Communicative & Integrative Biology 8:5, e1078041; September/October 2015; Published with license by Taylor & Francis Group, LLC

Taking a lipidation-dependent path toward endolysosomes

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Keywords: fluorescent markers/proteins, isoprenylation, lysosomes/endolysosomes, multivesicular bodies, organelle probe, palmitoylation, Rho proteins, subcellular localization

Abbreviations: BAEC, bovine aortic endothelial cells; ILV, intraluminal vesicles; MVB, multivesicular bodies; LTR, Lysotracker Red.

We recently reported that the isoprenylation and palmitoylation motif present at the C-terminus of human RhoB protein promotes intraluminal vesicle delivery of proteins in cells from organisms as phylogenetically apart as fungi and humans. Here we build on these observations by showing that chimeras of fluorescent proteins bearing this sequence, namely, CINCCKVL, which become isoprenylated and palmitoylated in cells, may be used to mark endolysosomes while preserving their morphology. Indeed, these chimeric proteins are devoid of the effects derived from overexpression of fluorescent constructs of full-length, active proteins widely used as endolysosomal markers, such as Lamp1 or Rab7, which cause lysosomal enlargement, or RhoB, which induces actin stress fibers. Moreover, the fact that lipidation-dependent endolysosomal localization of CINCCKVL chimeras can be ascertained in a wide variety of cells indicates that they follow a path toward endolysosomes that is conserved in diverse species. Therefore, CINCCKVL chimeras serve as robust tools to mark these late endocytic compartments

Newly synthesized proteins must reach specific destinations within the cell to carry out their biological functions. Correct cellular targeting of proteins is achieved by the concurrence of trafficking machineries as well as sorting motifs within the protein sequence itself. These sorting motifs can aid in the interaction with adaptor proteins or can *per se* target precise areas of the cell. In certain proteins, residues present in these motifs undergo posttranslational modifications that serve as anchors for specific membrane domains. Such is the case for most Ras superfamily GTPases, which undergo lipidation, specifically isoprenylation, at their C-terminal regions.[1] The particular moieties attached at these regions along with the surrounding sequence endow these proteins with a defined subcellular localization, such as the plasma membrane, e.g. K-Ras4B, or both the plasma membrane and Golgi for H-Ras (reviewed in[2]). Other isoprenylated proteins are further targeted to endocytic vesicles of varying types, as seen for different members of the Rab protein family. In the case of Rab7, its isoprenylation along with other interacting motifs give rise to a robust localization at late endosomes and lysosomes that has set this protein forth as a marker of these compartments.[3] Similarly, we recently showed that a Rho family protein, namely RhoB, is also targeted to endolysosomes, particularly to intraluminal vesicles (ILV) of multivesicular bodies (MVB).[4,5] This specific targeting to late endocytic compartments is dependent on full lipidation, i.e. isoprenylation and double palmitoylation, at cysteines of its C-terminus.

Taking advantage of sorting motifs derived from different proteins, markers for specific subcellular destinations have been developed by fusion of either full-length or partial sequences to fluorescent proteins. However, overexpression of these constructs is often accompanied by unwanted effects, including the enlarged lysosomes that accumulate at perinuclear regions upon transfection with Rab7 fluorescent constructs,[6] or the dilation and fusion of early endosomes observed upon overexpression of GFP-Rab5.[7] Fluorescent constructs of other endolysosomal constituents such as Lamp1, which have been extensively validated as endolysosomal markers,[9,11] can also display various biological effects when overexpressed in cells,[8] and give rise to enlargement, aggregation and/or rounding of these compartments[9] (see below).

Here we describe that chimeras of the last 8 amino acids of RhoB, which comprise the lipidation motif "-CINCCKVL," fused to different fluorescent proteins, can be used as robust markers of endolysosomes. Furthermore, these constructs do not induce

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Submitted: 06/24/2015; Revised: 07/21/2015; Accepted: 07/22/2015
http://dx.doi.org/10.1080/19420889.2015.1078041

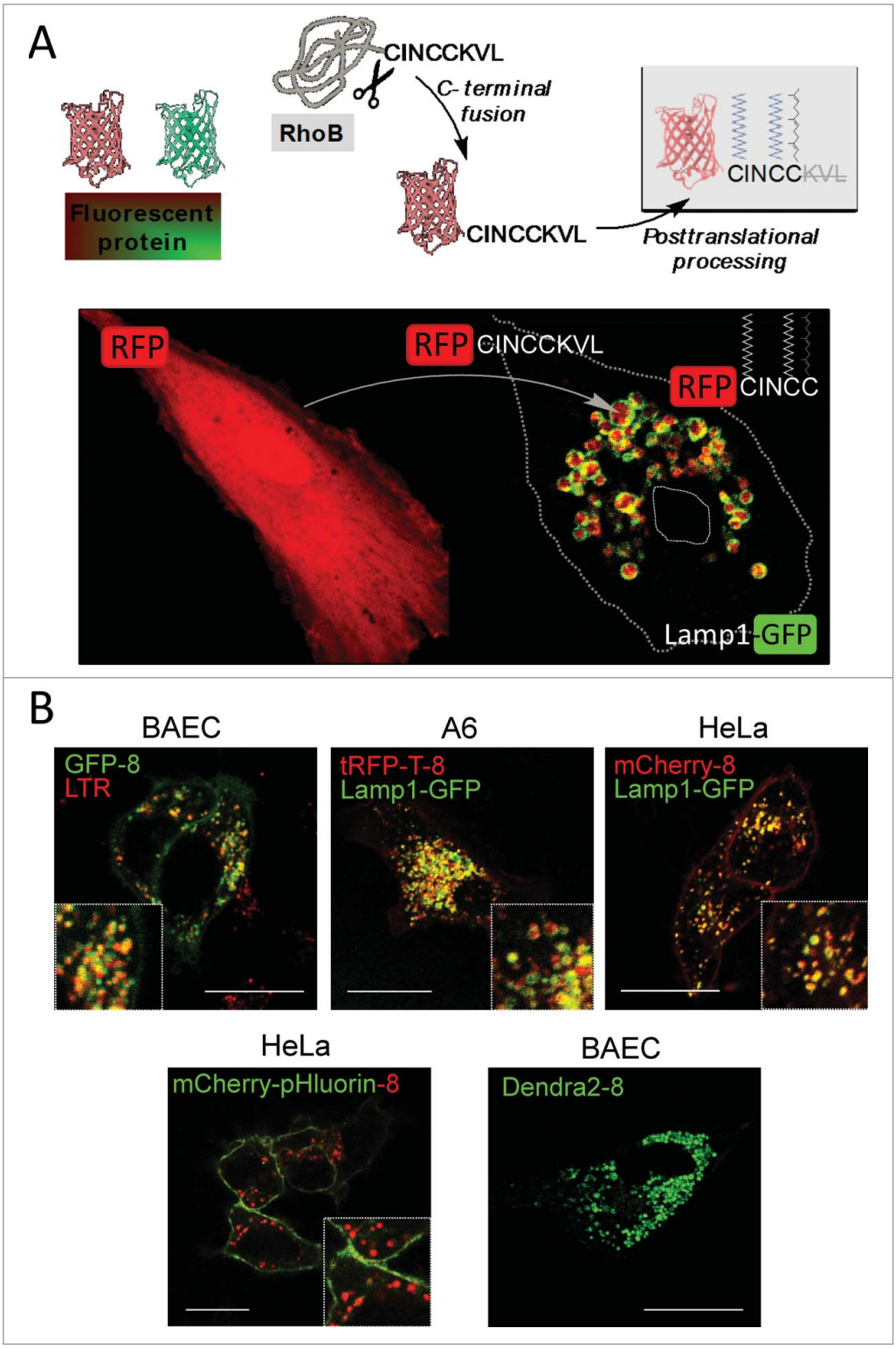

Figure 1. Generation of CINCCKVL chimeras. (**A**) Fluorescent proteins such as GFP or tRFP/mCherry, which are diffuse throughout the cell, were used to fuse the last 8 amino acids of RhoB, i.e., "CINCCKVL," to their C-terminus. These constructs were transfected into several cell types, where the lipidation machinery processes the sequence to give rise to the isoprenylated, doubly palmitoylated construct that localizes at endolysosomes, positive for GFP-Lamp1. (**B**) Examples of several fluorescent constructs transfected in several primary cells or cell lines in combination with Lamp1-GFP or after staining with LTR. Cells were observed live and single confocal sections are shown.

detectable artifacts such as alteration of late endosomal morphology or actin dynamics, as seen otherwise for overexpression of full-length chimeras.

Generation and expression of CINCCKVL chimeras

In a series of recent articles we have described that RhoB is targeted to endolysosomes in a variety of cell types and that, remarkably, just the last 8 amino acids of this protein are sufficient to confer this targeting.[4,5,10] Thus, this "-CINCCKVL" or "-8" sequence can be fused to the C-terminus of several fluorescent proteins to induce endolysosomal localization (**Fig. 1A**). As in full-length RhoB, this specific targeting requires posttranslational processing of the –CINCCKVL sequence by isoprenylation of the CAAX box cysteine and double palmitoylation at the cysteines directly upstream from it (**Fig. 1A**). Therefore, these posttranslational modifications elicit a vesicular localization for fluorescent chimeras, whose parent fluorescent proteins are fully cytosolic (**Fig. 1B**). The constructs colocalize with markers of late endocytic compartments such as LysoID,[11] LysoTracker Red (LTR), or Lamp1 constructs (**Fig. 1B**), though not with markers of early endosomes or autophagosomes, such as Rab5 or LC3 constructs, respectively.[5,11]

Special interest lies in the photoconvertible constructs Dendra2-8, which could facilitate studies on endosomal dynamics, as well as mCherry-SEpHluorin-8 (**Fig. 1B**). In the latter case, the pH sensitivity of the GFP component of the tandem construct results in loss of

green fluorescence in acidic environments; therefore, endolysosomal localization of this construct is demonstrated by its switch from green and red fluorescence in neutral environments to only red fluorescence upon entrance in acidic vesicles. We have observed that some of the fluorescent chimeras display a variable degree of membrane localization, which may depend on the nature of the parent protein. In general, the extent of membrane localization is higher for GFP-8, mCherry-8 and mCherry-SEpHluorin-8, with t-RFP-T-8 and Dendra2-8 showing almost exclusive endolysosomal localization.

Notably, the endolysosomal localization of these constructs not only takes place in normal cells but also in cancer cell lines and in primary cells with alterations in lysosomal biogenesis or distribution (**Fig. 2**). In particular, adrenocarcinoma SW13/cl.2 cells, which lack cytoplasmic intermediate filaments, display an abnormal distribution of lysosomes, which accumulate at one side of the nucleus.[12,13] In these cells, GFP-8 and t-RFP-T-8 chimeras also get trapped in a juxtanuclear localization, colocalizing with Lamp1 and LTR (**Fig. 2A**). In addition, several genetic human diseases display profound alterations in lysosomal or lysosomal-related organelle morphology and function. In the Chediak-Higashi Syndrome, caused by mutations of the lysosomal trafficking regulator (Lyst) gene,[14] late endosomal compartments are anomalously dilated and accumulate ceroid-like material. Remarkably, GFP-8 marks the edges of these dilated endolysosomes, into which, even LTR is poorly internalized (**Fig. 2B**). In cells from patients with Hermansky-Pudlak syndrome, which includes several diseases affecting lysosomal related organelles,[15] LTR staining shows accumulation of lysosomes, alterations which are also highlighted by the GFP-8 probe (**Fig. 2B**).

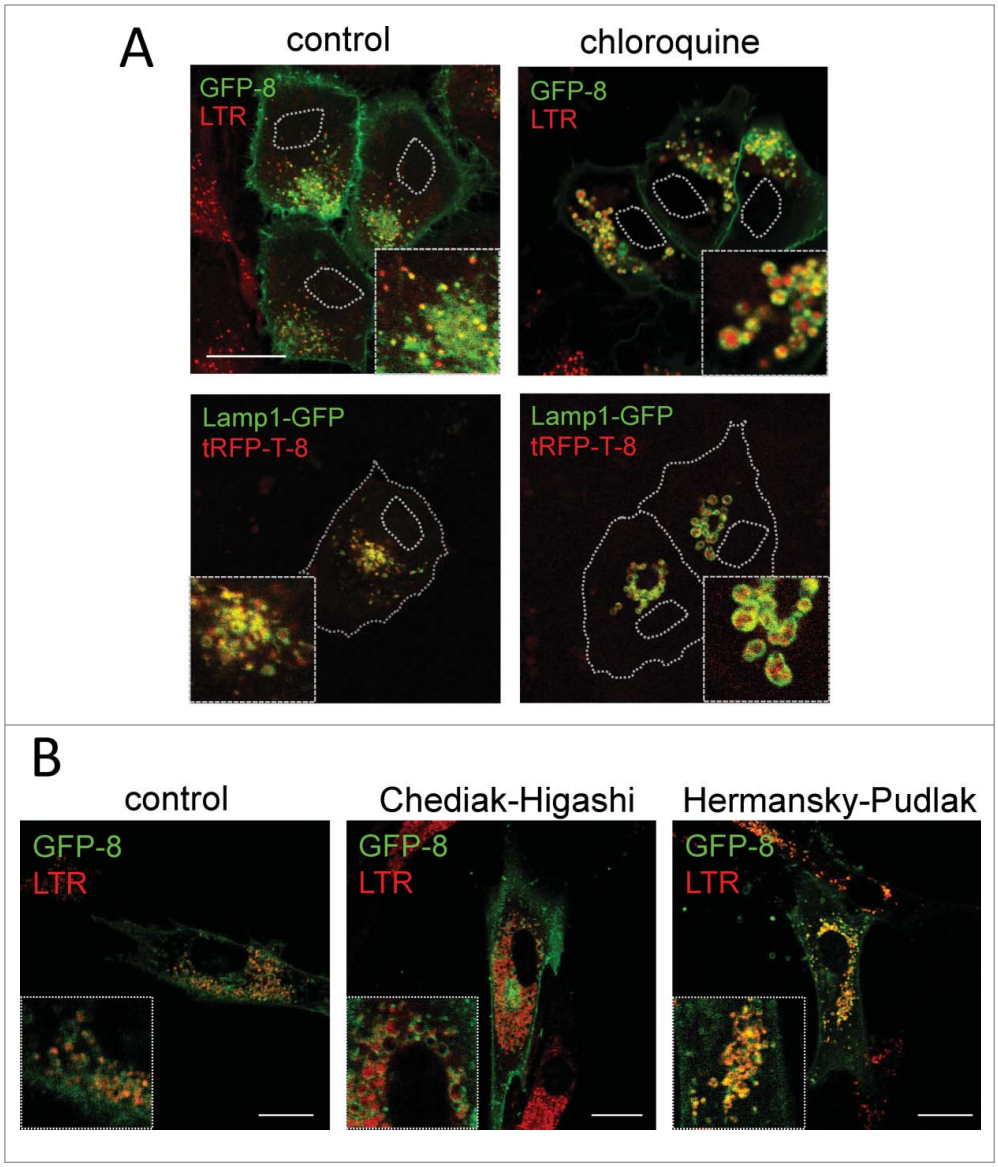

Figure 2. Localization of CINCCKVL constructs in cells bearing lysosomal alterations. (**A**) SW13/cl.2 cells were transfected with CINCCKVL constructs ("GFP-8") and stained with LTR prior to live visualization. Lower panels show cells after chloroquine treatment which induces lysosomal enlargement. (**B**) Human primary fibroblasts from patients with the indicated diseases were transfected with GFP-8 and stained with LTR.

CINCCKVL chimeras do not alter endolysosomal morphology or actin distribution

These results indicate that the –CINCCKVL targeting mechanism is preserved in several pathophysiological situations affecting lysosomes. Moreover, we have shown that this targeting pathway also operates in cells from phylogenetically distant species, including fungi, insects and humans.[5] This suggests that the RhoB sorting motif may be taking a highly conserved route toward endolysosomes, even though endogenous proteins taking this route have not yet been found in all of these organisms, or might dock at a conserved membrane microdomain of specific composition.

Given the fact that CINCCKVL chimeras only share the last 8 amino acids, and that this short sequence is unlikely to be endowed with biological activity, use of these constructs is not expected to significantly alter cell behavior. Indeed, we observed that, unlike Lamp1-GFP or GFP-Rab7, GFP-8 did not increase the size of

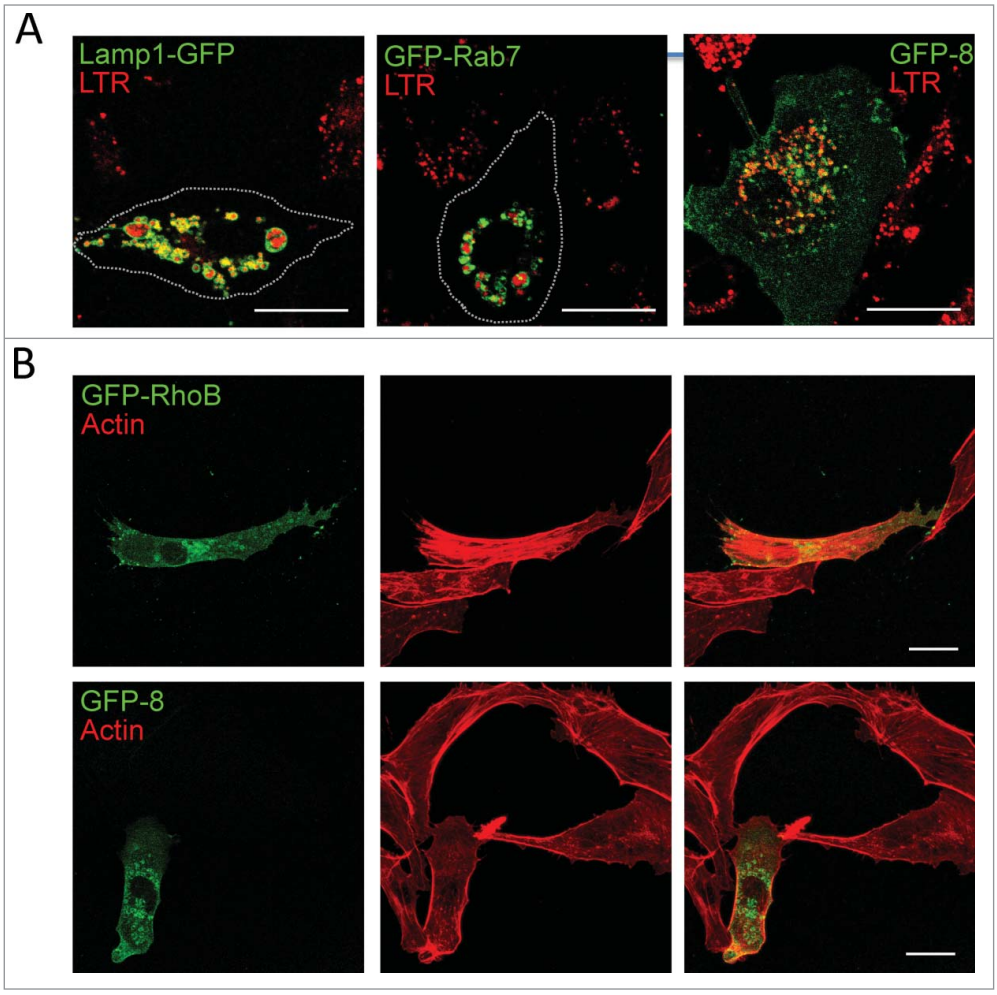

Figure 3. Absence of functional effects of GFP-8 compared to common endolysosomal probes. (**A**) BAEC were transfected with Lamp1-GFP, GFP-Rab7 or GFP-8 and stained with LTR. (**B**) BAEC were transfected with either GFP-RhoB (full-length protein) or GFP-8, fixed and stained with phalloidin-Alexa568 to detect the actin cytoskeleton.

LTR-positive compartments (**Fig. 3A**). GFP-RhoB itself has also been postulated as a marker for endosomal compartments. However, much like other Rho proteins, RhoB has been reported to induce stress fibers in various cell types.[16,17] In fact, expression of GFP-RhoB in bovine aortic endothelial cells (BAEC) resulted in a marked increase in stress fibers, as evidenced by staining of filamentous actin with Phalloidin-Alexa568 (**Fig. 3B**). In contrast, transfection of GFP-8 did not induce this effect. In addition, RhoB has been shown to be pro-apoptotic or anti-apoptotic depending on the experimental system,[18-20] and actively control endosomal traffic.[21,22] Therefore, taking into account these varied effects, use of a minimal targeting construct such as GFP-8 presents clear advantages.

From a practical perspective it should be taken into account that, given their requirement for lipidation, levels of CINCCKVL constructs should be kept low enough as to allow their full processing. This can be confirmed by assessing the fraction of construct associated with membranes, which should be close to 100%,[5] or by optical microscopy, in which the presence of cytosolic or nuclear GFP-8 may reflect incomplete isoprenylation and/or palmitoylation. In addition, this illustrates a remarkable feature of CINCCKVL constructs, namely, their potential as reporters of these lipidation pathways.[5]

In summary, we show here that the lipidation sequence derived from RhoB can be used as a robust endolysosomal marker without altering late endosomal morphology. CINCCKVL constructs appear at this specific subcellular localization in cells from a wide spectrum of species, pathophysiological conditions or experimental settings. The lipidation-dependent sorting of these chimeras highlights a direct pathway toward endolysosomes that can be traced in living cells to further characterize molecular players and late endosomal dynamics at these subcellular sites.

Materials and Methods

Cells and reagents

Primary human dermal fibroblasts from control subjects (AG10803) or patients from Chediak-Higashi (GM02075) or Hermanski-Pudlak Syndromes (GM17890) were obtained from the National Institute of General Medical Sciences (NIGMS) Human Genetic Cell Repository at the Coriell Institute for Medical Research (Candem, NJ), and were manipulated according to the instructions of the supplier. The following plasmids were generous gifts: Lamp1-GFP from Prof. J. Lippincott-Schwartz (National Institutes of Health), GFP-Rab7 from Prof. C. Bucci (University of Copenhagen). Fluorescent proteins were from Clontech (mCherry, Dendra2, GFP, RFP), Evrogen (tRFP) and Addgene (mCherry-SEpHluorin).

Experimental procedures

Generation of –CINCCKVL constructs was achieved by PCR essentially as described,[4,5] or by gene synthesis performed by Genewiz. Cell transfection and confocal microscopy of live cells have been previously described in detail.[4,5] Images shown are overlays of single sections. In some images cell contour and nuclei position are outlined by dotted lines. Bars, 20 μm.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank M. T. Seisdedos and G. Elvira for expert assistance with confocal microscopy and M.J. Carrasco for technical work.

Funding

This work has been supported by grants SAF2012-36519 from MINECO and RETIC RIRAAF (RD12/0013/0008) from ISCIII, Spain. C.L.O. has been the recipient of fellowship BES-2010–033718 from FPI, MINECO.

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