Transport of newly synthesized lysosomal membrane proteins from the TGN (trans-Golgi network) to the lysosomes is due to the presence of specific signals in their cytoplasmic domains that are recognized by cytosolic adaptors. p40, a hypothetical transporter of 372 amino acids localized in the lysosomal membrane, contains four putative lysosomal sorting motifs in its sequence: three of the YXXφ-type (Y6QLF, Y106VAL, Y333NGL) and one of the [D/E]XXX[L/I]-type (EQERL360L361). To test the role of these motifs in the biosynthetic transport of p40, we replaced the most critical residues of these consensus sequences, the tyrosine residue or the leucine–leucine pair, by alanine or alanine–valine respectively. We analysed the subcellular localization of the mutated p40 proteins in transfected HeLa cells by confocal microscopy and by biochemical approaches (subcellular fractionation on self-forming Percoll density gradients and cell surface biotinylation). The results of the present study show that p40 is mistargeted to the plasma membrane when its dileucine motif is disrupted. No role of the tyrosine motifs could be put forward. Taken together, our results provide evidence that the sorting of p40 from the TGN to the lysosomes is directed by the dileucine EQERL360L361 motif situated in its C-terminal tail.

Key words: dileucine signal, lysosome, membrane, p40, trafficking.
mutagenesis. The critical tyrosine residues have been replaced by alanine residues, and the two leucine residues (360–361) have been replaced by an alanine and a valine residue. The impact of these mutations on the localization of p40 has been analysed by confocal fluorescence microscopy and biochemically, using subcellular fractionation on self-forming Percoll density gradients and cell-surface biotinylation. The results of the present study demonstrate that the dileucine motif situated near the C-terminal end of p40 is an essential determinant for the targeting of this protein to the lysosomal membrane.

MATERIALS AND METHODS

Mutation of the potential lysosomal targeting signals

p40–GFP (green fluorescent protein) and GFP–p40 proteins were engineered as described in Boonen et al. [32]. Mutations of putative sorting motifs were introduced in wild-type p40–GFP by overlap extension using PCR. The tyrosine residues of the YQLF, Y106VAL and Y333NGL motifs were replaced by alanine residues, and the two leucine residues in the C-terminal EQERL_Leu motif were substituted by an alanine and a valine residue respectively (LL360A_V). The primers (sense and antisense) bearing the mutations (indicated in italics) of these mutations on the localization of p40 has been analysed by alanine residues, and the two leucine residues (360–361) have been replaced by alanine and a valine residue respectively (LL360A_V). The primers (sense and antisense) bearing the mutations (indicated in italics) were the following: Y6A-F, 5′-CTTGCGGCTTGTTCTTGCCG-3′; Y6A-R, 5′-CAATCCGGCTTGTTCTTGCCG-3′; Y106A-F, 5′-CATGCGGCTTGTTCTTGCCG-3′; Y106A-R, 5′-CCACCGCCATGCTGCTGCTGCCAGTCTC-3′; Y333A-F, 5′-GCCTCGCAATGAGGGGCGGTGCACGCCAGTCTC-3′; Y333A-R, 5′-CCCATTGCGGACGGCGCGTTGCCCATAGA-3′; LL360A-V-F, 5′-AGAGAGAGGGGTAGGTGACAGTCCG-3′; LL360A-V-R, 5′-CTGTACACATTGCCGTTCCTTGTCTCCCTCCCTCC-3′. Final mutated PCR products were cloned into pCMV-Sport6_p40–GFP by EcoRI/BamHI.

To introduce the dileucine mutation into wild-type p40 (without GFP), a linker containing an EcoRI restriction site and a stop codon (5′-GATGCCGGAGAATTCTCAGG-3′; EcoRI restriction site is underlined, the stop codon is in italics) was generated and inserted at the BamHI restriction site situated between p40 and the GFP in p40-LL360AV–GFP. The p40 fragment, bearing the dileucine mutation, was then excised by EcoRI and cloned in place of wild-type p40 in pCMV-Sport6_p40 to obtain pCMV-Sport6_p40-LL360AV. This construct was used to generate GFP–p40 bearing the dileucine substitution (LL360AV). The p40-LL360AV fragment was excised by BstXI and MluI and inserted into GFP–p40 via the same restriction enzymes.

A HA (haemagglutinin) tag was introduced at the C-terminal end of p40, by excision of the GFP moiety of p40–GFP and its replacement by a linker containing the HA tag, a stop codon and BamHI/NotI restriction sites. The linker was formed by the hybridization of the two following primers: 5′-GATCCGTACCAGCTACGACTGTTA-3′ and 5′-CTAGATCAAGGGTAGGAGCGTCGCGGCAGTACG-3′ (HA tag is underlined, the stop codon is in italics). The fusion of the HA tag at the C-terminal end of the dileucine mutant of p40 was performed by replacing the wild-type p40 portion of p40–HA by the same dileucine-mutated portion excised from p40-LL360AV–GFP. This was performed by enzymatic restriction with EcoRI and BamHI.

Cell culture and transient transfection

HeLa cells were cultured at 37 °C in a humidified atmosphere with 5% CO2 in glucose-rich DMEM (Dulbecco’s modified Eagle’s medium; LONZA) containing 10% fetal calf serum (Greiner Bio-one), 100 units/ml penicillin and 100 μg/ml streptomycin. Transient transfections of plasmids were performed with the FuGENETM transfection reagent (Roche Diagnostics) according to the manufacturer’s instructions.

Fluorescence labelling

At 48 h post-transfection with GFP constructs, HeLa cells were washed five times with PBS supplemented with 0.7 mM CaCl2 and 0.25 mM MgSO4 (PBS+-, pH 8). Cell-surface proteins were labelled by incubation with 1 mg/ml sulfo-NHS-SS-biotin [sulfo-succinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate] in PBS+- (Pierce) for 45 min on ice. Biotinylation was stopped by washing five times with ice-cold 0.05 mM glycine/PBS+- and cells were lysed with RIPA buffer [50 mM Tris/HCl (pH 7.4), 120 mM NaCl, 1% (v/v) Triton X-100, 0.1% SDS and 1% deoxycholate] containing protease inhibitors (Complete Mini Protease inhibitor cocktail tablets, Roche). Biotinylated proteins were precipitated with streptavidin–agarose beads (Pierce) by centrifugation at 4°C for 5 min at 5000 g in a bench top centrifuge and then eluted by

Inhibition of AP-2-dependent endocytosis

For the study on the intracellular trafficking of p40, HeLa cells were transiently co-transfected with either the wild-type or the dileucine-mutated p40 tagged with the HA epitope along with a dominant-negative construct of Eps15 (GFP–Eps15/295) (provided by Dr A. Benmerah, Department of Infectious Diseases, Institut Cochin, Paris, France). At 24 h post-transfection, cells were washed, fixed with 4% formaldehyde and mounted in Mowiol (Calbiochem).

Cell-surface biotinylation

Transient HeLa cells were washed twice with ice-cold PBS and five times with PBS supplemented with 0.7 mM CaCl2 and 0.25 mM MgSO4 (PBS+-, pH 8). Cell-surface proteins were labelled by incubation with 1 mg/ml sulfo-NHS-SS-biotin [sulfo-succinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate] in PBS+- (Pierce) for 45 min on ice. Biotinylation was stopped by washing five times with ice-cold 0.05 mM glycine/PBS+- and cells were lysed with RIPA buffer [50 mM Tris/HCl (pH 7.4), 120 mM NaCl, 1% (v/v) Triton X-100, 0.1% SDS and 1% deoxycholate] containing protease inhibitors (Complete Mini Protease inhibitor cocktail tablets, Roche). Biotinylated proteins were precipitated with streptavidin–agarose beads (Pierce) by centrifugation at 4°C for 5 min at 5000 g in a bench top centrifuge and then eluted by
incubation for 40 min at room temperature in Laemmli’s buffer containing 200 mM fresh DTT (dithiothreitol). One-tenth of the supernatant obtained after centrifugation, containing proteins unbound to streptavidin beads, and proteins eluted from the beads (bound), were separated on SDS/PAGE (12 % gel). Proteins were electrotransferred on to low fluorescence PVDF membranes (Amersham Hybond LFP, GE Healthcare) which were blocked for 10 min with 10 % (w/v) dried skimmed milk in 0.1 % Tween/ PBS. Detection of p40, p40–HA or GFP-tagged proteins was performed with a rabbit antiserum raised against the 15 C-terminal amino acids of mouse p40, with a rabbit polyclonal antibody anti-HA (Sigma–Aldrich) or with a rabbit polyclonal antibody directed against GFP (Santa Cruz Biotechnology) respectively. Membranes were then incubated for 1 h with an Alexa Fluor® 488-conjugated goat anti-rabbit antibody (Molecular Probes). Signal detection was performed using the Typhoon™ fluorescence scanning system (GE Healthcare) and intensities were quantified with the ImageQuant™ analysis software (GE Healthcare).

Self-forming Percoll density gradient

Confluent cells grown in three 100-mm Petri dishes were rinsed three times with PBS, then twice with ice-cold 0.25 M sucrose. Cells were scraped in 1 ml of ice-cold 0.25 M sucrose and homogenized by ten passages through a 25-gauge needle mounted on a 5 ml syringe. Cell homogenate was subjected to a centrifugation at 1000 g for 10 min at 4 °C in an International Centrifuge PR-J (International Equipment). The pellet was suspended in 2 ml of ice-cold 0.25 M sucrose, disrupted by ten passages through a 25-gauge needle and submitted to another centrifugation at 1000 g for 10 min at 4 °C. This second pellet corresponded to the nuclear fraction (N). The 4–5 ml post-nuclear supernatant was then centrifuged at 35 000 rev./min for 40 min in a Beckman 50Ti rotor (4 °C) in order to sediment the membrane compartments (MLP fraction). The MLP pellet was resuspended in 1 ml of ice-cold sucrose and mixed with 8 ml of an 18 % Percoll solution (18 % (v/v) Percoll, 0.25 M sucrose, 2 mM EDTA and 10 mM Tris/HCl (pH 7.4)). The solution (9 ml) was then layered on to 1 ml of a 2.2 M sucrose cushion and centrifuged for 1 h at 17 000 rev./min in a Beckman 50Ti rotor at 4 °C. Seven fractions were collected from the top to the bottom of the gradient. The β-galactosidase and alkaline phosphodiesterase enzyme activities were assayed in each fraction as described previously [33,34]. The distributions of the p40/p40–GFP and LAMP-1 proteins were assayed in each fraction as described previously [33,34]. Tyrosine residues at positions 6, 106 or 333 were replaced by an alanine and a valine respectively (mutation LL360A V) (Figure 1).

RESULTS

Mutation of the putative lysosomal sorting motifs of p40: effect on its localization in transfected cells

p40 contains four putative lysosomal sorting motifs: three of the tyrosine-type (Y(106)QFL, Y(333)VAL and Y(106)NGL) and one of the dileucine-type (EQQRL360L361) (Figure 1). Based on computational topology prediction models, it seems unlikely that the tyrosine motif at position 106 acts as a sorting signal since it appears to be localized in one of the 8–10 predicted transmembrane regions. Nevertheless, we analysed the role of each motif in the biosynthetic transport of p40 to lysosomes. We first engineered a GFP chimaeric protein in which the GFP was fused C-terminally to the mouse p40 protein (p40–GFP). Then, we mutated each putative signal separately in the p40–GFP protein. We replaced the critical tyrosine residues by alanine (mutations Y6A, Y106A or Y333A) and the two leucine residues of the EQERL360L361 sequence by an alanine and a valine respectively (mutation LL360AV) (Figure 1).

Figure 1 Mutation of the putative sorting motifs of p40

p40 is a protein of 372 amino acids containing four potential lysosomal membrane targeting signals. Three are of the YXXφ-type (Y(106)QFL, Y(333)VAL and Y(106)NGL) and one is of the dileucine-type (EQQRL360L361). Mutational analysis of these motifs was performed as described in the Materials and methods section. Tyrosine residues at positions 6, 106 or 333 were replaced by an alanine residue. The two leucine residues situated 11 and 12 residues upstream from the C-terminal end (360–361) were replaced by an alamine and a valine residue respectively.

The wild-type and mutated p40–GFP proteins were transfected into HeLa cells and their subcellular localization was observed 48 h post-transfection by confocal fluorescence microscopy. The late endosomal/lysosomal compartments were localized by immunofluorescence, using an antibody directed against LAMP-1 (Figure 2A). As previously shown [32], wild-type p40–GFP transfected in HeLa cells displayed a punctuated labelling throughout the cytoplasm, with a predominance in the perinuclear region, and extensively co-localized with LAMP-1 (Figure 2A). No signal was detected at the plasma membrane. A similar distribution was observed for p40 (without the GFP tag), and for p40 proteins with a GFP tag fused N-terminally (GFP–p40) or a HA tag fused C-terminally (p40–HA) (Figure 2A). These results suggest that the addition of a GFP or a HA tag does not interfere with the lysosomal localization of the protein.

The p40–GFP proteins mutated at one tyrosine motif (Y6A, Y106A or Y333A) exhibited the same distribution as that of wild-type p40–GFP, and thus extensively co-localized with LAMP-1 (Figure 2B). In contrast, substitution of the two leucine residues of the C-terminal EQERL360L361 motif induced the targeting of p40–GFP proteins to the plasma membrane (Figure 3A). Similar results were obtained after the introduction of the dileucine mutation in wild-type p40, in GFP–p40 or in p40–HA chimaeric proteins (Figure 3A). The partial diversion of the p40 dileucine mutant to the cell surface was also observed after transfection in BHK21 cells (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/414/bj4140431add.htm). Nevertheless, irrespective of the construct, a significant portion of the LL360AV mutated proteins still generated an intracellular signal and co-localized with LAMP-1 both in HeLa cells and BHK21 cells (Figure 3A and Supplementary Figure S1).

Cells transfected with p40–GFP or its dileucine-mutated form were incubated with Texas Red-dextran (Figure 3B). This molecule enters the cells by fluid-phase endocytosis and is transported through the early and late endosomes to the lysosomes where it accumulates owing to its resistance to hydrolysis. Incubation of the transfected cells with Texas Red-dextran for 6 h followed by a 2 h-chase in dextran-free medium to allow its accumulation in late endocytic compartments, revealed an extensive co-localization between the fluorescent probe and wild-type p40–GFP (Figure 3B). The intracellular signal observed for the dileucine mutant of p40–GFP also extensively co-localized with the...
fluorescent probe (Figure 3B), confirming its partial localization in a compartment of the endocytic pathway.

The observation of a dual intracellular/plasma membrane localization for the dileucine mutant raises the possibility of another functional sorting determinant which could mediate the targeting of p40 to lysosomes in the absence of the EQERL<sup>360</sup>L<sup>361</sup> motif. In order to test the putative contribution of the tyrosine motifs to the sorting of p40 when the dileucine signal is disrupted, these motifs were mutated in the LL360AV-mutated p40–GFP construct. All p40–GFP proteins bearing the LL360AV substitution, in addition to the mutation of one tyrosine motif (double mutants) or the three tyrosine motifs together (quadruple mutant), were partly associated with the plasma membrane (Figure 4), suggesting that the tyrosine mutations had no additional effect on the mistargeting of p40 proteins to the plasma membrane, compared with the single LL360AV mutation.

**Distribution of wild-type and mutated p40 proteins (LL360AV) on Percoll density gradients**

We tested the delivery of wild-type p40 and p40–GFP, with and without the LL360AV substitution, to dense lysosomes as identified by centrifugation on a self-forming Percoll density gradient. MLP fractions (the high-speed pellets of post-nuclear supernatants) were submitted to centrifugation in iso-osmotic self-forming Percoll density gradients. Measurements of β-galactosidase and alkaline phosphodiesterase activities were performed in the collected fractions in order to localize the lysosomes and the plasma membrane respectively. As expected, the majority of the β-galactosidase activity was recovered in the highest density fractions, whereas the plasma membrane marker was mainly associated with more buoyant fractions (Figure 5). Detection of p40 and p40–GFP proteins by Western blot analysis revealed their presence in all fractions, with a predominance in the light density fractions (~1.052–1.057 g/ml) and in the highest density fraction (~1.16 g/ml). This pattern was similar to the bimodal distribution profile of the late endosomal/lysosomal marker LAMP-1. By contrast, the dileucine mutants (LL360AV) with and without GFP, were not detected in the dense lysosome-containing fractions. This shift in p40 distribution observed on Percoll density gradients after substitutions of the two leucine residues at positions 360–361, confirms the crucial role of the dileucine motif in the targeting of p40 to lysosomes.

**Plasma-membrane association of the wild-type and mutated p40 proteins**

The relative abundance of the wild-type and mutated proteins at the plasma membrane was estimated by cell-surface
biotinylation. Transfected cells were incubated with sulfo-NHS-SS-biotin for 45 min on ice, lysed and incubated with streptavidin–agarose beads in order to separate the biotinylated (bound) from the non-biotinylated (unbound) proteins. The bound proteins, eluted from the beads, and one-tenth of the unbound proteins were resolved by SDS/PAGE. The proportion of each p40 construct recovered in the bound and unbound samples was estimated by Western blot analysis using the fluorescence staining of p40 proteins mutated in one of the tyrosine motifs (Y6A, Y106A or Y333A) were very poorly biotinylated (%), supporting their presence not only in an intracellular compartment but also at the cell surface. We asked whether p40 molecules passed through the plasma membrane on their way to the endo/lysosomal compartments, as described for other lysosomal membrane proteins, including the LAMPS [35]. To answer this question, we overexpressed p40 in HeLa cells together with a dominant-negative form of Eps15, which is known to inhibit AP-2-dependent endocytosis by sequestering AP-2 in the cytosol [36]. First, we checked the ability of this EH (Eps homology domains)-deleted form of Eps15 tagged with GFP to block AP-2-dependent endocytosis by measuring the uptake of an Alexa Fluor® 568-conjugated transferrin, a well-known marker of clathrin-mediated endocytosis [36,37]. We observed that the uptake of transferrin was effectively inhibited although to very different extents in individual cells. The most prominent inhibition of transferrin uptake was observed in cells expressing a high level of GFP–Eps15 EΔ95/295 (Figure 7A). Then, we analysed the distribution of wild-type p40 in co-transfected cells expressing high levels of GFP–Eps15 EΔ95/295. As shown in Figure 7, the immunofluorescent staining of p40 remained in the perinuclear area similar to cells expressing p40 alone (Figure 2A), and never appeared at the cell surface (Figure 7B). This result supports the idea that wild-type p40 proteins gain access to the endo/lysosomal compartment without passing through the plasma membrane. The same experiment was carried out with the dileucine-mutated p40 proteins and showed that the staining was present not only intracellularly but also at the cell surface (Figure 7C), as described above for the cells not transfected with the dominant-negative form of Eps15.

**DISCUSSION**

The present study on the importance of the four putative lysosomal sorting motifs of p40 (Y6QLF, Y106VAL, Y333NGL, EQERL360L361) in its biosynthetic transport to the lysosomes has revealed a key role of the C-terminal EQERL360L361 motif. Indeed, replacement of the leucine pair by other amino acids (LL360AV) induced a mistargeting of the protein to the plasma membrane, as judged by confocal fluorescence microscopy and by cell-surface intracellular localization. The mutation of the dileucine signal (LL360AV) in p40–GFP induced the appearance of 21 ± 3% (n = 6) of the molecules in the biotinylated fraction attesting of their exposure to the extracellular medium. Mutation of the tyrosine motifs at positions 6, 106 or 333, together with the dileucine signal (double and quadruple mutants) did not increase the percentage of proteins associated with the bound fraction, providing further evidence that the tyrosine motif mutations have little or no additional impact on the mistargeting of the dileucine mutant to the plasma membrane.

Wild-type p40, the C-terminally HA- and N-terminally GFP-tagged p40 constructs yielded very low percentages of proteins in their biotinylated form (7 ± 2%, 2 ± 0% and 3 ± 1% respectively, with n ≥ 3) (Figure 6B). When the dileucine mutation was introduced in p40–HA and GFP–p40, 24 ± 4% (n = 3) and 22 ± 7% (n = 3) of p40 proteins were detected among the biotinylated proteins respectively (Figure 6B), which was not different from the values obtained with the mutated p40–GFP protein (21 ± 3%; n = 6) (Figure 6A). By contrast, in the absence of any tag, 49 ± 11% (n = 7) of dileucine mutants were biotinylated (Figure 6B), showing that the presence of a GFP or a HA tag has an effect on the association of the mutated protein with the cell surface.

**Intracellular trafficking of the wild-type and mutated p40 proteins**

In the steady-state, only a low level of wild-type p40 appears at the cell surface. We asked whether p40 molecules passed through the plasma membrane on their way to the endo/lysosomal compartments, as described for other lysosomal membrane proteins, including the LAMPS [35]. To answer this question, we overexpressed p40 in HeLa cells together with a dominant-negative form of Eps15, which is known to inhibit AP-2-dependent endocytosis by sequestering AP-2 in the cytosol [36]. First, we checked the ability of this EH (Eps homology domains)-deleted form of Eps15 tagged with GFP to block AP-2-dependent endocytosis by measuring the uptake of an Alexa Fluor® 568-conjugated transferrin, a well-known marker of clathrin-mediated endocytosis [36,37]. We observed that the uptake of transferrin was effectively inhibited although to very different extents in individual cells. The most prominent inhibition of transferrin uptake was observed in cells expressing a high level of GFP–Eps15 EΔ95/295 (Figure 7A). Then, we analysed the distribution of wild-type p40 in co-transfected cells expressing high levels of GFP–Eps15 EΔ95/295. As shown in Figure 7, the immunofluorescent staining of p40 remained in the perinuclear area similar to cells expressing p40 alone (Figure 2A), and never appeared at the cell surface (Figure 7B). This result supports the idea that wild-type p40 proteins gain access to the endo/lysosomal compartment without passing through the plasma membrane. The same experiment was carried out with the dileucine-mutated p40 proteins and showed that the staining was present not only intracellularly but also at the cell surface (Figure 7C), as described above for the cells not transfected with the dominant-negative form of Eps15.
At 48 h post-transfection with p40–GFP proteins harbouring at least the dileucine substitution, combined to the mutation of one (double mutants: Y6A, Y106A or Y333A+LL360AV), or of the three tyrosine motifs (quadruple mutant: Y6A-Y106A-Y333A-LL360AV), cells were fixed and LAMP-1 was detected as described in Figure 2.

biotinylation. Using this method, it appeared that, in steady-state, up to 49% of the untagged dileucine mutants were detected at the cell surface. Considering that a significant portion of dileucine-mutated p40 proteins still co-localized with LAMP-1 and with the fluid-phase endocytosis tracer Texas Red-dextran after a 2 h-chase period, we searched for another functional sorting signal in the p40 sequence which could act in combination with the dileucine motif at the same or another step in the p40 transport towards lysosomes. With this in mind, we mutated the other putative targeting motifs found in p40 (Y6QLF, Y106VAL, Y333NGL) together with the dileucine signal mutation. No additional impact on the mistargeting of p40 to the plasma membrane was observed when compared with the single dileucine mutant, suggesting that the tyrosine motifs would not be implicated in p40 targeting to lysosomes. In the case of LAMP-1, it was shown that 95% of newly synthesized LAMP-1 proteins mutated in their tyrosine-based sorting signal were expressed at the cell surface within 30 min after pulse labelling, suggesting that the tyrosine motif was the only lysosomal-sorting signal, despite the presence of only 39% of tyrosine-mutated proteins at the cell surface in steady-state [38].

For practical reasons, probably linked to the hydrophobic nature of p40, we failed to determine the percentage of newly synthesized p40 expressed at the cell surface. However, our results clearly demonstrate that, unlike the tyrosine motifs, the EQERL<sup>360</sup>L<sup>361</sup> motif plays a crucial role in the lysosomal sorting of p40. In the future, it should be worthwhile to evaluate whether some of the numerous transmembrane domains of p40 contribute or not to its targeting and delivery to lysosomes, as has been described for several integral membrane proteins [39,40]. The lower percentage of GFP- and HA-tagged mutated p40 proteins at the plasma membrane (21–24%) could be the consequence of a less efficient biosynthetic transport. Alternatively, the possible misfolding of the dileucine mutants fused to HA or GFP tags could trigger their ubiquitinylation and their sorting from the TGN or from the plasma membrane to the endosomal compartments [41,42]. This process could result in a lower cell surface to intracellular ratio when compared with untagged proteins.

Membrane proteins can be targeted to the endolysosomes either directly from the TGN or indirectly, passing first by the plasma membrane before being directed toward lysosomes along the endocytic pathway. The biosynthetic transport of LAP provides an example of indirect traffic. Indeed, its precursor cycles several times between early endosomes and the plasma membrane before reaching lysosomes [43]. Trafficking through a direct pathway has been described for lysosomal membrane proteins such as LIMP-II, GLUT (glucose transporter)-8, mucolipin 1 and LAMP-1 [20,38,44–48]. Nevertheless, it has previously been suggested that a significant portion of LAMP-1 and GLUT-8 is also transported by an indirect pathway, since the knockdown of the plasma membrane adaptor AP-2 in HeLa cells by siRNA (small interfering RNA) has led to a substantial accumulation of these proteins at the cell surface [10,35]. In contrast, the sorting of mucolipin 1 and LIMP-II to lysosomes has been attributed to AP-1 or AP-1 and AP-3 respectively, supporting the idea of their trafficking by an intracellular (direct) route [20,46,47]. The results of the present study lend support to a direct transport of wild-type p40 from the TGN to the lysosomes. Indeed, the co-expression of wild-type p40–HA and a dominant-negative form of Eps15, known to inhibit clathrin-mediated endocytosis by interacting with AP-2 [36], did not result in the appearance of a detectable
Figure 5 Analysis of the subcellular distribution of p40 and p40–GFP dileucine mutants by fractionation in self-forming Percoll density gradients

HeLa cells were transfected with p40, p40–GFP, p40 with the dileucine mutation (p40-LL360AV) or the dileucine mutant of p40–GFP (p40-LL360AV–GFP). Later (48 h), MLP fractions were prepared and fractionated on self-forming Percoll density gradients as described in the Materials and methods section. Seven fractions were collected and enzyme activities of β-galactosidase (●) and alkaline phosphodiesterase (◆) were measured to localize the lysosomes and the plasma membrane respectively. Values on the histogram represent the average percentage (means ± S.D.) of the enzyme activity in each of the seven fractions, calculated from all of the experiments performed (n = 12). The open circles (○) represent the density of Percoll. The distributions of the late endosomal/lysosomal marker LAMP-1 and of the p40 proteins were analysed by Western blotting. Blots correspond to one representative experiment.

amount of p40 proteins at the plasma membrane. Therefore it seems likely that newly synthesized wild-type p40 molecules reach the endosomal compartment primarily by the direct pathway following the interactions of the dileucine motif with either AP-1 or AP-3 adaptor complexes. The GEQERLL sequence from p40 can potentially be recognized by each of them by interactions with isolated subunits [9–13], but most likely with γ/σ1 or δ/σ3 hemicomplexes [14,15]. However, it does not fulfill the criteria described for optimal recognition by AP-3, i.e. the presence of a proline residue at position −1, a positively charged amino acid at position −3 and acidic residues at positions −4 and −5 from the first leucine residue [49]. Nevertheless, like p40, the yeast LAP which is known to interact with AP-3, contains an arginine residue at position −1 [1,49]. In addition, it seems that the proline residue of the ERAPLI motif from LIMP-II is dispensable for the interaction with the δ–σ3A, and δ–σ3B hemicomplexes [14]. Therefore both AP complexes could, in theory, recognize the dileucine motif of p40.

The observation of a dual localization of p40-LL360AV at the cell surface and intracellularly, where it co-localizes with LAMP-1, could give rise to several interpretations. Part of the intracellular signal could represent molecules transiting along with LAMP-1 in biosynthetic compartments. Nevertheless, the extensive co-localization of the dileucine mutant with endocytosed Texas Red-dextran after a 2 h-chase seems rather to indicate that the bulk of the mutated proteins present inside the cells resides in organelles of the endocytic pathway. The presence of p40-LL360AV in late endosomal compartments could be the consequence of its impaired binding to AP-1 and/or AP-3 adaptor complexes at the TGN, resulting in its diversion to the plasma membrane by default [1,20,46,49–51]. Once at the cell surface, due to the absence of a functional dileucine motif, p40-LL360AV would be slowly internalized by a clathrin-dependent or independent pathway [37,38,50]. On the other hand, taking into account the partial localization of the dileucine mutant in endo/lysosomal structures, and its exclusion from dense lysosomes, as shown on Percoll density gradients, we cannot rule out the possibility that the dileucine signal would be involved in trafficking of the protein from late endosomes to dense lysosomes. It is commonly admitted that transport between these compartments occurs by a default pathway, but the existence of a signal-mediated pathway has already been proposed to explain the accumulation of a mutant form of LAMP-1 in a prelysosomal compartment [52]. Finally, we cannot rule out the possibility that the p40 dileucine mutant was not detected in the densest fractions of the Percoll gradient because it is more prone to lysosomal
Figure 6  Relative abundance of the p40 constructs at the plasma membrane in HeLa cells

(A) At 48 h post-transfection with p40–GFP constructs, HeLa cells were incubated on ice with a biotinylation reagent (sulfo-NHS-S-S-biotin), as described in the Materials and methods section. Biotinylated (bound, B) proteins were separated from non-biotinylated ones (unbound, U) by absorption on streptavidin–agarose beads. Biotinylated proteins and one-tenth of the non-biotinylated fraction were resolved by SDS/PAGE and the p40 proteins were detected by ECL-Plex™ Western blotting. Signals were quantified with the ImageQuant™ software and the abundance of the biotinylated fraction relative to the total p40 proteins (bound + unbound signals) was calculated. The histogram represents means ± S.D., with n ≥ 3. EEA1 was used as a negative control. (B) HeLa cells transfected with the wild-type or the dileucine-mutated forms (LL360AV) of p40, p40–HA or GFP–p40 were subjected to the cell-surface biotinylation assay. Proteins were detected among the biotinylated and non-biotinylated fractions (one-tenth) by Western blotting. Values on the histograms represent the percentage of the biotinylated signal relative to the total amount of p40 proteins (means ± S.D., n ≥ 3).

degradation than the wild-type p40. Disruption of the different AP complexes by specific siRNA would be required to clarify the intracellular trafficking of p40-LL360AV.

As previously mentioned, the elevated number of predicted transmembrane domains of p40 and the similarity of part of its sequence to Golgi nucleotide-sugar transporters raise the issue of a putative transport activity of p40 in the lysosomal membrane [32]. Small-molecular-mass molecules produced by the intralysosomal hydrolysis are transferred from the lumen of the organelle to the cytosol by membrane transporters. To date, only a few transporters have been identified in lysosomes although transport activities have been described or suggested for a large number...
of molecules [53–56]. Among the most extensively characterized lysosomal transporters are those in charge of the translocation of cystine (cystinosin) and of sialic acid (sialin), whose deficiency leads to lysosomal storage disorders [57–59]. The discovery of the sorting determinants of these membrane proteins has been of particular interest in the study of their transport activities. Indeed, expression of sialin and cystinosin at the plasma membrane, induced by the mutations of their sorting signals, has permitted the design of efficient transport assays [5,60]. The results of the present study, demonstrating the role of the p40EQERL 1 sequence in its biosynthetic routing, could therefore represent a valuable step toward the implementation of similar methods in an attempt to unravel the function of this protein.

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SUPPLEMENTARY ONLINE DATA
A dileucine signal situated in the C-terminal tail of the lysosomal membrane protein p40 is responsible for its targeting to lysosomes

Marielle BOONEN, Roberta REZENDE de CASTRO, Gaëlle CUVELIER, Isabelle HAMER and Michel JADOT

URPhiM, Laboratoire de Chimie Physiologique, FUNDP, B-5000 Namur, Belgium

Figure S1 Subcellular localization of wild-type p40 and its dileucine-mutated form (LL360AV) in BHK-21 cells

At 48 h after transfection with the wild-type or the dileucine-mutated forms of p40 and p40–GFP, BHK-21 cells were fixed and permeabilized. Cells were incubated with a mouse anti-hamster LAMP-1 monoclonal antibody (4A1), provided by Dr J. Gruenberg (Department of Biochemistry, University of Geneva, Geneva, Switzerland), followed by an Alexa Fluor® 588-conjugated goat anti-mouse secondary antibody (red). p40 was detected with a specific antibody directed against the last 15 amino acids of the protein followed by an Alexa Fluor® 488-conjugated anti-rabbit antibody (green), and p40–GFP was visualized directly with GFP autofluorescence. Fluorescent signals were observed by confocal fluorescence microscopy.

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1 To whom correspondence should be addressed (email michel.jadot@fundp.ac.be).