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AN UNCONVENTIONAL DILEUCINE AND A NOVEL CYTOSOLIC MOTIF ARE REQUIRED FOR THE LYSOSOMAL/MELANOSOMAL TARGETING OF OA1

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SUMMARY

The protein product of the gene responsible for ocular albinism type 1, named OA1, is a pigment cell-specific membrane glycoprotein, displaying features of G protein-coupled receptors, yet exclusively localized to late endosomes/lysosomes and melanosomes. To dissect the signals responsible for the intracellular localization of OA1 we generated LAMP1/OA1 chimeras and OA1 mutants at the cytosolic domains of the protein. By this approach we identified two separate sorting signals that are both necessary and sufficient for intracellular retention and lysosomal/melanosomal localization in melanocytic and non-melanocytic cells: an unconventional dileucine motif within the third cytosolic loop and a novel motif, characterized by a tryptophan-glutamic acid doublet, within the C-terminal tail. Both motifs must be mutated to promote the plasma membrane localization of OA1, suggesting that they can independently drive its intracellular targeting. In addition, both motifs act similarly as lysosomal sorting signals in non-melanocytic cells, but appear to carry different specificities in melanocytic cells. Our findings indicate that OA1 contains multiple unconventional signals responsible for its lysosomal/melanosomal localization, and reveal a remarkable and unforeseen complexity in the regulation of polytopic protein sorting to specialized secretory organelles.

Keywords

Ocular albinism; Melanosome; Lysosome; GPCR; Sorting

INTRODUCTION

Ocular albinism type 1 (OA1), the most common form of ocular albinism, is an X-linked inherited disorder, characterized by foveal hypoplasia with severe reduction of visual acuity, nystagmus, strabismus, photophobia, iris translucency, hypopigmentation of the retina, and misrouting of the optic tracts, resulting in loss of stereoscopic vision (King et al., 1995). Patients affected with the disorder also show the presence of melanosomal abnormalities, including giant melanosomes (macromelanosomes), in the pigment cells of the skin and eyes (melanocytes and retinal pigment epithelium, RPE), suggesting an underlying defect in

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melanosome biogenesis (O'Donnell et al., 1976; Garner and Jay, 1980; Wong et al., 1983). The OA1 protein is an integral membrane glycoprotein, specifically expressed in melanocytes and RPE, and displaying features of G protein-coupled receptors (GPCRs) (Schiaffino et al., 1996; Schiaffino et al., 1999). In fact, OA1 shows seven transmembrane domains and homologies with several members of the GPCR superfamily, including residues highly conserved in most GPCRs, some of which are the site of albinism-causing mutations (Schiaffino et al., 1999; d'Addio et al., 2000). In addition, OA1 binds heterotrimeric Gi, Go and Gq proteins by co-immunoprecipitation and in vitro binding assays (Schiaffino et al., 1999).

However, at variance with canonical GPCRs, OA1 is not localized to the cell surface, but at steady-state is exclusively detectable on the membrane of intracellular organelles. In fact, previous morphological and biochemical studies performed by us and others established that the endogenous OA1 in melanocytes is excluded from the plasma membrane and is mainly localized to late-endosome/lysosomes and melanosomes (Schiaffino et al., 1996; Schiaffino et al., 1999; Samaraweera et al., 2001; Basrur et al., 2003). The latter represent the specialized subcellular organelles of pigment cells devoted to the synthesis, storage and transport of melanins. Genetic, biochemical and functional evidences suggest that they are related to the lysosomes (Orlow, 1995; Dell'Angelica et al., 2000). Nevertheless, melanosomes also differ from lysosomes, since they are unique to pigment cells, are characterized by specific resident proteins and display a distinctive structure, being classified into four maturation stages based upon melanin content and ultrastructural morphology (stage I to III correspond to immature melanosomes, while stage IV to mature organelles) (Seiji et al., 1963; Marks and Seabra, 2001). In addition, in pigment cells melanosomes co-exist with lysosomes and are inaccessible to fluid phase markers even at long chase times, thus appearing distinct from endocytic organelles (Raposo et al., 2001).

Little is known about the sorting mechanisms that retain OA1 at intracellular locations and drive it to its bipartite lysosomal/melanosomal compartment. Glycosylation does not seem to be involved (Shen and Orlow, 2001) and missense mutations identified in patients with ocular albinism can lead to protein misfolding and endoplasmic reticulum (ER) retention, but apparently do not provoke other kind of sorting defects (d'Addio et al., 2000; Shen et al., 2001). In addition, as other melanosomal proteins including tyrosinase and TRP1 (Winder et al., 1993), when heterologously expressed in non-melanocytic cells OA1 is efficiently delivered to the lysosomes, indicating that it contains trafficking information recognized by the lysosomal sorting machinery even in the absence of melanosomes (Schiaffino et al., 1999; Shen et al., 2001). However, OA1 does not contain any canonical tyrosine or dileucine-based motifs, which often direct the traffic of lysosomal and melanosomal membrane proteins (Setaluri, 2000; Bonifacino and Traub, 2003). To shed light on the targeting determinants of OA1, we generated a series of chimeric and mutant OA1 proteins and analyzed their subcellular distribution when expressed in melanocytic and nonmelanocytic cells.

MATERIALS AND METHODS

cDNA Constructs

Full-length rat LAMP1 and C-terminus deleted rat LAMP1 were obtained by RT-PCR, using primers LAMP/F2-LAMP/R2 and LAMP/F2-LAMP/R5, respectively, to amplify a rat insulinoma line (RIN5F) cDNA prepared with the Superscript Preamplification System kit (Gibco-BRL). The amplified LAMP1 cDNA was then digested with HindIII and BamHI and cloned into the same sites of the mammalian expression vector pCR3 (Invitrogen). LAMP/OA1 chimeras were generated by digesting the C-terminus deleted rat LAMP1 construct with BamHI and EcoRI and by cloning into these sites the cytosolic regions of OA1, previously amplified using specific primers carrying BamHI and EcoRI flanking sites. For primers and

PCR conditions used to generate LAMP1/OA1 chimeras see Supplementary Fig. 1A. OA1-mycHis (full-length wild-type) and OA1CT2-mycHis (deleted of the C-terminal amino acids 340-404) constructs were generated by amplifying the human OA1 cDNA in the presence of 5% DMSO with primers OA1/FXba-OA1/RHind and OA1/FXba-OA1CT2/Hind, respectively. Intramolecular deletions or mutations of OA1 (OA1/ΔSY, deleted of amino acids 222-231; OA1/LL→AA, mutated at amino acids 223-224; and OA1/WE→AA, mutated at amino acids 329-330) were generated by using a two-step PCR method (Deminie and Emerman, 1993) using specific primers. Briefly, 1st step amplifications were performed using two pair of primers, A and B, in two separate reactions. The products of 1st step amplifications A and B were subsequently purified and utilized together as template in 2nd step amplifications, which were performed with primers OA1/FXba-OA1/RHind and OA1/FXba-OA1CT2/Hind, as described above. Wild type and mutant OA1 amplification products were then digested with XbaI and HindIII and cloned into the same sites of the mammalian expression vector pcDNA 3.1/myc-His (−) C (Invitrogen). For primers and PCR conditions used to generate OA1 mutants see Supplementary Fig. 1B. Proof-reading Pfu DNA polymerase (Promega) was used to avoid synthesis errors in all amplifications. DNA sequences of all constructs were confirmed by sequencing (Bio Molecular Research, DNA sequencing service at the University of Padua, Italy; http://bmr.cribi.unipd.it/). Plasmid DNA was purified using the Qiagen Midi kit (Qiagen) and DNA concentration was double checked by ethidium bromide and spectro-photometric analyses.

Cell Culture and Transfection

HeLa cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (Gibco, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Euroclone, Ltd, UK) and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin). MNT-1 cells (gift of PierGiorgio Natali, Regina Elena Institute, Rome, Italy) were maintained in DMEM containing 10% AIM-V medium (Gibco, Invitrogen), 20% FBS, 0,1 mM non-essential amino acids (Gibco, Invitrogen), 1 mM sodium pyruvate (Gibco, Invitrogen) and antibiotics. HeLa cells were transiently transfected on glass coverslips placed in 24-well plates using FuGENE 6, according to the manufacturer instructions (Roche). To avoid undesirable overexpression, each well was transfected with 60 ng of “expressor” plasmid DNA and the balance was made up with empty vector up to 600 ng total. This protocol was adapted from previous studies on human tyrosinase (Calvo et al., 1999) and allowed detectable expression of wild type LAMP1 or OA1 without significant overexpression (revealed by appearance of the proteins at the cell surface). MNT-1 cells were transiently transfected on glass coverslips placed in 24-well plates using LipoFectamine 2000 reagent, according to the manufacturer instructions (Invitrogen). To avoid undesirable overexpression, each well was transfected with 270 ng of ”expressor” plasmid DNA and the balance was made up with empty vector up to 800 ng total. For transfections with OA1CT2 constructs, DNA quantities were kept higher due to the lower expression level of the recombinant proteins (120/600 and 800/800 ng of “expressor” plasmid/empty vector in HeLa and MNT1 cells, respectively). Transfection efficiencies with the different constructs were comparable, as assessed in each experiment by the detection of similar numbers of transfected cells expressing the recombinant proteins.

Immunofluorescence Analysis

In all experiments, 24 hours following transfection, cells were incubated 1 hour with cycloheximide (50 μg/ml as final concentration) and fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), pH 7.4. Fixed cells were blocked and permeabilized 1 hour with 0.2% saponin (Sigma) and 10% porcine serum (Sigma) in PBS. Primary and secondary antibodies were incubated 2 and 1 hour, respectively, with 0.1% saponin and 1% porcine serum in PBS. We used the following primary antibodies: W7 anti-OA1, rabbit anti-human OA1 polyclonal antiserum (Schiaffino et al., 1996); H4A3, mouse anti-human LAMP1 mAb

(developed by August J. T. and Hildreth J. E. K. and obtained from the Developmental Studies Hybridoma Bank, University of Iowa, Department of Biological Sciences, Iowa City, IA); LY1C6, mouse anti-rat LAMP1 mAb (Stressgen Biotechnologies); HMB45, mouse anti-Pmel17 mAb (DAKO corporation, CA); TA99, mouse anti-human TRP1 mAb (kind gift of A. Houghton); anti-EEA1, mouse anti-EEA1 mAb (BD Biosciences); anti-EEA1, rabbit anti-EEA1 polyclonal antiserum (Affinity Bioreagents); anti-His_6, mouse anti-His_6 mAb (Roche). For lysosomal labeling, cells were pulsed 1 hour at 37°C with medium supplemented with 4 mg/ml of Lucifer Yellow CH (LY, Sigma) and, following extensive washing (at least five times) with warm medium, were chased 1 additional hour at 37°C with normal medium, to accumulate the tracer into the late endocytic pathway (Miller et al., 1983). For plasma membrane labeling, cells were either immunodecorated prior to permeabilization with mouse anti-Na^+/K^+ ATPase mAb (clone C464.6, Upstate inc.), or transfected with a vector encoding farnesylated GFP (pEGFP-F, Clontech) that, thanks to the farnesylation signal from c-Ha-Ras, accumulates specifically at the inner face of the plasma membrane. Indeed, farnesylated GFP was found to colocalize extensively with endogenous Na^+/K^+ ATPase both in HeLa and MNT1 cells (data not shown). Secondary antibodies were immunoadsorbed Cy2-conjugated donkey anti-rabbit IgG and Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories) or Alexa Fluor 488/594-conjugated donkey anti-rabbit IgG and Alexa Fluor 488/594-conjugated donkey anti-mouse IgG. Labeled coverslips were mounted using Mowiol 4-88 reagent (Calbiochem) and visualized using either an Axiophot microscope equipped with a charge-coupled device camera (Carl Zeiss, Jena, Germany) for subcellular distribution analyses or a TCS-SP2 AOBS confocal microscope (Leica, Mannheim, Germany) for colocalization studies.

Data Analysis

Adobe Photoshop and Microsoft Excel softwares were used for image processing and statistical analyses, respectively. Quantitative immunofluorescence analysis of the subcellular distribution displayed by LAMP/OA1 chimeras or OA1 mutants was performed by counting in each transfection experiment the number of cells showing vesicular, plasma membrane or mixed staining patterns. Classification of cells into the three categories was according to the following criteria. Vesicular distribution: bright cytoplasmic vesicular staining more prominent in the perinuclear area on a black background, cell margins are not detectable. Plasma membrane distribution: diffuse and often finely granular staining throughout the cell, intense labeling at margins and membrane spikes, cell margins are clearly visible; occasionally few vesicles may be present, likely due to the normal protein turnover from the cell surface. Mixed vesicular-plasma membrane distribution: vesicular pattern comprising lots of bright vesicles, superimposed to a diffuse staining throughout the cell, with labeling of cell margins and membrane spikes. Cell surface distribution was also confirmed by colocalization with plasma membrane markers (see above). A minor number of cells (less than 5%) that could not be classified into these categories (because out-of-focus, too confluent or superimposed, etc.) were omitted from the count. Quantitative immunofluorescence analysis of the colocalization between endogenous/recombinant OA1 or LAMP/OA1 chimeras and various markers was performed on digital confocal micrographs by marking within individual cells several (20-50) distinct single OA1 or LAMP1 positive vesicles and subsequently assessing their colocalization with the markers. Vesicles were chosen randomly throughout the cells, with the exception of areas overcrowded with organelles (perinuclear areas, tips of dendrites in melanocytes). The latter were avoided because of the risk of false positives despite the confocal analysis (see Supplementary Figs 2, 3). Quantitative immunofluorescence analyses were performed in blind (without knowing the name of constructs analyzed) by the first author and counterchecked for verification by a second independent investigator.

RESULTS

OA1 localizes to lysosomes in HeLa cells and to lysosomes and melanosomes in MNT1 cells

We and others previously reported that, when heterologously expressed at moderate levels in COS7 cells, OA1 is sorted to late endosomes and lysosomes, showing extensive colocalization with Cathepsin D, LAMP2 and DAMP, a basic congener of dinitrophenol that accumulates in acidic compartments (Schiaffino et al., 1999; Shen et al., 2001). We obtained similar results in HeLa cells transiently expressing a mycHis-tagged OA1 (OA1-mycHis). In fact, the recombinant OA1-mycHis displays a vesicular cytosolic distribution and colocalizes largely with the fluid phase marker Lucifer Yellow, subjected to 1 hour of chase prior to fixation (Fig. 1A). This time interval allows accumulation of the tracer into the late endocytic pathway, particularly into lysosomes, as in these chase conditions its staining overlaps extensively with DAMP and LAMP1 (data not shown). In melanocytes, the endogenous OA1 has been localized to late-endosome/lysosomes (Samaraweera et al., 2001) and to melanosomes (Schiaffino et al., 1996; Schiaffino et al., 1999; Schiaffino et al., 2002; Basrur et al., 2003), however quantitative studies with multiple markers have never been performed.

To determine the steady-state localization of endogenous OA1 in melanocytes, we took advantage of the pigmented human melanoma cell line MNT1, which has been previously utilized as a suitable model for melanosome biogenesis (Kushimoto et al., 2001; Raposo et al., 2001). The subcellular distribution of OA1 was analyzed by indirect immunofluorescence and confocal microscopy, using markers of different subcellular compartments: LAMP1, a marker of late-endosome/lysosomes; Pmel17, a marker of immature melanosomes; TRP1, a marker of mature melanosomes; EEA1, a marker of early endosomes; and Lucifer Yellow at 1 hour chase, to label the lysosomes. Previous quantitative ultrastructural studies have shown that in MNT1 cells some degree of overlap exists among LAMP1, Pmel17 and TRP1 (Raposo et al., 2001). In particular, LAMP1 is also present to a minor extent on immature and mature melanosomes, while Pmel17 labels stage I to III melanosomes and TRP1 stage III to IV melanosomes (Raposo et al., 2001). By contrast, melanosomes were found inaccessible to fluid phase markers (Raposo et al., 2001), therefore Lucifer Yellow staining is expected to label the late endocytic pathway only.

The quantitation of colocalization was performed by assessing on confocal micrographs the percent of OA1 positive vesicles that were also positive for the different markers (Fig. 1B and Supplementary Fig. 2). This statistical analysis showed that endogenous OA1 mostly co-distributes with LAMP1 (88%) and with Pmel17 (74%). Essentially identical results were obtained in MNT1 cells transiently transfected with the OA1-mycHis expression vector and immunodecorated with anti-His_6 antibodies. Exogenous OA1 colocalizes by 82% ± 0.1 with LAMP1 (vesicles counted: 305; cells counted: 18) and 74% ± 0.2 with Pmel17 (vesicles counted: 490; cells counted: 26). Minor colocalizations were found between endogenous OA1 and either TRP1 (21%) or EEA1 (6%), while about one third (31%) of the OA1 compartment corresponds to that labeled by Lucifer Yellow (Fig. 1B).

The third cytosolic loop and the C-terminal tail of OA1 contain lysosomal sorting signals

To establish which domains of OA1 are sufficient for intracellular targeting, we generated protein chimeras consisting of the N-terminus and transmembrane domain of rat LAMP1 and the cytosolic regions of OA1. LAMP1 is a type 1 lysosomal membrane glycoprotein, which contains specific sorting signals within its cytosolic C-terminal tail. In fact, previous studies showed that, when deleted of its C-terminus, LAMP1 accumulates instead at the plasma membrane by means of the default pathway (Williams and Fukuda, 1990) and hence can be conveniently used as “neutral” reporter (Simmen et al., 1999; Storch et al., 2004). In our case, if any of the cytosolic domains of OA1 contain lysosomal targeting signals, when fused to the

C-terminus deleted LAMP1 they are expected to re-direct targeting of the protein from the cell surface to the lysosomes. The precise regions of OA1 utilized to generate LAMP/OA1 chimeras are indicated in Fig. 2A. Since the limits of transmembrane domains are not predictable with certainty in OA1, few additional amino acids were included over the putative cytosolic loops. For the same reason, the stop transfer signal RKR at the end of the transmembrane domain of rat LAMP1 was retained in the constructs, in order to guarantee proper insertion of the chimeric proteins within the membrane. Nevertheless, similar results were obtained in the presence or in the absence of the RKR tripeptide either in HeLa or Cos-7 cells (data not shown).

We first analyzed the subcellular distribution of LAMP/OA1 chimeras in transiently transfected HeLa cells by indirect immunofluorescence, using an antibody directed specifically against LAMP1 of rat origin and showing no cross-reaction with the protein from other species. Although LAMP/OA1 chimeras did not show any evident ER retention, to eliminate the pool of newly synthesized proteins, cells were treated with cycloheximide 1 h prior to fixation (see Material and Methods). The results of this analysis are shown in Fig. 2B. Interestingly, we observed that addition of the first or the second cytosolic loops of OA1 to the C-terminus deleted LAMP1 (LAMP/i1 and LAMP/i2) does not interfere with its plasma membrane localization, visible as a diffuse cell staining more intense at margins and membrane spikes and corroborated by colocalization with farnesylated GFP (Fig. 2B and not shown). Conversely, chimeric proteins carrying the third cytosolic loop or the C-tail of OA1 (LAMP/i3 and LAMP/CT) showed a complete or partial vesicular cytoplasmic distribution, respectively (Fig. 2B), and colocalized with Lucifer Yellow at 1 hour chase (not shown), suggesting that these regions contain lysosomal sorting signals.

Dissection of the critical regions sufficient for lysosomal targeting in HeLa cells

To further dissect these signals, we generated chimeric proteins carrying progressive deletions of the third cytosolic loop (i3) or the C-tail (CT) of OA1 and expressed them in HeLa cells. This strategy allowed to restrict the signal-containing regions to the 19 N-terminal amino acids of i3 (i3FY; Fig. 2A) and to the 25 N-terminal amino acids of CT (CT2; Fig. 2A), since the corresponding fusion proteins (LAMP/i3FY and LAMP/CT2) were promptly delivered to the lysosomes (Figs 3, 4). In contrast, the remaining part of i3 (18 C-terminal amino acids of i3) does not appear to contain any signal for intracellular localization, since the protein chimera carrying this portion, LAMP/i3ΔFY, displayed plasma membrane distribution and colocalization with farnesylated GFP (Fig. 3B). Similarly, the remaining part of CT (65 C-terminal amino acids of CT) was found to induce significant cell surface accumulation of the corresponding chimera, LAMP/CTΔCT2, even though in this case a mixed vesicular/plasma membrane distribution pattern was observed, suggesting that this region of OA1 might contain further signals (Fig. 4B; see below for further description).

Next, we took advantage of the interspecies amino acid conservation of the i3FY and CT2 sequences to generate chimeric proteins carrying progressive deletion of conserved blocks (LAMP/i3FG and LAMP/i3FA; LAMP/CT2M5 and LAMP/CT2M11) (Figs 3A, 4A). In addition, we mutated to alanine a double-leucine in i3FY (LAMP/i3FY LL→AA; Fig. 3A), since dileucine-based motifs have been implicated in the sorting of lysosomal and melanosomal membrane proteins (Bonifacino and Traub, 2003), and a tryptophan-glutamic acid in CT2 (LAMP/CT2M5 WE→AA; Fig. 4A), since despite poor conservation of this region these two residues are preserved in all species tested. We expressed these chimeras in HeLa cells and observed that LAMP/i3FA distributes predominantly to the cell surface, while both LAMP/i3FY LL→AA and LAMP/i3FG display a mixed distribution, characterized by a vesicular/lysosomal pattern colocalizing with Lucifer Yellow, superimposed to a diffuse plasma membrane staining (Fig. 3B and not shown). As mutation of the dileucine does not result in a complete plasma membrane displacement, which is obtained instead with deletion of additional

eight contiguous amino acids in the LAMP/i3FA chimera, it is likely that the relevant signal-containing sequence in i3 is represented by SLLKGRQGIY ("S-Y" sequence). Furthermore, we found that LAMP/CT2M5 shows a lysosomal pattern comparable to that of LAMP/CT2. In contrast, LAMP/CT2M5 WE→AA and LAMP/CT2M11 were mostly delivered to the plasma membrane (Fig. 4B and not shown), revealing a critical role of the WE doublet.

Four different missense mutations were identified within i3 distally to the double-leucine in patients with ocular albinism, including G229V, T232K, E235K and I244V (Fig. 2A) (Schnur et al., 1998;Bassi et al., 2001). The role of these mutations in the pathogenesis of the disease remains to be established, since previous studies showed that in the context of the native OA1 they do not determine ER retention of the protein nor other apparent sorting defects (d'Addio et al., 2000;Shen et al., 2001). Since the presence of additional targeting signals in OA1 might have masked the effect of these mutations, we introduced each of them within the LAMP/i3 chimera; moreover, we introduced the G229V mutation within LAMP/i3FY, and the T232K, E235K and I244V mutations within LAMP/i3ΔFY. Upon expression in HeLa cells, we found no difference between the distribution pattern of the original chimeras and that of the mutation-carrying chimeras, i.e. either in the absence or in the presence of missense mutations LAMP/i3 and LAMP/i3FY showed a lysosomal distribution, while LAMP/i3ΔFY was delivered to the plasma membrane (data not shown). These findings support the idea that, except for ER retention, missense mutations in ocular albinism do not cause other kinds of trafficking defects and that G229 within the "S-Y" sequence is not a critical component of the sorting signal.

Dissection of the critical regions sufficient for lysosomal/melanosomal targeting in MNT1 cells

To determine whether the dileucine-like motif contained within the "S-Y" sequence and the "WE" signal are also relevant for lysosomal/melanosomal targeting in melanocytic cells, we transiently expressed each chimera in MNT1 cells. Since in this cell type immunofluorescence patterns were more variable than in HeLa cells, both within the same and among different experiments, we performed a statistical analysis of multiple transfection experiments by evaluating the relative number of cells showing plasma membrane, vesicular or mixed distribution of the chimeric proteins (Fig. 5A and Supplementary Fig. 3; see Material and Methods for details). Similarly to the results obtained in HeLa cells, this analysis revealed that most of MNT1 cells transfected with LAMP/i3ΔFY display cell surface localization of the recombinant protein, while most of cells transfected with LAMP/i3FY, LAMP/CT2 and LAMP/CT2M5 show vesicular distribution (Fig. 5A and Supplementary Fig. 3). LAMP/i3FA, LAMP/CT2M5 WE→AA and LAMP/CT2M11 were predominantly found on the plasma membrane with or without a superimposed vesicular staining. In contrast, LAMP/i3FY LL→AA and LAMP/i3FG were characterized by a high percentage of cells displaying mixed and vesicular labeling, respectively, with only about 10% of cases showing complete cell surface localization (Fig. 5A).

To investigate the specificity of these signals for the OA1 compartment, we colocalized LAMP/OA1 chimeras with endogenous OA1 in transiently transfected MNT1 cells. The quantitation of colocalization was performed by assessing on confocal micrographs the percent of rat LAMP1 positive vesicles that were also positive for endogenous OA1. Interestingly, as shown in Fig. 5B, over 80% of intracellular vesicles enriched in LAMP/CT2 also contain endogenous OA1, indicating that CT2 exhibits a significant ability to direct the C-terminus deleted LAMP1 toward the OA1 compartment. This capacity is maintained by LAMP/CT2M5, but is abolished by mutation or deletion of the WE doublet, since LAMP/CT2M5 WE→AA and LAMP/CT2M11 are mostly delivered to the cell surface and their residual intracellular vesicles co-segregate with endogenous OA1 by less than 30% (Fig. 5B and Supplementary Fig. 3). Modest

levels of colocalization with endogenous OA1 compared to LAMP/CT2 were also observed with LAMP/i3FY and its mutants (Fig. 5B and Supplementary Fig. 3).

The dileucine-like and "WE" motifs are necessary for the intracellular targeting of OA1

To establish whether the peptide motifs identified above are relevant for the sorting of the native OA1, we generated missense or deletion mutants of full-length mycHis-tagged OA1 and explored their localization in transiently transfected HeLa and MNT1 cells. In particular, we produced a mutant in which the entire "S-Y" sequence in i3 was deleted (OA1/ΔSY; Fig. 2A, round brackets in i3), a mutant carrying a LL→AA amino acid substitution in i3 (OA1/LL→AA; Fig. 2A, arrowheads in i3), and a mutant carrying a WE→AA amino acid substitution in CT (OA1/WE→AA; Fig. 2A, arrowheads in CT). In addition, we created two double mutants, carrying either the ΔSY deletion or the LL→AA substitution in combination with the WE→AA mutation (OA1/ΔSY/WE→AA and OA1/LL→AA/WE→AA, respectively). In order to obtain a quantitative comparison among the distributions of different mutants, transiently transfected HeLa and MNT-1 cells were counted and classified into three categories, based on the vesicular, plasma membrane or mixed distribution of the recombinant proteins, as performed previously with LAMP/OA1 chimeras (see Material and Methods for details). The results of this analysis are shown in Figs 6, 7.

Both in transfected HeLa and MNT-1 cells, we observed that neither the deletion of the "S-Y" sequence nor the mutation of the dileucine or of the "WE" motif alone result in significant missorting of the corresponding OA1 variants to the plasma membrane (Figs 6, 7). In contrast, the concomitant abrogation of the "S-Y" sequence and of the "WE" motif results in a clear plasma membrane displacement of the double mutant, which showed labeling of the cell surface in 76% and 45% of transfected HeLa and MNT1 cells, respectively (Figs 6, 7). Plasma membrane localization was confirmed by colocalization with farnesylated GFP or Na^+/K^+ ATPase and was usually accompanied by a persistent vesicular staining (Figs 6A, 7A), so that most cells displayed a mixed distribution pattern. Similar, although less striking results were obtained with the OA1/LL→AA/WE→AA double mutant, confirming that other amino acids of the "S-Y" sequence in addition to the double-leucine are relevant for sorting.

The distal portion of the C-tail (last 65 residues) is not required for the intracellular targeting of OA1

The LAMP/CTΔCT2 chimera, carrying the last 65 amino acids of CT fused to LAMP1, displays a mixed vesicular/plasma membrane distribution when expressed in HeLa cells (Fig. 4B), suggesting that this region of OA1 might contain additional signals for intracellular localization. Thus, we tested its contribution to the sorting of the full-length protein by generating a deletion mutant of OA1 lacking the 65 C-terminal amino acids (OA1CT2-mycHis; Fig. 2A, bar in CT). When expressed in HeLa cells, OA1CT2-mycHis showed a subcellular distribution indistinguishable from the full-length OA1 and colocalized extensively with Lucifer Yellow at 1 hour chase (Fig. 8A). In transiently transfected MNT1 cells, OA1CT2 displayed a vesicular distribution and could be compared to the endogenous OA1 staining, since it is not recognized by the W7 anti-OA1 antibody (tested by western blotting and immunofluorescence analyses, not shown). OA1CT2 colocalizes by 76% with endogenous OA1 and to a significant extent with LAMP1, Pmel17 and Lucifer Yellow (Fig. 9A and Supplementary Fig. 4), displaying a behavior very similar to that of the endogenous and exogenous full-length OA1 (Fig. 1B).

In order to test whether deletion of the distal portion of the C-tail would hamper the intracellular localization of OA1/ΔSY, OA1/LL→AA or OA1/WE→AA single mutants, we introduced within OA1CT2 the same mutations generated within the full-length OA1 and expressed the resulting mutants in HeLa and MNT1 cells. As previously observed with full-length OA1

mutants, despite the absence of the 65 C-terminal residues, only OA1CT2/ΔSY/WE→AA and to a minor extent OA1CT2/LL→AA/WE→AA double mutants displayed cell surface localization, while none of the single mutants did (Figs 8B, 9B). In addition, both in HeLa and MNT1 cells, full-length OA1 and OA1CT2 double mutants showed plasma membrane displacement in a comparable percentage of cells (compare Fig. 6B to Fig. 8B and Fig. 7B to Fig. 9B).

Finally, to test whether abrogation of the dileucine-like or "WE" motifs might interfere with the specific subcellular localization of OA1 in melanocytic cells, we colocalized OA1CT2 mutants with endogenous OA1 in MNT1 cells. Similarly to OA1CT2 (Fig. 9A), OA1CT2/LL→AA and OA1CT2/ΔSY displayed a high degree of colocalization with endogenous OA1 (OA1CT2/LL→AA: 81% ± 0.1; OA1CT2/ΔSY 83% ± 0.1) (vesicles counted: 1100; cells counted: 40). In contrast, OA1CT2/WE→AA revealed a significantly lower ability to redistribute to the endogenous OA1 compartment, since its colocalization with OA1 dropped to 43% ± 0.2 (vesicles counted: 1000; cells counted: 40) (see Supplementary Fig. 5), suggesting that the "WE" motif plays a critical role in the specialized sorting of the protein.

DISCUSSION

In this study, we sought to identify the determinants responsible for the intracellular distribution of OA1 in melanocytic and non-melanocytic cells. In order to solve previous discrepancies on the localization of the endogenous protein in melanocytes, we performed a quantitative confocal immunofluorescence analysis in the human melanoma line MNT1. By using two distinct antibodies, anti-OA1 to label the endogenous protein and anti-His_6 to detect the exogenous one, OA1 was found to colocalize extensively with markers of late endosomes/lysosomes (LAMP1, Lucifer Yellow at 1 hour chase) and melanosomes (Pmel17, TRP1). Thus, OA1 appears equally distributed along both the lysosomal and the melanosomal sorting pathways, however the high level of cosegregation with LAMP1 and Pmel17 suggests that a major fraction of the protein localizes to an intermediate late endosomal/immature melanosomal compartment. This subcellular distribution most likely depends on direct sorting from the trans-Golgi network, since OA1 is only minimally found in EEA1-positive early endosomes (Fig. 1) and does not localize to transferrin-labeled recycling endosomes, nor is detected at the cell surface, even in dynamin K44A-transfected MNT1 cells (our unpublished results), supporting the notion that the protein does not normally transit through the plasma membrane.

To search for the sorting signals responsible for the lysosomal/melanosomal targeting of OA1 we utilized both LAMP1/OA1 chimeras and OA1 mutants and analyzed their steady-state distribution in melanocytic and non-melanocytic cells. By this strategy we identified two independent signals that are necessary and sufficient for lysosomal/melanosomal localization in HeLa and MNT1 cells. The first signal is contained within the third cytosolic loop and is characterized by a double-leucine, although additional adjacent residues included in the highly conserved sequence SLLKGRQGIY ("S-Y" sequence) appear to be important (Fig. 2A). Even if dileucine-based motifs are well known lysosomal/melanosomal sorting signals, the one carried by OA1 appears unconventional, since it is located in a cytosolic loop instead of a C-tail and it is not preceded by proline and acidic residues (Setaluri, 2000;Bonifacino and Traub, 2003). In addition, at variance with typical dileucine-based signals, mutation of a single leucine of the doublet within a LAMP/OA1 chimera (LAMP/i3FY LL→LA) does not interfere with its intracellular localization (our unpublished results). Finally, the double-leucine itself seems to play only a partial role, since entire deletion of the "S-Y" sequence determines a more dramatic missorting effect both in the context of LAMP/OA1 chimeras and of the native OA1 either in HeLa or MNT1 cells.

The second signal identified is located within the C-tail and is characterized by a tryptophan-glutamic acid doublet ("WE" motif; Fig. 2A). This motif appears novel and differs from all previously reported lysosomal/melanosomal sorting signals. Given that adjacent residues are poorly conserved and their deletion in LAMP/OA1 chimeras does not result in evident sorting effects (LAMP/CT2M11 behaves as LAMP/CT2M5 WE→AA), it is likely that the WE dipeptide represents the relevant component of the motif. Interestingly, sequence analysis of known melanosomal membrane proteins revealed that tryptophanglutamic acid doublets conserved in human and mouse are also present within the cytosolic domains of the P/pink-eyed dilution protein. Both dileucine-like and "WE" motifs do not seem to be dependent on membrane distance or topology, as previously reported for tyrosine or dileucine-based signals (Rohrer et al., 1996;Bonifacino and Traub, 2003). In fact, the dileucine-like signal works similarly when inserted in a cytosolic tail in LAMP/OA1 chimeras or in a loop in the native OA1. Deletion of the RKR stop transfer signal in LAMP/OA1 chimeras did not change the ability of dileucine-like and "WE" motifs to determine lysosomal sorting in HeLa cells; similarly, addition or deletion of four consecutive residues at the N-terminus of the "WE" motif did not perturb its ability to determine intracellular localization of the LAMP/CT2 chimera in HeLa or MNT1 cells (our unpublished results).

Both motifs are sufficient and necessary on their own to mediate lysosomal sorting in HeLa cells, thus functioning in a similar way as lysosomal sorting signals in non-melanocytic cell types. However, the "WE" motif, but not the dileucine-like signal, showed a significant capacity of redirecting the corresponding LAMP/OA1 chimera toward OA1 positive compartments in MNT1 cells. Moreover, abrogation of the "WE" motif in the OA1CT2 construct resulted in a significant reduction of its colocalization with endogenous OA1, while mutation of the dileucine or deletion of the entire "S-Y" sequence produced essentially no effect. Therefore, although we could not demonstrate by confocal immunofluorescence analysis a lysosomal versus melanosomal specificity of any of these signals, our results suggest that they might mediate the targeting of OA1 to different subcompartments in melanocytic cells and that the novel "WE" motif plays a key role in the intracellular transport of OA1.

The dileucine-like and "WE" motifs must be mutated simultaneously in order to observe a substantial plasma membrane appearance of the native OA1, suggesting that they are able of independently facilitating the intracellular targeting of the protein and compensate for each other. However, at variance with the findings obtained using LAMP/OA1 chimeras, a "pure" plasma membrane distribution was rarely observed with OA1 double mutants, which mostly show cell surface localization associated to a persistent vesicular staining. Thus, although the two motifs identified are clearly implicated in its intracellular targeting, OA1 might contain additional sorting determinants that could justify the incomplete cell surface misrouting of double mutants. Furthermore, the definitively minor plasma membrane displacement exhibited by double mutants in MNT1 compared to HeLa suggests that melanocytic cells possess specific sorting machineries, possibly based on specialized adaptor proteins.

Our screening was restricted to the cytosolic domains of OA1 because they would be accessible to adaptor binding during transit along the secretory pathway, as previously described for canonical tyrosine- or dileucine-based motifs, typically located in the cytosolic domains of single-spanning lysosomal and melanosomal membrane proteins (Setaluri, 2000; Bonifacino and Traub, 2003). However, additional signals involved in the subcellular targeting of OA1 might be located in other regions, including transmembrane domains and lumenal loops. It is also possible that some other signals lie within the cytosolic loops and were not detected, because they are either spread into multiple loops or dependent on the precise topology of the protein, which we could not reproduce in LAMP/OA1 chimeras. Nevertheless, critical signals responsible for the steady-state distribution of OA1 do not appear confined within the distal portion of the C-tail, since our results show that this region is not necessary for the efficient

intracellular retention and specific subcellular targeting of the protein in HeLa or MNT1 cells. The C-terminus of OA1 is not highly conserved among species and is enriched in serines and threonines. Although its deletion in the OA1CT2 construct does not result in major sorting defects, the mixed vesicular/plasma membrane distribution displayed by the LAMP/CTΔCT2 chimera (Fig. 4B) and the slight reduction of Pmel17 colocalization showed by OA1CT2 (Fig. 9A) suggest that it might contain some supplementary trafficking information, possibly dependent on the receptor's activity and regulated by phosphorylation or other post-translational modifications, as typically observed in canonical GPCRs (Marchese et al., 2003).

In summary, we show here that, similarly to other recently characterized polytopic lysosomal proteins (Cherqui et al., 2001; Kyttala et al., 2004) and in contrast to single-spanning lysosomal/melanosomal proteins (which are typically targeted by unique motifs in their cytosolic tails), OA1 contains at least two sorting signals that are responsible for its efficient intracellular retention and delivery to lysosomes and melanosomes. These signals are unconventional, lie in topologically separate protein domains and function independently as lysosomal sorting signals in non-melanocytic cells, nevertheless they might play different and more specialized roles in melanocytic cells. Our findings provide additional evidence that OA1 is a resident lysosomal/melanosomal GPCR, since it possesses specific signals for these subcellular compartments, supporting the idea that a putative ligand should come from the organelle lumen. The identification of such OA1 activator/s might hopefully be facilitated by the present study, which provides the means to efficiently deliver OA1 to the plasma membrane and therefore to take advantage of the widely used second-messenger activation assays requiring cell surface localization of the receptors.

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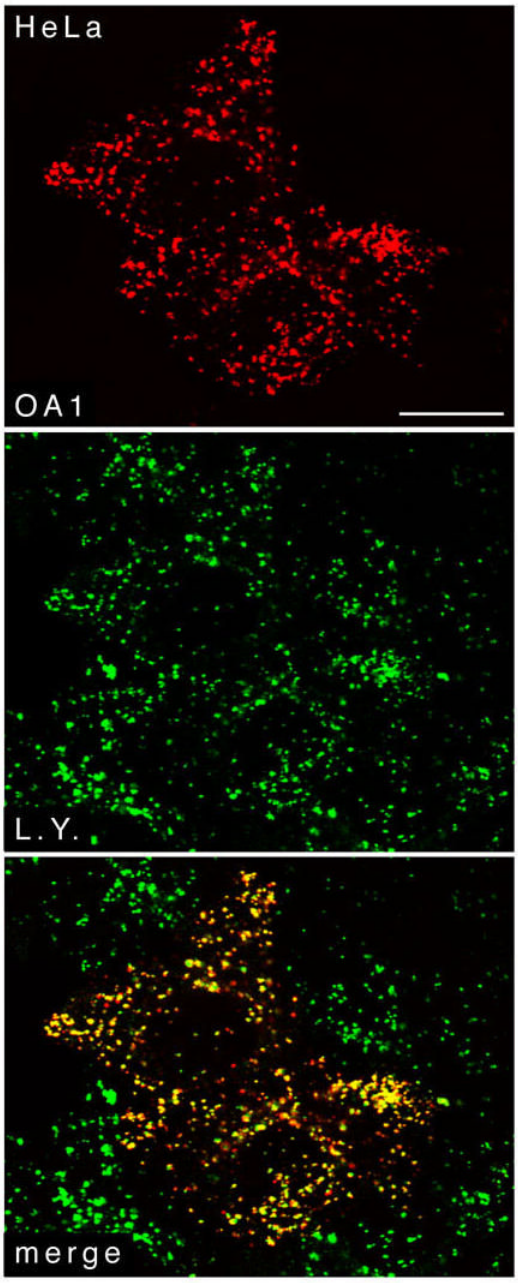


Fig. 1.

OA1 is targeted to lysosomes in HeLa cells and to lysosomes and melanosomes in MNT1 cells. (A) Immunofluorescence analysis of HeLa cells transiently transfected with an expression vector for wild-type mycHis-tagged OA1. Exogenous OA1 was immunodecorated with anti-His_6 antibodies and lysosomes were labeled using the fluid phase marker Lucifer Yellow at 1 hour chase prior to fixation (L.Y.). In the merge, areas of colocalization can be observed in yellow/orange. Bar, 10 μm. (B) Quantitative immunofluorescence analysis of the colocalization between endogenous OA1 and markers of various intracellular compartments in MNT-1 cells. Cells were double-labeled with anti-OA1 antibodies and with antibodies to LAMP1, Pmel17, TRP1, EEA1, or with Lucifer Yellow at 1 hour chase. Results are expressed as the percent of double-positive vesicles out of all OA1-positive compartments per cell and represent the mean ± s.d. of the data pooled from one or two independent experiments. The number of vesicles and cells counted is indicated.

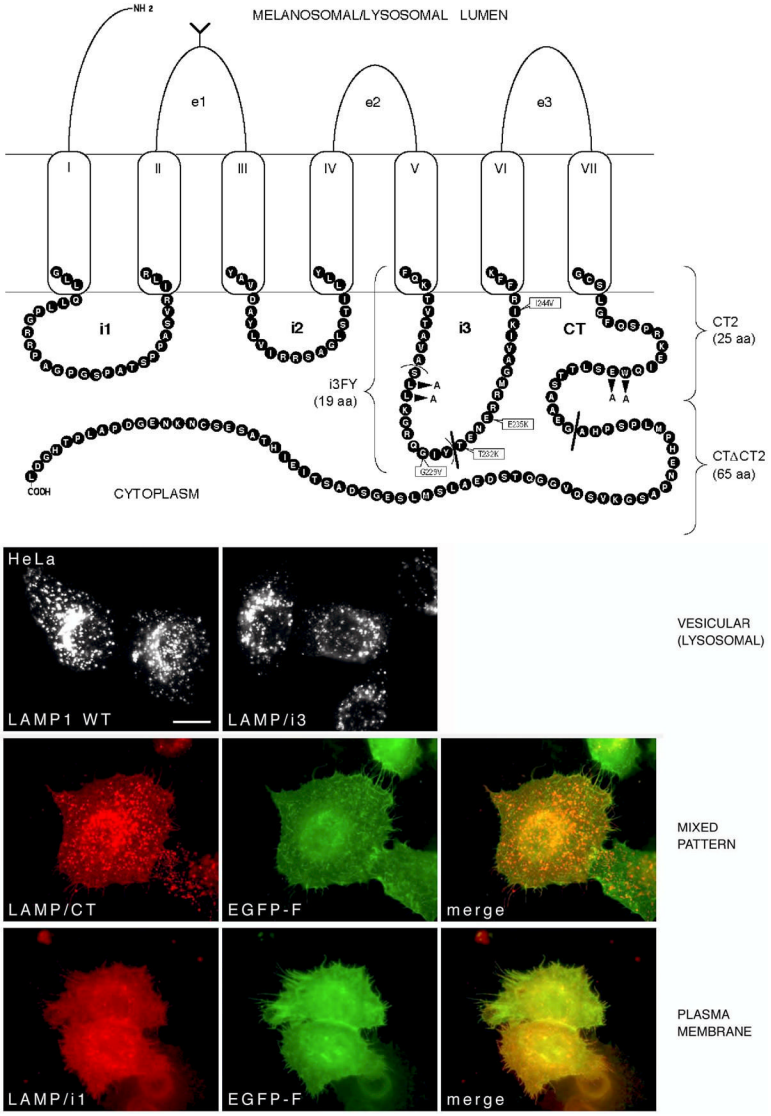


Fig. 2.

The third cytosolic loop (i3) and C-terminal tail (CT) of OA1 contain lysosomal sorting signals. (A) Schematic representation of the predicted heptahelical topology of OA1, as supported by previous bioinformatic, biochemical and morphological studies (Schiaffino et al., 1999). The position of transmembrane domains (I-VII), hydrophilic lumenal (e1-3) and cytosolic (i1-3) loops, and C-terminal tail (CT) is indicated. The portions of the protein utilized to generate LAMP/OA1 chimeras are depicted as black circles and missense mutations previously described in i3 are shown. The black bar in i3 delimits the 19 N-terminal amino acids of the third cytosolic loop, named i3FY and containing the dileucine-like motif; in between round brackets is the “S-Y” sequence, deleted in the OA1/ΔSY mutant. The black bar in CT separates the 25 N-terminal amino acids of the C-tail, named CT2 and containing the “WE” motif, from the 65 C-terminal amino acids of the C-tail, named CTΔCT2 and deleted in the OA1CT2 mutant. Arrowheads indicate the LL or WE residues, which represent the core of the corresponding motifs and are mutated to AA in the OA1/LL→AA or OA1/WE→AA mutants, respectively. (B) Immunofluorescence analysis of HeLa cells transiently transfected with expression vectors for LAMP/OA1 chimeras and immunodecorated with anti-rat LAMP1 antibodies. Cells were cotransfected with an expression vector for farnesylated GFP to label

the plasma membrane (EGFP-F). In the merge, areas of colocalization can be observed in yellow/orange. Distribution patterns of recombinant proteins are indicated on the right. LAMP/i2 behaves as LAMP/i1 (not shown). Bar, 10 μm.

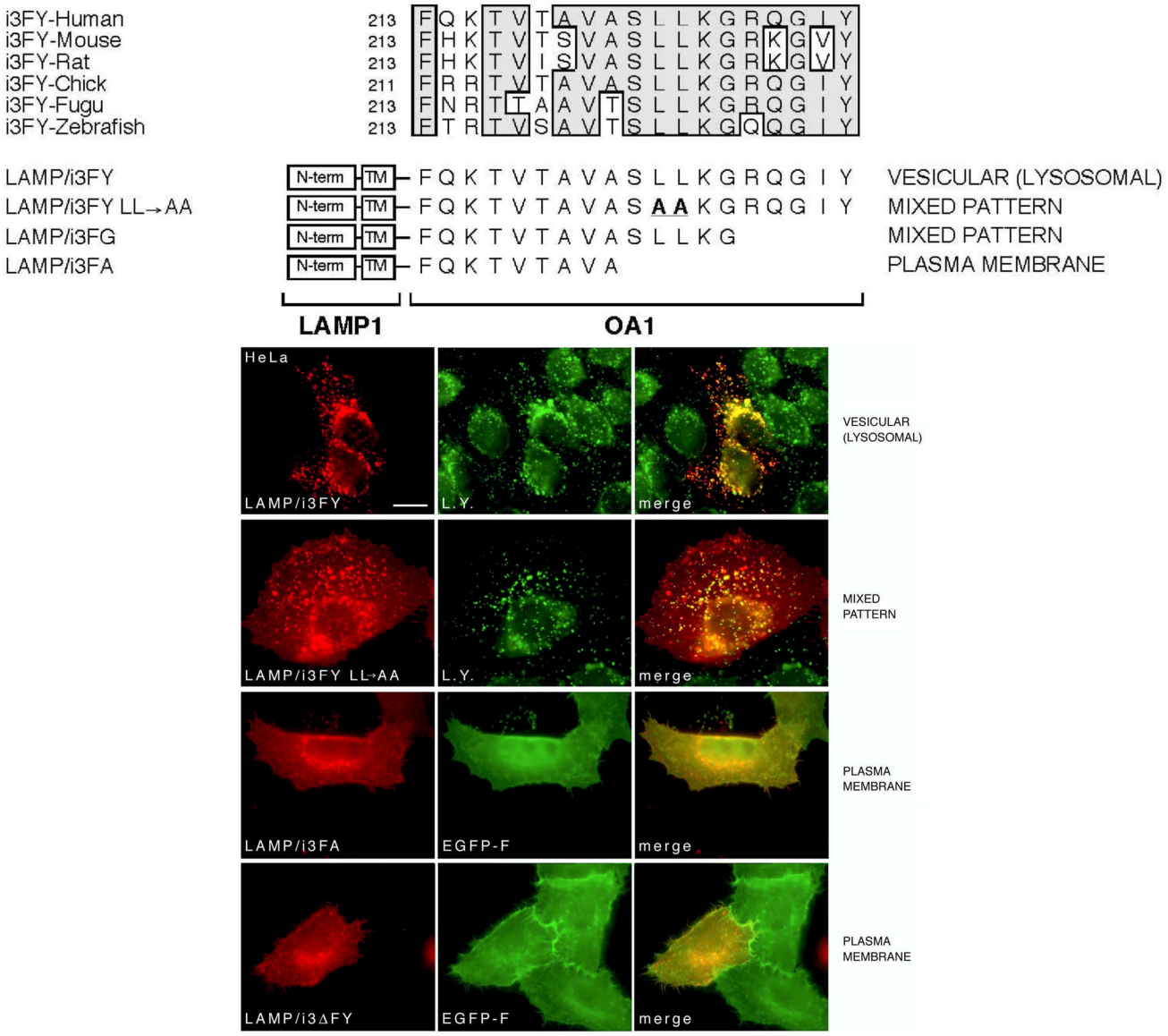


Fig. 3.

The dileucine-like motif in i3 functions as a signal sufficient for lysosomal sorting in HeLa cells. (A) Structure, sequence and cross-species conservation of the LAMP/i3FY construct, carrying the 19 N-terminal amino acids of i3, and of its deletion and mutagenesis variants. N-term and TM, N-terminus and transmembrane domain of rat LAMP1 that were fused with the cytosolic loop of OA1. (B) Immunofluorescence analysis of HeLa cells transiently transfected with expression vectors for LAMP/i3 chimeras and immunodecorated with anti-rat LAMP1 antibodies. Cells were counterstained with the fluid phase marker Lucifer Yellow at 1 hour chase prior to fixation to label the lysosomes (L.Y.), or were cotransfected with an expression vector for farnesylated GFP to label the plasma membrane (EGFP-F). In the merge, areas of colocalization can be observed in yellow/orange. LAMP/i3FG behaves as LAMP/i3FY LL→AA (not shown). LAMP/i3ΔFY, LAMP/OA1 chimera carrying the 18 C-terminal amino acids of i3. Bar, 10 μm.

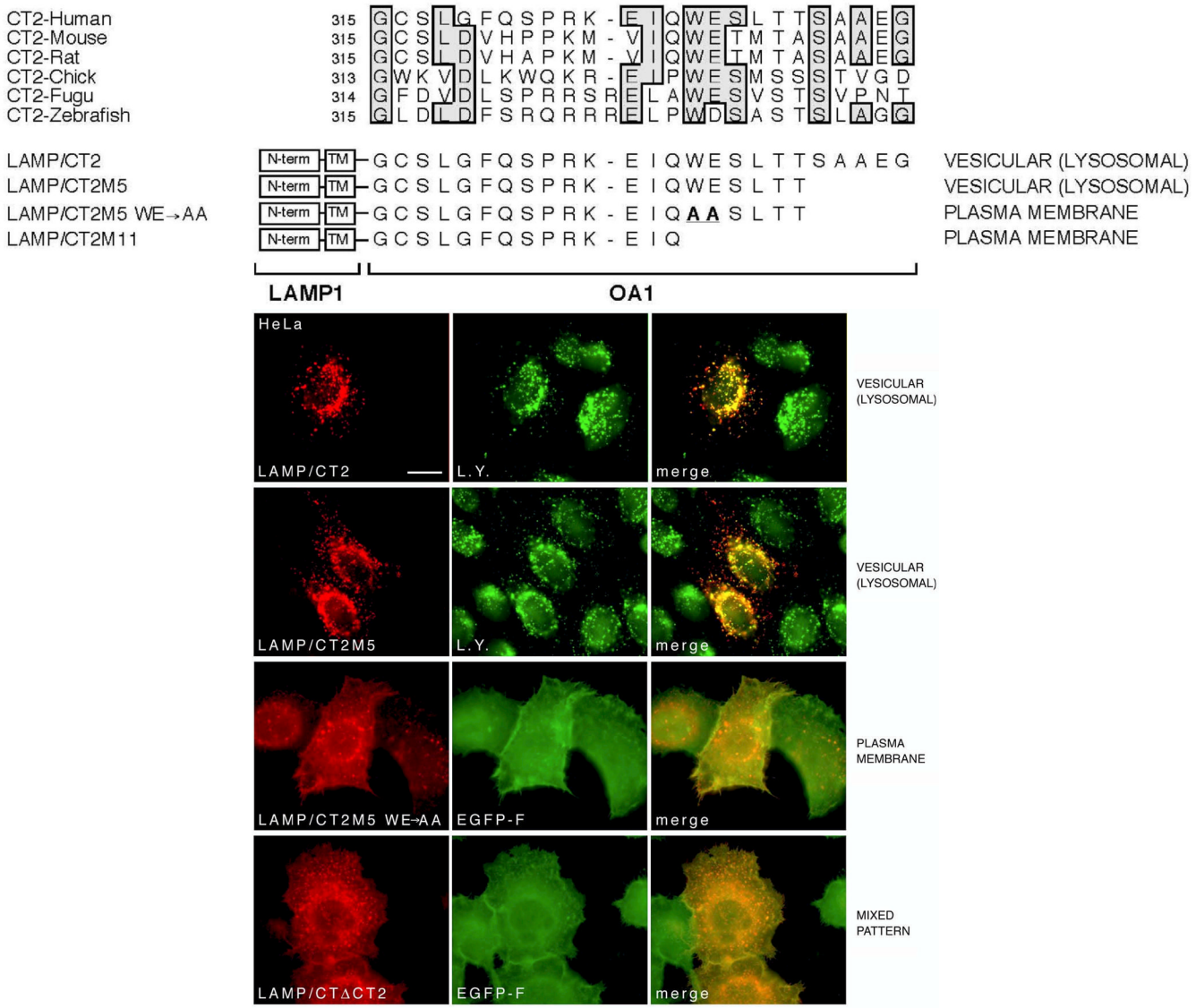


Fig. 4.

The “WE” motif in CT functions as a signal sufficient for lysosomal sorting in HeLa cells. (A) Structure, sequence and cross-species conservation of the LAMP/CT2 construct, carrying the 25 N-terminal amino acids of CT, and of its deletion and mutagenesis variants. N-term and TM, N-terminus and transmembrane domain of rat LAMP1 that were fused with the C-tail of OA1. (B) Immunofluorescence analysis of HeLa cells transiently transfected with expression vectors for LAMP/CT chimeras and processed as in Fig. 3B. In the merge, areas of colocalization can be observed in yellow/orange. LAMP/CT2M11 behaves as LAMP/CT2M5 WE→AA (not shown). LAMP/CTΔCT2, LAMP/OA1 chimera carrying the 65 C-terminal amino acids of CT. Bar, 10 μm.

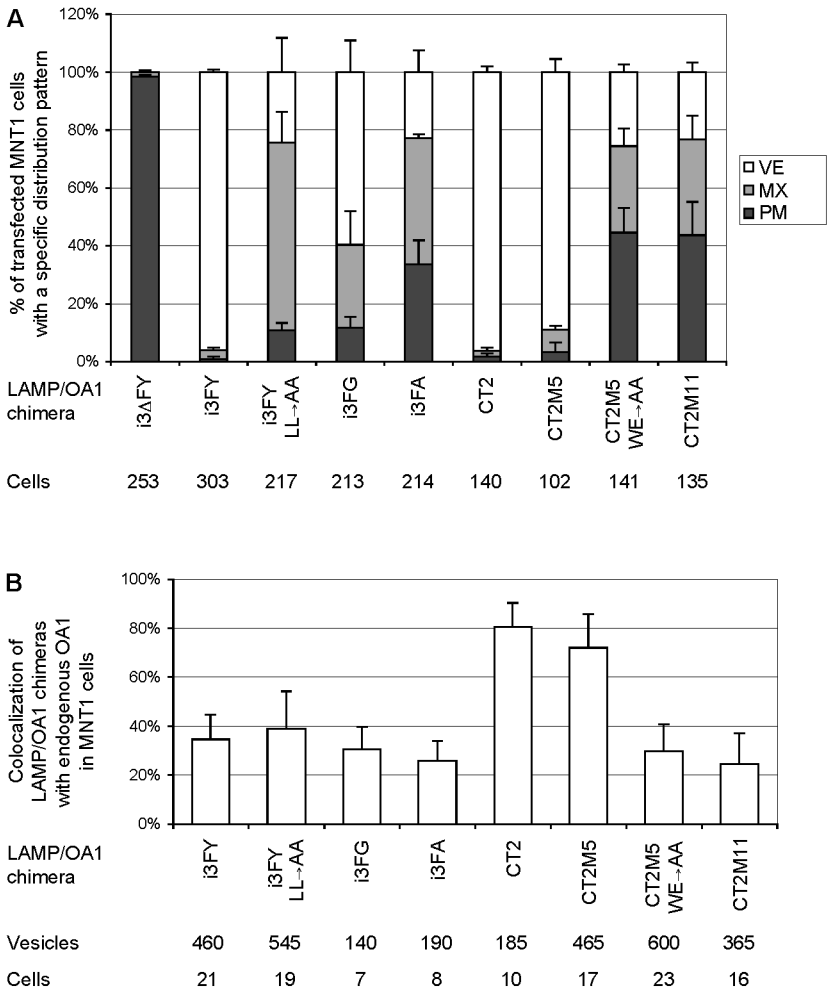
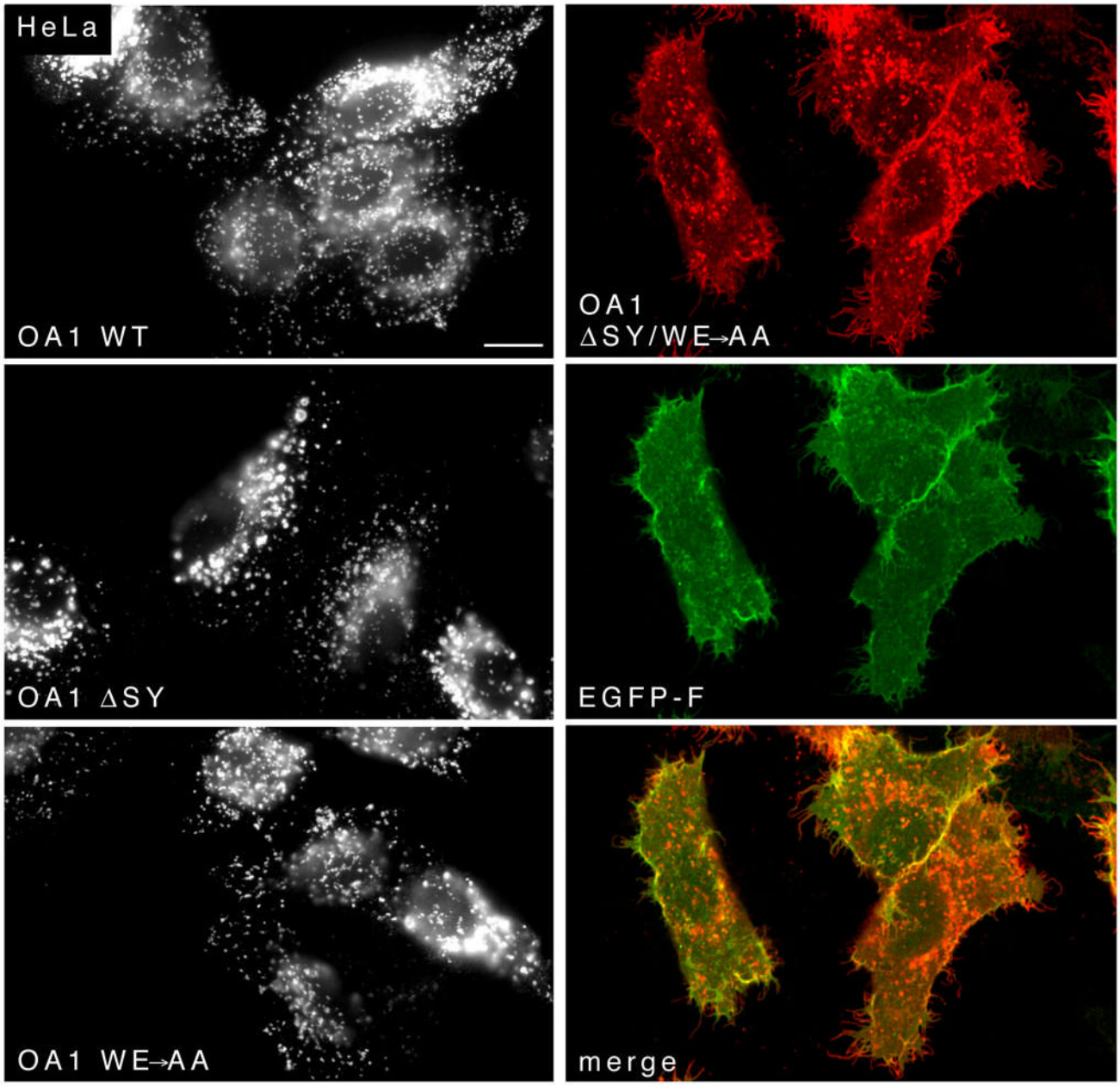


Fig. 5.

The dileucine-like and "WE" motifs are sufficient for lysosomal/melanosomal targeting in MNT1 cells. (A) Quantitative immunofluorescence analysis of the subcellular distribution displayed by LAMP/OA1 chimeras in transiently transfected MNT1 cells. Following immunodecoration with anti-rat LAMP1 antibodies, transfected cells were counted and classified into three categories, based on the vesicular (VE), plasma membrane (PM) or mixed (MX) distribution of the recombinant proteins (see Materials and Methods for details). Results are expressed as the percent of cells showing a specific distribution pattern and represent the mean ± s.e.m. of 3-5 independent experiments. The total number of cells counted is indicated. (B) Quantitative immunofluorescence analysis of the colocalization between LAMP/OA1 chimeras and endogenous OA1 in transiently transfected MNT-1 cells. Cells were double-labeled with anti-rat LAMP1 and anti-OA1 antibodies. Results are expressed as the percent of double-positive vesicles out of all rat LAMP1-positive compartments per cell and represent the mean ± s.d. of the data pooled from 2-5 independent experiments. The number of vesicles and cells counted is indicated. No colocalization differences were noted between cells displaying vesicular or mixed distribution patterns of the recombinant proteins, thus all data concerning each chimera were pooled together.



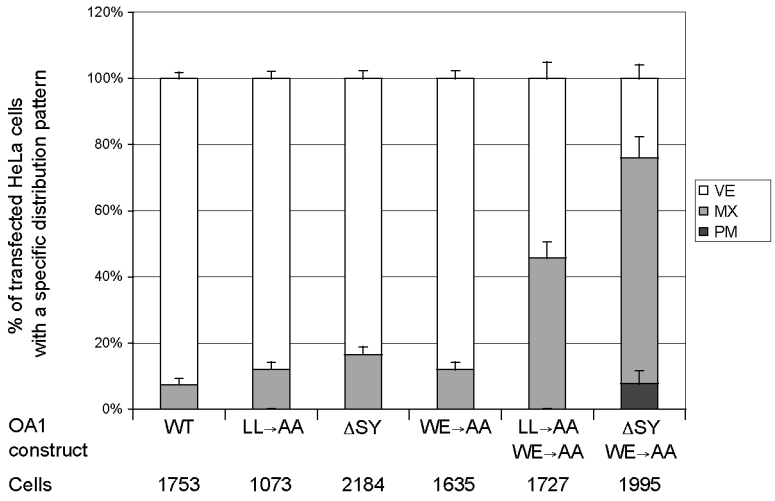
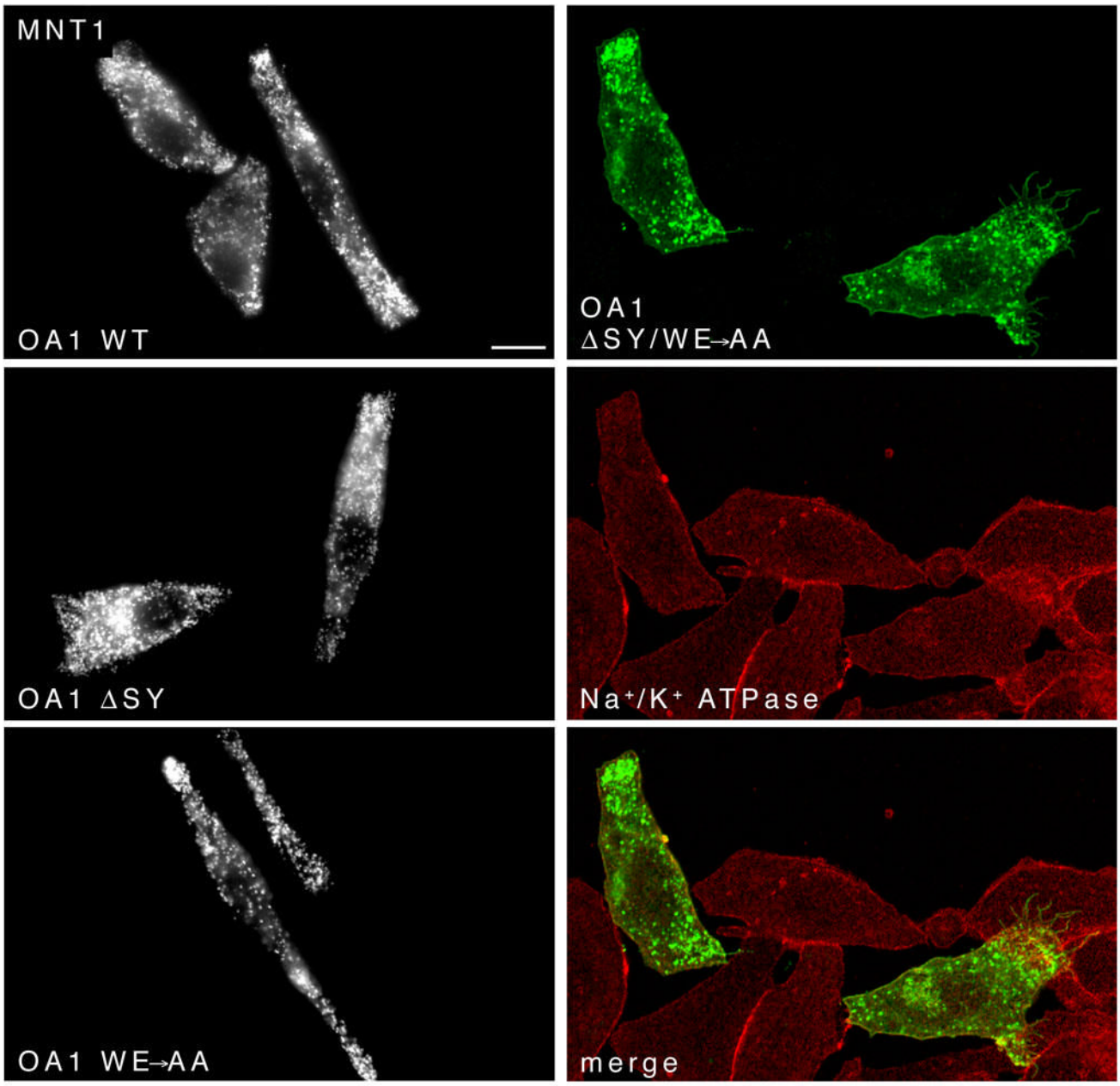


Fig. 6.

The dileucine-like and "WE" motifs are necessary for intracellular targeting of OA1 in HeLa cells. (A) Immunofluorescence analysis of HeLa cells transiently transfected with expression vectors for mycHis-tagged OA1 wild-type (OA1/WT), single mutant (OA1/ΔSY and OA1/WE→AA) or double mutant (OA1/ΔSY/WE→AA) and immunodecorated with anti-His_6 antibodies. OA1/ΔSY/WE→AA was colocalized by confocal microscopy with co-expressed farnesylated GFP (EGFP-F), which labels the plasma membrane. In the merge, areas of colocalization can be observed in yellow/orange. Bar, 10 μm. (B) Quantitative immunofluorescence analysis of the subcellular distribution displayed by full-length OA1 constructs in transiently transfected HeLa cells. Following immunodecoration with anti-His_6 antibodies, transfected cells were counted and classified as described in Fig. 5A. Results represent the mean ± s.e.m. of 5-10 independent experiments. The total number of cells counted is indicated.



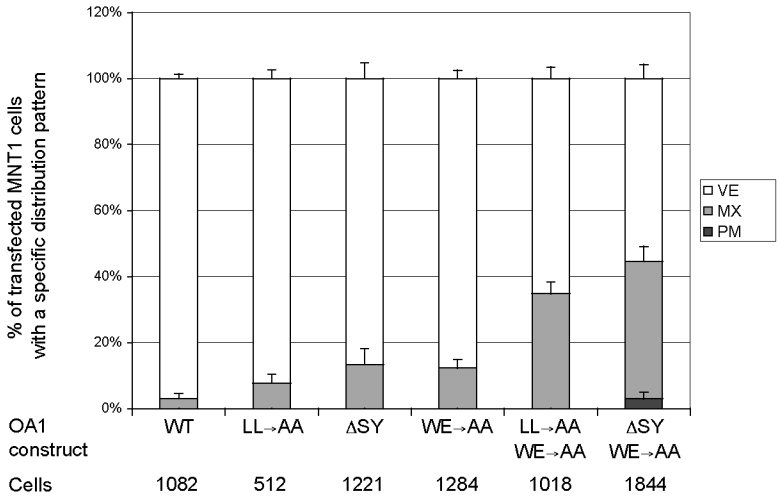
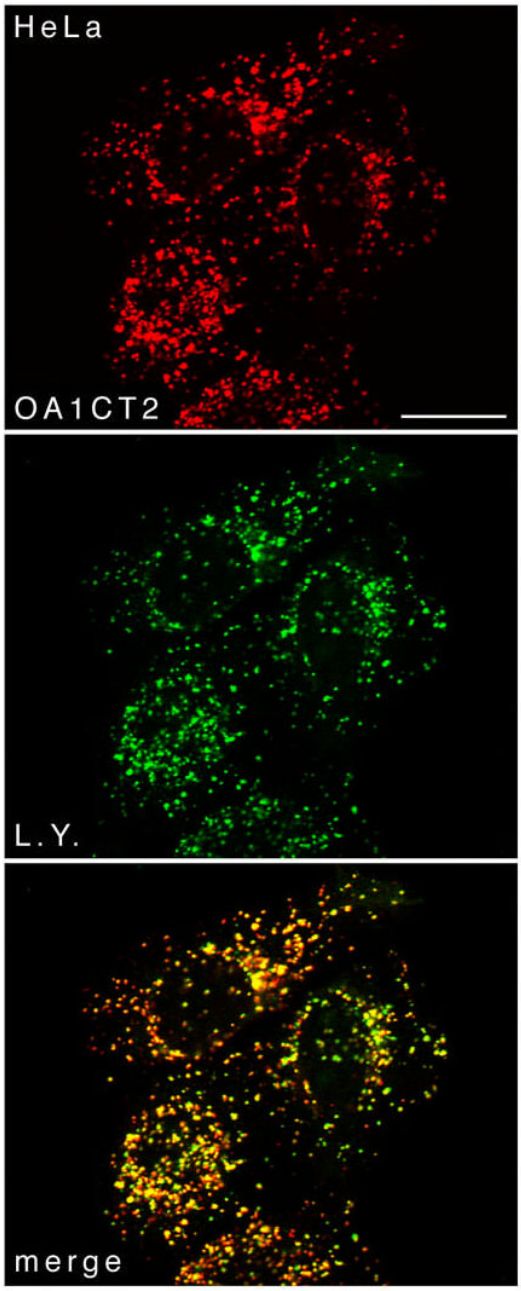


Fig. 7.

The dileucine-like and "WE" motifs are necessary for intracellular targeting of OA1 in MNT1 cells. (A) Immunofluorescence analysis of MNT1 cells transiently transfected with expression vectors for mycHis-tagged OA1 wild-type (OA1/WT), single mutant (OA1/ΔSY and OA1/WE→AA) or double mutant (OA1/ΔSY/WE→AA) and immunodecorated with anti-His_6 antibodies. OA1/ΔSY/WE→AA was colocalized by confocal microscopy with endogenous Na^+/K^+ ATPase, a marker of the plasma membrane. In the merge, areas of colocalization can be observed in yellow/orange. Bar, 10 μm. (B) Quantitative immunofluorescence analysis of the subcellular distribution displayed by full-length OA1 constructs in transiently transfected MNT1 cells. Following immunodecoration with anti-His_6 antibodies, transfected cells were counted and classified as described in Fig. 5A. Results represent the mean ± s.e.m. of 3-10 independent experiments. The total number of cells counted is indicated.



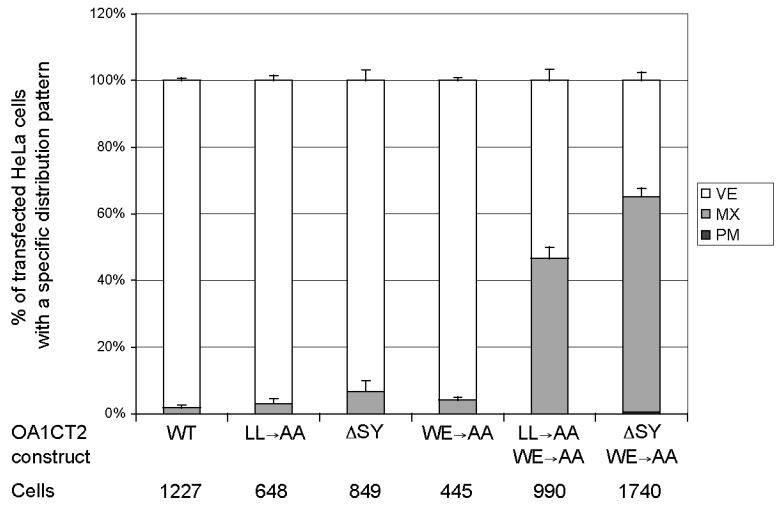


Fig. 8.

The distal portion of the C-tail is not necessary for lysosomal targeting of OA1 in HeLa cells. (A) Immunofluorescence analysis of HeLa cells transiently transfected with an expression vector for mycHis-tagged OA1CT2, which lacks the 65 C-terminal amino acids of CT. Exogenous OA1 was immunodecorated with anti-His_6 antibodies and lysosomes were labeled using the fluid phase marker Lucifer Yellow at 1 hour chase prior to fixation (L.Y.). In the merge, areas of colocalization can be observed in yellow/orange. Bar, 10 μm. (B) Quantitative immunofluorescence analysis of the subcellular distribution displayed by OA1CT2 constructs in transiently transfected HeLa cells. Following immunodecoration with anti-His_6 antibodies, transfected cells were counted and classified as described in Fig. 5A. Results represent the mean ± s.e.m. of 3-8 independent experiments. The total number of cells counted is indicated.

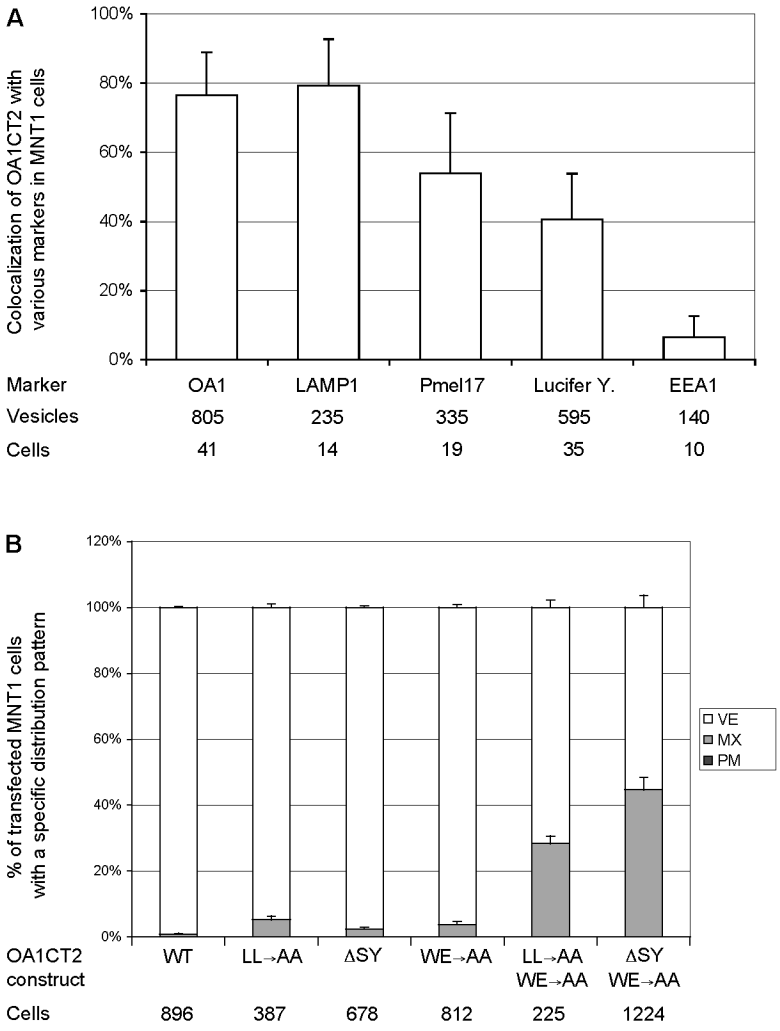


Fig. 9.

The distal portion of the C-tail is not necessary for lysosomal/melanosomal targeting of OA1 in MNT1 cells. (A) Quantitative immunofluorescence analysis of the colocalization between exogenous mycHis-tagged OA1CT2 and markers of various intracellular compartments in transiently transfected MNT-1 cells. Cells were double-labeled with anti-His_6 antibodies and with antibodies to OA1 (that recognize the endogenous protein, but not OA1CT2), LAMP1, Pmel17, EEA1, or with Lucifer Yellow at 1 hour chase. Results are expressed as the percent of double-positive vesicles out of all His_6-positive compartments per cell and represent the mean ± s.d. of the data pooled from 1-6 independent experiments. The number of vesicles and cells counted is indicated. (B) Quantitative immunofluorescence analysis of the subcellular distribution displayed by OA1CT2 constructs in transiently transfected MNT1 cells. Following immunodecoration with anti-His_6 antibodies, transfected cells were counted and classified as described in Fig. 5A. Results represent the mean ± s.e.m. of 3-10 independent experiments. The total number of cells counted is indicated.