Christoforidis, S., Gaullier, J.M., Brech, A., Callaghan, J., Toh, B.H., Murphy, C., Zerial, M. and Stenmark, H. (1998) EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. *Nature*, **394**, 494-498.

Spiro, D.J., Boll, W., Kirchhausen, T. and Wessling-Resnick, M. (1996) Wortmannin alters the transferrin receptor endocytic pathway in vivo and in vitro. *Mol Biol Cell*, **7**, 355-367.

Swanson, J.A. and McNeil, P.L. (1987) Nuclear reassembly excludes large macromolecules. *Science*, **238**, 548-550.

Yarrow, J.C., Feng, Y., Perlman, Z.E., Kirchhausen, T. and Mitchison, T.J. (2003) Phenotypic screening of small molecule libraries by high throughput cell imaging. *Comb Chem High Throughput Screen*, **6**, 279-286.