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Interactions of y^+LAT1 and 4F2hc in the y^+L amino acid transporter complex: consequences of lysinuric protein intolerance-causing mutations

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Abstract. Lysinuric protein intolerance (LPI) is an inherited aminoaciduria caused by recessive mutations in the *SLC7A7* gene encoding y^+L amino acid transporter 1 (y^+LAT1), which combines with 4F2hc to generate an active transporter responsible for the system y^+L amino acid transport. We have previously shown that the y^+LAT1 proteins with point mutations are expressed in the plasma membrane, while those with frame shift mutations are retained in the cytoplasm. This finding has prompted us to study whether the difference in localization is due to the inability of the structurally altered mutant y^+LAT1 proteins to heteromerize with 4F2hc. For this purpose, we utilized FACS technique to reveal fluorescence resonance energy transfer (FRET) in cells expressing wild type or LPI-mutant CFP-tagged y^+LAT1 and YFP-tagged 4F2hc. The heteromerization of y^+LAT1 and 4F2hc within the cell is not disrupted by any of the tested LPI mutations. In addition, the expression rate of the LPI mutant y^+LAT1 proteins was significantly lower and cellular mortality was markedly increased than that of the wild type y^+LAT1 in transfected samples. Our results indicate that the FACS-FRET method provides an alternative approach for screening of potential protein associations.

Key words: Fluorescence activated cell sorting — Fluorescence resonance energy transfer — Heteromerization — Lysinuric protein intolerance — y^+LAT1

Introduction

Lysinuric protein intolerance (LPI; OMIM #222700) is a rare recessively inherited disorder in which the transport of cationic amino acids lysine, arginine and ornithine through the epithelium of small intestine and proximal kidney tubules is defective. This results in a variety of clinical symptoms including nausea, vomiting and diarrhoea following protein-rich meals, leading to protein aversion at an early age. Children with LPI thrive poorly after weaning, show growth failure, osteoporosis and hepatosplenomegaly and may suffer from immunological abnormalities and pulmonary alveolar proteinosis. Excessive protein intake may lead to a hyperammonemic crisis due to a urea cycle failure caused by the lack of arginine and ornithine (Simell 2001). The incidence of LPI is exceptionally high in Finland (1:76 000) (Sperandeo et al. 2008), but the local incidence is also high e.g. in some restricted areas of Italy and northern Japan (Koizumi et al. 2000; Sperandeo et al. 2000). Sporadic cases are found worldwide.

The transport defect in LPI is caused by mutations in the *SLC7A7* gene, which encodes transporter protein y^+LAT1 (y^+L amino acid transporter 1). It forms a heteromeric protein complex together with the heavy chain of the cell surface antigen 4F2 (4F2hc; CD98) and contributes to the basolateral efflux of cationic amino acids and influx of neutral amino acids together with Na^+(Borsani et al. 1999; Torrents et al.

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1998; 1999). The Finnish LPI patients all harbour the same founder mutation, LPI_{Fin}, but a spectrum of mutations has been reported in patients from other countries (Borsani et al. 1999; Sperandeo et al. 2008; Torrents et al. 1999). We have previously observed that various mutant proteins, despite of their varying subcellular location, have an equally reduced y^+L transport activity (Mykkänen et al. 2000; Toivonen et al. 2002).

y^+LAT1 belongs to the family of catalytic subunits or light chains of heterodimeric amino acid transporters (LSHATs), which comprise altogether seven ~40 kDa polypeptides, out of which six (LAT1, LAT2, y^+LAT1, y^+LAT2, xCT, asc1) heteromerize with 4F2hc to form a functioning transporter complex (Yan et al. 2008). 4F2hc is also involved in several other cellular functions, in addition to its role as a chaperone, which brings the transporter to the plasma membrane (Nakamura et al. 1999; Fernandez et al. 2006). 4F2hc contributes to for example cell proliferation (Haynes et al. 1981; Papetti and Herman 2001), malignant transformation of cells (Hara et al. 1999; Shishido et al. 2000; Henderson et al. 2004), cell fusion (Ohgimoto et al. 1995), as well as β-integrin interaction (Fenczik et al. 2001; Kolesnikova et al. 2001), which promotes integrin-dependent cell signalling (Feral et al. 2005) and cellular adhesion functions (Ohta et al. 1994).

Previously, we have successfully used different microscopy techniques in studying the localization and interaction of wild type or mutated y^+LAT1 and 4F2hc (Toivonen et al. 2002; Kleemola et al. 2007). Since the y^+LAT1 proteins carrying LPI-causing amino acid substitutions are localized at the plasma membrane whereas the frameshift mutant transporters are retained in the cytoplasm, we studied whether this difference was caused by the inability of the structurally altered transporter protein to interact with 4F2hc. To study the heteromerization between various LPI mutant y^+LAT1 and 4F2hc, we chose to apply flow cytometry (FACS) (Siegel et al. 2000; Chan et al. 2001) in detecting FRET from y^+LAT1-ECFP to 4F2hc-EYFP in transfected mammalian cells. Moreover, since 4F2hc has an essential role in the cell growth and proliferation (Haynes et al. 1981; Edinger 2007), the mutated y^+LAT1 could potentially affect the cell viability *via* the interaction of transporter subunits. Therefore, we also studied and quantified the effects y^+LAT1-EGFP carrying LPI mutations on cell proliferation by the means of FACS.

Materials and Methods

The construction of the fusion protein plasmids

The y^+LAT1 and 4F2hc open reading frames (ORFs) were cloned in frame into the FRET donor (cyan fluorescent protein, CFP) and acceptor (yellow fluorescent protein, YFP) expression vectors, respectively, to generate plasmid combinations for transfection (Table 1). The expression vector backbones pECFP-C1 (enhanced CFP) and pEYFP-C1 (enhanced YFP) were purchased from Living Colors®, BD Biosciences Clontech, Palo Alto, CA, USA. To generate the ECFP-y^+LAT1 fusion protein expression plasmids, ORFs for the wild type and mutated y^+LAT1 sequences carrying LPI_{Fin} (Borsani et al. 1999; Torrents et al. 1999) [c.895-2A>T,p.T299IfsX10 (nomenclature in parentheses according to (den Dunnen and Antonarakis 2001))], 1548delC (c.1262delC, p.P421RfsX98), or G54V (c.161G>T[b],447G>T) mutation (Mykkänen et al. 2000), were amplified by PCR. All LPI mutations used in the study abolish the y^+LAT1 transporter activity completely (Mykkänen et al. 2000). Since the frameshift mutants, LPI_{Fin} and 1548delC, are translated as truncated or aberrantly prolonged polypeptides, respectively, all the y^+LAT1 mutant fusion proteins carried the fluorescent label at their N terminus, i.e. were cloned in frame into the pECFP-C1 plasmid. To amplify the open reading frame for the insertion in C1 plasmids, the forward primer corresponding to nucleotides 287-304 of y^+LAT1 cDNA (GenBank accession NM_003982) extended at its 5' end by adding a *Hind*III cleavage site [5' gcaagcttcc (atggttgacagcactgag) 3'] and the reverse primer corresponding to the end of the coding region (nucleotides 1799-1819 of cDNA) extended by a *Sal*I digestion site added at the 5' end of the primer [5' CGCGTCGAC(GTTAGACTTGGGA TCCCGTTG)3'] were used. The PCR products were ligated

Table 1. Fusion protein constructs and the transfected plasmid combinations used in the FACS-FRET experiments

CFP	YFP	FRET combination
pECFP-C1 vector	pEYFP-C1 vector	negative FRET control
CFP-YFP tandem[a]	—	forced FRET, positive control
ECFP-y^+LAT1[b]	EYFP-4F2hc[b]	FRET

[a] A linker peptide of nine amino acids separates CFP and YFP; the vector construct kindly provided by Anton Sandqvist, originally from Kim et al. (2002). [b] The y^+LAT1 constructs used in the control and FRET experiments expressed the wild type, $LPI_{Fin,}$ G54V or 1548delC cDNA sequences; 4F2hc sequence was wild type in all transfections.

to pGEM-T plasmids (Promega Corporation, Madison, WI) and digested with the appropriate restriction enzymes (New England Biolabs, Ipswich, MA) to isolate the insert. The gel purified inserts were introduced to the final vectors to create y+LAT1-ECFP plasmids encoding an N-terminally CFP tagged, wild type or mutant y+LAT1.

The 4F2hc-EYFP plasmid from our previous work was used (Kleemola et al. 2007). All the final plasmid constructs were verified by sequencing the insert. In addition, the fluorescent fusion protein expression in transfected cells was tested by fluorescence microscopy of transfected HEK293 cells. The y+LAT1-EGFP fusion expression vectors from our previous work (Toivonen et al. 2002) were utilized in the cell viability assays. The CFP-YFP tandem expression plasmid used as the positive FACS-FRET control is a kind gift from Anton Sandqvist, and is originally from (Kim et al. 2002).

Cell culture, transfection and flow cytometry FRET

The HEK293 cells (human embryonic kidney, ECACC #85120602) were maintained as described (Toivonen et al. 2002). To prepare the FACS-FRET samples, the cells were seeded on six-well plates 24 h prior to the transfection. The sub-confluent cells were then transfected with 2 μg of each fusion protein plasmid using the FuGENE6 transfection reagent in 1:3 DNA: transfection reagent ratio according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, Ind) except for the mock-transfection control, which was treated with FuGENE6 alone. The transfected fusion protein expression plasmids and their combinations for FRET controls and experiments are described in Table 1. The y+LAT1 constructs expressed the wild type, LPI_{Fin}, G54V or 1548delC ORF sequences tagged with ECFP at the amino terminus. 4F2hc was expressed as N-terminal EYFP fusions to ensure the intracellular localization of the FRET acceptor. We combined it with an N-terminally tagged y+LAT1-ECFP for the FACS-FRET assays, because this combination of the FRET fluorochromes gave the highest net donor fluorescence intensity change in the acceptor photobleaching FRET microscopy analysis in our previous study on wt y+LAT1 and 4F2hc heterodimerization (Kleemola et al. 2007). The cell cultures were incubated for 48h post-transfection, trypsinized, washed with PBS and suspended in 500 μl of EMEM with HEPES buffering. The cell samples were then immediately analyzed with an LSR II flow cytometer (BD Biosciences, San Jose, CA, USA).

The cell concentration during the analysis was ~2×10^6/ml. The cells were exposed to a 405 nm violet laser, followed by the immediate collection of the emitted CFP and YFP signals by detectors with 480/40BP filter and 585/42BP filter, respectively. 20,000 and 50,000 cells were analyzed from each control and test sample, respectively.

Since the 405 nm laser does not excite YFP, the background signal for the YFP detector is very weak. Thus, FRET was observed as an average increase of the acceptor YFP signal and the decrease of the donor CFP signal following exposure to the 405 nm laser light in the double-transfected cell populations compared to the single transfected, either y+LAT1-ECFP or 4F2hc-EYFP expressing samples.

The CFP fluorescence intensity decrease was visualized by CFP *vs.* YFP dot plots and the YFP fluorescence intensity by histograms from the YFP (blue laser) channel. The dot plots were utilized for both the detection of FRET and quantitation the CFP positive cells as described in section Results.

Quantification of GFP positive cells and cell mortality with FACS

Sub-confluent HEK293 cells were transfected using GFP-tagged wild type and mutant y+LAT1 and non-tagged 4F2hc expression plasmids (1 μg each) as described (Toivonen et al. 2002) and incubated for 48 hours post-transfection. The cells were harvested and the cell suspension samples for the FACS analysis were prepared as described in section Materials and methods. Propidium iodide (PI, 2 μg/ml) was added to the cell suspensions immediately prior to the FACS run on the FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA) to discriminate the dead cell population. The cells were analyzed by exposing them to the 488 nm blue laser and the emitted GFP and PI fluorescence was detected on 530/30 or 585/42 nm channels, respectively. The cell population was visualized on a GFP *vs.* PI fluorescence dot plot in which the cells fell into four groups according to their GFP expression (positive/negative) and PI intake (live/dead).

Statistical analysis

Poisson regression analysis was used to compare the cell viability assay and fusion protein expression rates from FACS runs between wild type and mutant y+LAT1 proteins. The results are expressed using relative risk (RL) with a 95% confidence interval (CI). The FRET population sizes were compared using t test. *p*-values lower than 0.05 were considered statistically significant. Statistical analysis was done with the SAS System for Windows, release 9.1 SP 4 (SAS Institute, Cary, NC).

Results

Heteromerization of the wild type and LPI mutant-expressing cells

Interaction between wt and LPI mutant y+LAT1-ECFP and 4F2hc-EYFP was detected using FACS-FRET. In FACS, FRET is visualized in the CFP *vs.* YFP dot plot by the distri-

bution of the CFP-fluorescent cell population which, if interaction occurs, shows a decrease in the mean fluorescence intensity while the YFP fluorescence emission increases when exposed to the 405 nm laser light. This quenching of the CFP emission resulting from FRET was detected at the CFP *vs.* YFP dot plots in wt and all LPI mutant y^+LAT1-ECFP/4F2hc-EYFP-expressing samples (LPI_{Fin}, G54V, 1548delC); in each of them, the y^+LAT1-ECFP-positive cell population shifted towards the left compared to the negative controls (Figure 1). In the CFP *vs.* YFP dot plots,

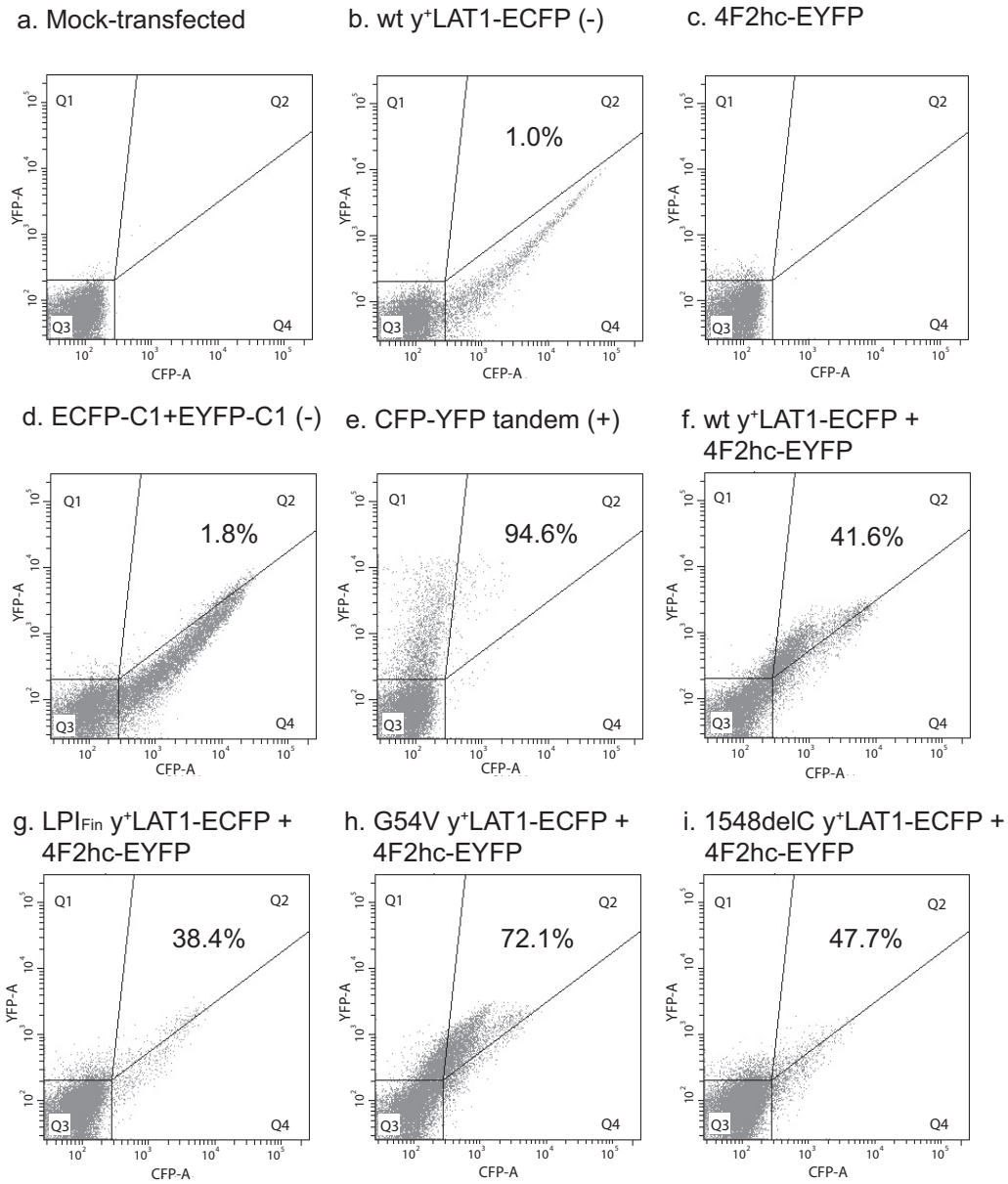

Figure 1. CFP *vs.* YFP dot plots (in the calculations, the cells in Q1 and Q2 were considered as FRET positive, Q3 as non-fluorescent cells and Q4 as CFP positive, FRET negative cells). When FRET occurs, the mean CFP (donor) fluorescence of the cell population decreases as the fluorescence energy is transferred to the acceptor (YFP). Thus, the CFP-positive population moves to left and down in the dot plot. The dot plots of the mock-transfected negative control (a) as well as wt y^+LAT1-ECFP alone (b), 4F2hc-EYFP alone (c) and the double-transfected ECFP-C1/EYFP-C1 vectors (d) delineate the negative baseline of CFP fluorescence and FRET, respectively. The tandem CFP-YFP (e) is the FRET-positive control, as it indicates the effect of near the maximal, forced FRET in the expressing cell population. Wt y^+LAT1-ECFP (f), LPI_{Fin} y^+LAT1-ECFP (g), G54V y^+LAT1-ECFP (h) and 1548delC y^+LAT1-ECFP (i), all co-transfected with 4F2hc-EYFP show a FRET-positive cell population, but the frameshift mutant transfections result in a smaller population of y^+LAT1-ECFP positive cells.

Table 2. The FRET positive cell population sizes in CFP expressing subpopulations

Transfected plasmid constructs [a]	CFP-positive cells (%) [b]	FRET-positive cells (%) [c]
y+LAT1-ECFP	13.3 (8.4–16.5)[e]	1.0 (0.2–3.2)[e]
ECFP-C1/EYFP-C1 (negative control)	29.2 (25.2–36.9)	1.8 (0.4–3.3)
CFP-YFP -tandem (forced FRET; positive control)	12.2 (6.7–17.9)	94.6 (93.2–98.4)
wt y+LAT1-ECFP-C1/4F2hc-EYFP-C1	12.3 (6.6–27.5)	41.6[d] (12.9–72.7)
LPI_{Fin} y+LAT1-ECFP-C1/4F2hc-EYFP-C1	2.6 (1.3–4.8)	38.4[d] (31.9–63.2)
G54V y+LAT1-ECFP-C1/4F2hc-EYFP-C1	10.5 (5.4–30.9)	72.1[d] (32.3–89.5)
1548delC y+LAT1-ECFP-C1/4F2hc-EYFP-C1	3.2 (2.3–5.6)	47.7[d] (28.5–62)

In three independent FRET-FACS runs, the CFP-positive cells were counted from the parent population of 50 000 living cells in each assay. The percentages represent the total amount of CFP and/or FRET positive cells in the combined results. [a] The plasmid construct combinations correspond to those in Figure 1. [b] The cells from Q1, Q2 and Q4 (see Figure 1) were considered as CFP-positive. [c] The CFP-positive cells from Q1 and Q2 were considered as FRET-positive. [d] Each sample had a significantly higher percentage of FRET-positive cells than the negative controls ($p < 0.0001$). [e] The percentages in parentheses represent the observed variation in CFP or FRET positive cells.

the quarters Q1 and Q2 contain the FRET-positive cells, Q3 the non-fluorescent cells and Q4 the CFP-positive but FRET-negative cells (Figure 1 and Table 2).

As controls for the FRET measurements in y+LAT1-ECFP/4F2hc-EYFP double-transfected cells, we performed the same FACS-FRET runs using mock-transfected, single wt y+LAT1-ECFP or 4F2hc-EYFP transfected cells, co-transfected pECFP-C1 and pEYFP-C1 vectors as negative controls and CFP-YFP tandem as a positive FRET control. When the wt y+LAT1-ECFP fusion protein alone or the samples double-transfected with ECFP-C1 and EYFP-C1 vectors were exposed to the 405 nm laser light, the CFP fluorescence intensity was higher than in the ECFP-y+LAT1/EYFP-4F2hc double-transfected cells or the positive control cells, confirming the quenching effect of FRET on the donor CFP fluorochrome in the wt or any of the LPI mutant y+LAT1-ECFP/4F2hc-EYFP double-transfected samples. The forced FRET produced as the result of CFP-YFP tandem construct expression, i.e., the FRET-positive control was represented by a significantly higher number of FRET-positive cells compared to the y+LAT1-ECFP/4F2hc-EYFP FRET-positive population size, most likely due to the optimal distance and 1:1 ratio of donor and acceptor fluorochromes in the tandem CFP-YFP construct. However, the difference between the wt and LPI mutant y+LAT1-ECFP/4F2hc-EYFP double-transfected samples and the FRET-negative controls was clear.

Cells expressing LPI-mutant y+LAT1 fusion proteins proliferate less efficiently than the wt y+LAT1 fusion protein positive cells

The wt y+LAT1 fluorescent fusion proteins were expressed in a larger population of transfected cells than the studied LPI mutant y+LAT1 fusions were (Table 3 and Figure 2 for the y+LAT1-EGFP results, Table 2 for the y+LAT1-ECFP results). Following the wt y+LAT1-EGFP transfections, the proportion of GFP-positive cells was 20.7% (12.3% for y+LAT1-ECFP) and in the LPI mutant transfected samples the proportion varied from 5.2% to 10.9% (from 2.6% to 10.5% for y+LAT1-ECFP). The EGFP and ECFP positive population sizes can be compared only within the same

Table 3. The y+LAT1-EGFP fusion protein expression levels and their effect on cell mortality

y+LAT1 Construct	GFP+ cells (%)	RL (95% CI)	Mortality GFP- (%)	Mortality GFP- RL (95% CI)	Mortality GFP+ (%)	Mortality GFP+ *p*-value [c]	Mortality GFP+ RL (95% CI) [c]	Mortality difference GFP-*vs.* GFP+: *p*-value [d]	Mortality difference RL (95% CI) [d]
WT	20.7 [a]	1	4.2	1	3.6		1	<0.0001	1.17 (1.08–1.26)
LPI_{Fin}	5.2	0.25 (0.24–0.26)	2.0 [b]	0.48 (0.46–0.51)	3.2	ns	0.88 (0.75–1.04)	<0.0001	0.64 (0.55–0.75)
G54V	8.0	0.38 (0.38–0.39)	1.7 [b]	0.41 (0.38–0.43)	1.6	<0.0001	0.44 (0.36–0.53)	ns	1.09 (0.91–1.30)
1548delC	10.9	0.53 (0.51–0.54)	2.9 [b]	0.70 (0.66–0.73)	4.1	0.042	1.13 (1.00–1.26)	<0.0001	0.72 (0.66–0.80)

Results are combined from three independent FACS-runs of 90 000 cells each. CI, confidence interval; RL, relative risk; ns, not statistically significant. [a] The number of cells expressing wild type y+LAT1 is significantly higher ($p < 0.0001$) than that of any LPI mutations. [b] The mortality rate of any LPI-mutant GFP- cell population is significantly lower ($p < 0.0001$) than the wild type GFP- cell population. The mortality rate of each mutant-GFP-positive cell population was compared to the wild type GFP-positive population mortality. Mortality in the GFP-negative and GFP-positive subpopulations of each sample was compared.

fluorescent protein group, i.e. EGFP or ECFP, due to the different transfection protocol: the EGFP transfections were performed using only one fluorescent fusion protein (y^+LAT1-EGFP) whereas the FACS-FRET transfections used two (y^+LAT1-ECFP and 4F2hc-EYFP). Also, the latter required twice as much of each expression construct than the former (2 µg for ECFP *vs.* 1 µg for EGFP constructs, respectively) to obtain a sufficient amount of fluorescent protein positive cells.

In the FACS analysis, the difference between the number of y^+LAT1-EGFP-positive cells in the wt y^+LAT1-EGFP transfected cells and any of the LPI mutant y^+LAT1-EGFP was also significant ($p < 0.0001$). The observed variation in the y^+LAT1-EGFP-positive group size between LPI mutants was not directly explained by the mortality rates among the mutant fusion protein positive groups, e.g. 1548delC had the highest amount of GFP-positive cells (10.9%) among the LPI mutants, despite the high mortality in that group.

When each transfected sample was divided into GFP-positive and GFP-negative subgroups and the mortality rates of the subgroups were compared, the 1548delC y^+LAT1-EGFP-positive subgroup had significantly higher proportion of dead cells than the wt y^+LAT1-EGFP-positive subgroup ($p = 0.042$; Table 3). On the other hand, the mortality of G54V y^+LAT1-EGFP-positive cells was significantly lower than that of the wt y^+LAT1-EGFP-positive population. LPI_{Fin} mutation had a similar effect on the cell viability as did the wt y^+LAT1-EGFP expression.

The comparisons between the LPI_{Fin} and 1548delC y^+LAT1-EGFP-positive and negative subgroups showed a significant increase in the mortality rates within the positive subgroup, whereas the mortality in the G54V y^+LAT1-EGFP transfected cell population did not increase due to mutant fusion protein expression ("Mortality difference" in Table 3). In addition, the wt y^+LAT1-EGFP-positive cells had significantly lower mortality than the GFP-negative cells

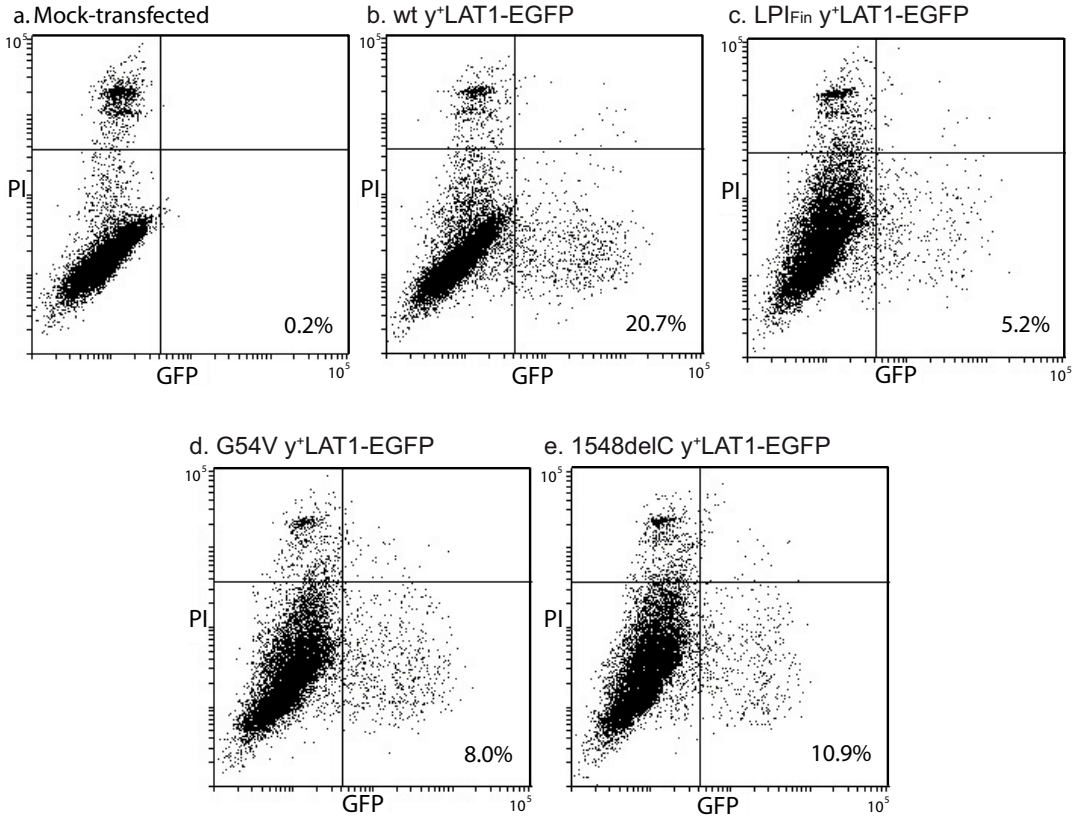

Figure 2. GFP *vs.* PI dot plots. y^+LAT1-EGFP/4F2hc transfected cells were treated with PI to detect the dead cells and immediately run on FACSCalibur^{TM} flow cytometer. In the dot plots, the quadrants represent cell groups as follows: Upper left: PI positive, GFP negative; lower left: PI negative, GFP negative; upper right (UR): PI positive, GFP positive; lower right (LR): PI negative, GFP positive. The dot plot from mock-transfected cells (a) delineates the GFP negative population; all cells fall into left side quadrants. The double-transfected cells (b-e) all have a GFP positive cell population on the right side quadrants, which is further divided between the PI positive (UR) and negative (LR) quadrants. The figures in each panel represent the average percentage of the GFP positive cells (UR and LR quadrants).

from the same sample. In conclusion, the LPI frameshift mutant y+LAT1 expression increased cellular mortality, the effect not seen in the studied point mutant transfections. In contrast to that of the LPI mutant y+LAT1-EGFP, wt y+LAT1-EGFP expression decreased the mortality rate compared with wt y+LAT1-EGFP negative cells in the sample, but the wt y+LAT1-GFP transfected cells without detected GFP expression also had the highest mortality among the GFP negative groups.

Discussion

Dimer formation is not disrupted by y+LAT1 mutations causing LPI

Previously, we have shown using acceptor photobleaching FRET microscopy that wt y+LAT1/4F2hc heteromeric complex is formed directly after the biosynthesis in endoplasmic reticulum (Kleemola et al. 2007). In the present study we demonstrate using FACS-FRET that y+LAT1 proteins carrying LPI-causing mutations LPI_{Fin}, G54V and 1548delC are able to associate with 4F2hc (Figures 1 and 2, Table 2). In fact, the point mutant G54V y+LAT1-ECFP transfection produced a larger FRET-positive sub-population (72.1%; i.e. cells in Q1 and Q2 in the dot blot in Figure 1) of CFP-positive cells than the wt y+LAT1-ECFP transfection (41.6%). The frameshift mutants, LPI_{Fin} y+LAT1-ECFP and 1548delC y+LAT1-ECFP, resulted in a smaller proportion of both CFP-positive (2.6% and 3.2%, respectively) and FRET-positive cells (38.4% and 47.7% of the CFP positive population, respectively).

The interaction between the heterodimeric amino acid transporter subunits is essential for the correct trafficking process of the transporter complex to the cell surface, since all LSHATs require the association with a heavy subunit in order to be trafficked in the plasma membrane (Nakamura et al. 1999). Similarly, in the case of 4F2hc homologue rBAT, the complete glycosylation and maturation of the heavy subunit is dependent on the rBAT/$b^{0,+}$AT transporter heterodimer formation (Sakamoto et al. 2009).

In our previous study on the effect of LPI mutants on the subcellular localization of y+LAT1 (Toivonen et al. 2002), we discussed that one hypothetical explanation for the false trafficking of the frameshift and nonsense mutant y+LAT1 proteins was their inability to dimerize with 4F2hc, which would prevent their correct localization to the plasma membrane. Thus, it is interesting that the ability of the light subunit to interact with 4F2hc is not abolished by the LPI mutations although they change the structure and properties of y+LAT1 enough to prevent the membrane trafficking and subsequently the transport activity of the transporter (Mykkänen et al. 2000). The current report shows that interaction between the transporter subunits occurs even though the light subunit is potentially abnormally folded. The fact that none of the studied mutations alters the intracellular N-terminal tail of y+LAT1 suggests that it may be needed in the successful interaction with the transporter subunits rather than the C-terminal tail. Since the three-dimensional structure of any of the mammalian SLC7 proteins has not yet been solved, the transporter subunit structure resulting from a mutation in the *SLC7A7* gene can only be speculated upon. A heteromeric complex composed of a frameshift mutant y+LAT1 together with a wild type 4F2hc may also be less stable than the wild type heteromer, and therefore maybe unable to pass the protein quality control to be sorted to the plasma membrane. Alternatively, the heteromer may have an abnormal structure due to the mutation, which leads the complex to be degraded by the cell.

y+LAT1 mutations affect the expression of fusion proteins

The transfection of cells with the LPI mutant fusion protein constructs constantly resulted in fewer CFP and GFP-positive cells when compared to the transfections with the wild type constructs (Table 2 for CFP, Table 3 and Figure 2 for GFP). Generally, the GFP transfections resulted in a larger GFP positive group than the CFP transfections, with the exception of G54V y+LAT1-ECFP. Most likely the better expression rates in the GFP-transfected cells are due to the more challenging experimental setting compared to the CFP-transfections: the GFP fusion proteins were expressed together with non-tagged 4F2hc, whereas the y+LAT1-ECFP constructs were co-transfected with equal amounts of EYFP-tagged 4F2hc resulting in a mixture of two fluorescent fusion proteins within expressing cells.

The difference in the y+LAT1-EGFP-positive group size between the wild type and any of the LPI mutants in this study was significant, indicating a disadvantage on the proliferation of LPI mutant expressing cells. We hypothesize, that the effect underlying the smaller y+LAT1 fusion protein-positive populations following the transfections with LPI mutants is the dimerization of dysfunctional and, in the case of the truncating frameshift and nonsense mutants, falsely targeted y+LAT1 with 4F2hc (Mykkänen et al. 2000; Toivonen et al. 2002), which may scavenge the latter from being targeted to the plasma membrane. 4F2hc is, in addition to amino acid transport, involved in multiple processes linked to promotion of cell division and growth. Thus, the relative 4F2hc depletion at the cell surface caused by the intracellular heterodimer formation and the subsequent mistargeting of the mutant holotransporter complex may prevent these cellular processes from occurring, leading to reduced mitotic rate and thus, in the lower proportion of the frameshift mutant y+LAT1/4F2hc-positive cells in transfected samples. In concordance with

the 4F2hc scavenging hypothesis as a cause for increased mortality in the truncated y^+LAT1 positive group, we observed significantly reduced mortality in the wild type y^+LAT1-GFP positive group compared to that of the GFP-negative cells (3.6% *vs*. 4.2%, $p < 0.0001$; Table 3), suggesting that the wt y^+LAT1 fusion protein-expressing cells would benefit over the non-GFP-expressing cells either on the increased amount of functional, nutrient providing transporter complex on the plasma membrane as such and/or on the proliferation-promoting and anti-apoptotic signalling mediated by 4F2hc. However, in contrast to this, the overall cell mortality in the GFP negative group after the wt y^+LAT1-GFP transfection is significantly higher than that of caused by any of the LPI mutation y^+LAT1-GFP transfections (4.2% *vs*. 1.7–2.9%; Table 3). This contradicts to the seemingly beneficial effect of wt y^+LAT1-GFP expression when comparing the mortality of GFP positive and negative groups after transfection. In the GFP positive group this difference is less clear, as the mortality rate is second highest in the wt y^+LAT1-GFP positive (Table 3). The latter, a less prominent effect can be explained by the stress caused by the production of highly over-expressing fusion proteins as such. On the other hand, the higher mortality among the GFP negative group in the wty$^+$LAT1-GFP transfected samples must be due to factors other than over-expression of fusion proteins. For example, the higher confluency prior to harvesting due to faster proliferation rate of the wt y^+LAT1-GFP positive cell population, may cause inhibition of cell proliferation or induction of apoptosis in other cells, subsequently detected as increased mortality within the GFP negative group.

Interestingly, the G54V y^+LAT1-GFP positive cells show no significant change in the mortality rate compared to the GFP negative group (1.6% *vs*. 1.7%, respectively). As previously shown, the point mutant y^+LAT1 transporters such as G54V localize to the plasma membrane (Mykkänen et al. 2000; Toivonen et al. 2002). Thus, the finding suggests that also the transporter complex may be targeted to the plasma membrane in the GFP positive group. If so, the cells with plasma membrane targeted 4F2hc benefits from the provided signalling for cell survival and proliferation. Unlike the wt y^+LAT1, the dysfunctional G54V mutated light subunit does not provide nutritional benefits for the expressing cells, which explains the lower than wild type amount of GFP-positive cells (8.0% *vs*. 20.7%, $p < 0.0001$). However, no conclusions can be made of the subcellular localization of y^+LAT1/4F2hc interaction within a single cell using the FACS-FRET method.

In conclusion, we have expressed the CFP-tagged wild type and LPI-mutant y^+LAT1 proteins together with YFP-tagged 4F2hc, and used these fusion proteins in FACS-FRET measurements to analyze the heteromerization between the y^+LAT1 transporter complex subunits. Our results reveal that all differently mutated y^+LAT1 transporters studied dimerize with 4F2hc. Thus, the cellular trafficking machinery uses the transporter dimer as the major selective determinant for protein quality control, instead of the light chain y^+LAT1 prior to the heteromerization. Therefore, the trafficking defect of the frameshift LPI mutant y^+LATs is not caused by the inability of the transporter subunits to interact. Instead, our data suggest that the false subcellular targeting of the frameshift LPI-mutant transporters may contribute to the reduced proliferation rate of the cells expressing them *via* the scavenging of 4F2hc, thus preventing it from performing its anti-apoptotic and cell division-promoting tasks. However, based on the current study, the precise subcellular localization of LPI-mutated y^+L amino acid transporter protein complexes remains elusive, as the FACS-FRET method detects FRET signal at the cell population level. Therefore, the subcellular consequences of LPI-mutations on the y^+LAT1/4F2hc complex formation and their effects on other 4F2hc-interacting proteins need further investigation.

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