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Ypk1, the yeast orthologue of the human serum- and glucocorticoid-induced kinase, is required for efficient uptake of fatty acids

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Summary

Fatty acids constitute an important energy source for various tissues. The mechanisms that mediate and control uptake of free fatty acids from the circulation, however, are poorly understood. Here we show that efficient fatty-acid uptake by yeast cells requires the protein kinase Ypk1, the orthologue of the human serum- and glucocorticoid-induced kinase Sgk1. *ypk1Δ* mutant cells fail to grow under conditions that render cells auxotrophic for fatty acids, show a reduced uptake of radiolabelled or fluorescently labelled fatty acids, lack the facilitated component of the uptake activity, and have elevated levels of fatty acids in a bovine serum albumin (BSA) back-extractable compartment. Efficient fatty-acid uptake and/or incorporation requires the protein-kinase activity of Ypk1, because a kinase-dead point-mutant allele of *YPK1* is defective in this process. This function of Ypk1 in fatty-acid uptake and/or incorporation is functionally conserved, because expression of the human Sgk1 kinase rescues *ypk1Δ* mutant yeast. These observations suggest that Ypk1 and possibly the human Sgk1 kinase affect fatty-acid uptake and thus energy homeostasis through regulating endocytosis. Consistent with such a proposition, mutations that block early steps of endocytosis display reduced levels of fatty-acid uptake.

Key words: Fatty-acid uptake, Sgk1 kinase, Endocytosis, Plasma membrane, *Saccharomyces cerevisiae*

Introduction

Fatty acids constitute a major energy source for tissues such as heart, liver and skeletal muscle, which take up fatty acids from the circulation. The molecular mechanisms that mediate and control this fatty-acid uptake are not well understood. Elevated plasma levels of fatty acids, however, are sufficient to induce insulin resistance in animal models, which emphasizes the importance of this lipid-uptake pathway in the progression of the metabolic syndrome (Boden et al., 2005).

Uptake of fatty acids in mammalian cells is mediated by two separate components: a passive component that is a linear function of the concentration of free fatty acids and a saturable component that exhibits the characteristics of a protein-facilitated process (Abumrad et al., 1998; Hajri and Abumrad, 2002). The relative contribution of these two components is tissue dependent and subject to hormonal regulation (Bonen et al., 2007; Kampf and Kleinfeld, 2007).

After or concomitant with translocation across the plasma membrane, the free fatty acid is activated to its acyl-CoA ester. This activation step might trap the fatty acid in the cytosol and makes it available to the different acyl-CoA-consuming processes such as lipid synthesis or fatty-acid β-oxidation. Efficient uptake of fatty acids might thus be coupled to their esterification by acyl-CoA synthetases, a process originally proposed for uptake of fatty acids by *Escherichia coli* and termed 'vectorial acylation' (Black and DiRusso, 2003; Klein et al., 1971).

From studies in animal cells, four different proteins have been implicated in fatty-acid uptake: the fatty-acid transport proteins (FATPs) (Watkins, 2008), the class B scavenger receptor FAT (also known as CD36) (Febbraio et al., 2001), the plasma-membrane fatty-acid-binding protein (FABPpm) (Isola et al., 1995) and caveolin (Meshulam et al., 2006). Whether these proteins are genuine fatty-acid transporters or whether they facilitate uptake more indirectly, however, remains to be established (Hajri and Abumrad, 2002).

In yeast, uptake of fatty acids is efficient and rescues mutants that are blocked in their de novo synthesis, such as mutants in the fatty-acid synthase (*FAS1* and *FAS2*) or those in fatty-acid desaturation (*OLE1*) (Knobling and Schweizer, 1975; Stukey et al., 1989).

Yeast contains two FATP homologues, Fat1 and Fat2 (also known as Pcs60). Fat2 is localized to the lumen of peroxisomes but is not required for growth under conditions in which peroxisomal β-oxidation of fatty acids provides the sole carbon source (Hiltunen et al., 2003). Fat1 has been found to be localized to internal membranes and contains an acyl-CoA-synthetase activity, which is important for regulation of very-long-chain fatty-acid levels and composition (C20-C26) (Choi and Martin, 1999; Watkins et al., 1998). Fat1 is not required for cells that are auxotrophic for fatty acids to grow on media supplemented with saturated long-chain fatty acids, indicating that Fat1 function is not strictly required for uptake of fatty acids, but the protein might increase uptake efficiency (Choi and Martin, 1999; Faergeman et al., 1997).

The two major cytosolic long-chain acyl-CoA synthetases, Faa1 and Faa4, by contrast, are required for auxotrophic cells to grow on fatty-acid-supplemented media, indicating that activation of long-chain fatty acids to their acyl-CoA esters is required either for uptake, retention and/or metabolic utilization of exogenous fatty acids, and that this function of Faa1 and Faa4 is not provided by Fat1 (Choi and Martin, 1999; Faergeman et al., 2001; Johnson et al., 1994; Knoll et al., 1995) (for review, see Black and DiRusso, 2007).

Here we identify Ypk1, the yeast orthologue of the human serum- and glucocorticoid-induced kinase Sgk1, as an important component for efficient uptake of fatty acids. Cells lacking Ypk1 fail to grow under fatty-acid auxotrophic conditions and lack the facilitated and/or saturable component of the uptake activity. Ypk1-dependent fatty-acid uptake requires the protein kinase activity of Ypk1 and is functionally conserved in human Sgk1. Ypk1 is required for the internalization step of endocytosis, suggesting that membrane internalization is required for efficient uptake of exogenous fatty acids. Consistent with a model in which fatty acids are taken up through membrane internalization, we find that other mutations that block early steps in endocytosis display a reduced efficiency of fatty-acid uptake.

Results

We have previously identified mutants that fail to grow in the absence of oxygen, conditions that render *Saccharomyces cerevisiae* auxotrophic for unsaturated fatty acids and sterols because the synthesis of these lipids requires molecular oxygen (Andreasen and Stier, 1954). This screen allowed us to identify components required for uptake and transport of sterols (Reiner et al., 2006). We also noted, however, that some of the mutants might fail to grow under anaerobic conditions because of a possible defect in uptake or transport of unsaturated fatty acids. One mutant that exhibited a strong growth defect under anaerobic conditions but that did not have altered uptake of sterols compared with wild type was deficient in the protein kinase Ypk1. *YPK1* encodes a sphingoid base-regulated serine-threonine kinase and is the yeast orthologue of mammalian Sgk1 (Casamayor et al., 1999; Sun et al., 2000). Ypk1 is required for both receptor-mediated and fluid-phase endocytosis, but not for receptor phosphorylation and ubiquitylation (deHart et al., 2002).

Ypk1 is required for growth under fatty-acid auxotrophic conditions

To test whether Ypk1 is required for uptake of fatty acids under aerobic conditions, we examined whether the *ypk1Δ* mutant could grow under conditions in which endogenous synthesis of fatty acids is inhibited by cerulenin, an irreversible inhibitor of the fatty-acid synthase (Omura, 1981). On cerulenin-containing media, cells can only grow if they can take up and activate exogenous fatty acids, which requires the long-chain acyl-CoA-synthetase activity of either Faa1 or Faa4 (Johnson et al., 1994). Examination of the growth of the *ypk1Δ* mutants on cerulenin- and fatty-acid-containing media revealed a strong growth defect (Fig. 1A). On plates containing only the fatty-acid supplementation, the *ypk1Δ* mutant grew well, indicating that the growth defect in the presence of cerulenin and fatty acids was not due to the detergent action of the fatty acids. On plates containing only cerulenin but lacking fatty acids, cells were unable to grow, confirming drug action. The *faa1Δ faa4Δ* double mutant also failed to grow in the presence of cerulenin and fatty acids, consistent with the requirement of these acyl-CoA synthetases for activation of the imported fatty acids (Johnson et al., 1994).

To examine whether uptake of unsaturated fatty acids also depends on Ypk1, we tested whether Ypk1 is required for growth of a conditional mutant in the sole essential fatty-acid desaturase, *OLE1*. Temperature-sensitive alleles in this fatty-acid desaturase have previously been isolated and the conditional growth phenotype of these mutants can be rescued by the addition of unsaturated fatty acids to the growth medium (Stewart and Yaffe, 1991). Similar to

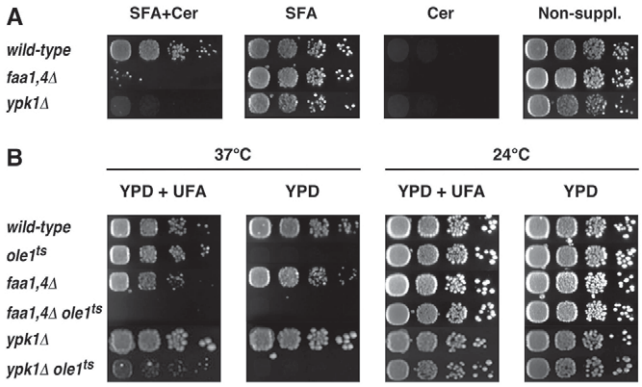

Fig. 1. Growth of the *ypk1Δ* mutant under fatty-acid auxotrophic conditions. (**A**) Ypk1 is required for growth in the presence of cerulenin. Cells of the indicated genotype were serially diluted tenfold and spotted onto YPD media containing either palmitic and stearic acid as a saturated fatty acid (SFA) supplement (0.5 mM each) and cerulenin (10 µg/ml), SFA only, cerulenin only, or neither cerulenin nor SFA (Non-suppl.). Plates were incubated for 4 days at 30°C. (**B**) Ypk1 is required for growth of a conditional fatty-acid-desaturase (*ole1ts*) mutant. Cells of the indicated genotype were serially diluted tenfold and spotted onto YPD media containing oleic acid as an unsaturated fatty acid (UFA) supplement, or onto YPD without lipid supplementation. Plates were incubated at nonpermissive (37°C) or permissive (24°C) conditions for 4 days.

activation of imported saturated fatty acids, that of unsaturated fatty acids also requires a functional acyl-CoA synthase provided by either *FAA1* or *FAA4*. Under non-permissive conditions, growth of the *faa1Δ faa4Δ ole1ts* triple mutant was completely blocked (Fig. 1B). By comparison, growth of the *ypk1Δ ole1ts* double mutant was strongly impaired, but not completely blocked, suggesting that some residual uptake of oleic acid is still occurring. Taken together, these two growth phenotypes indicate that Ypk1 is required for efficient growth under conditions that render cells auxotrophic for saturated or unsaturated fatty acids and thus indicate that Ypk1 is important for fatty-acid uptake and/or incorporation into more complex lipids.

Fatty-acid uptake is temperature dependent

To examine a possible function of Ypk1 in fatty-acid uptake more directly, a biochemical assay was established to monitor uptake and metabolic conversion of exogenously added radiolabelled fatty acids over time. Therefore, cells were first cultivated in media containing fatty acids to metabolically prime them for uptake of fatty acids from the outside and to saturate possible fatty-acid-binding sites on the cell exterior. Exponentially growing cells were then concentrated and incubated in media containing 1 mM oleic acid spiked with 0.03% radiolabelled [^{3}H]oleic acid. Samples were taken over a 60-minute time period. Cells were metabolically poisoned by the addition of sodium azide and sodium fluoride, washed, and lipids were isolated and analyzed by thin-layer chromatography (TLC) to separate free fatty acids from those that have been incorporated into complex lipids. Under these conditions, radiolabelled lipids accumulated linearly during the 60-minute uptake assay (Fig. 2A). Extracellular fatty acids were not depleted during the 60-minute time period (Fig. 2B) and intracellular levels of free fatty acids remained at a constant low level (Fig. 2C). Fatty acids that were taken up, however, were rapidly metabolized and incorporated into phospholipids and neutral lipids (Fig. 2D).

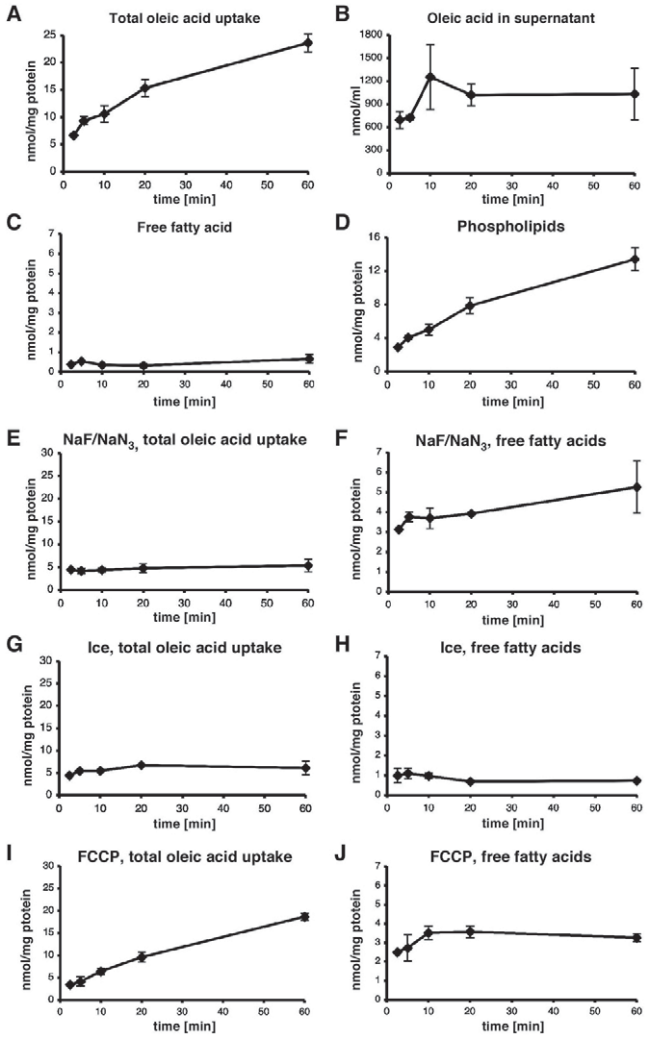

Fig. 2. Uptake of unsaturated fatty acids by wild-type cells. (**A-D**) Fatty-acid uptake is linear. Wild-type cells were cultivated overnight in YPD medium containing oleic acid (1 mM) and 0.1% Brij 58. Exponentially growing cells were collected, concentrated and uptake of [H^3]oleic acid was analyzed over time as described in the Materials and Methods. Graphs display total uptake of the radiolabelled fatty acid (A), fatty acids remaining in the extracellular media (B), cell-associated free fatty acids (C) and fatty acids incorporated into phospholipids (D). (**E-J**) Uptake of fatty acids is dependent on both energy and temperature. Wild-type cells were metabolically poisoned by incubation with NaN_3 and NaF (20 mM each; E,F) or FCCP (20 µM; I, J), or placed on ice for 30 minutes prior to initiation of the uptake assay (G,H), and total incorporation of fatty acids and levels of free fatty acids were determined. Values represent means and standard deviations of two independent experiments.

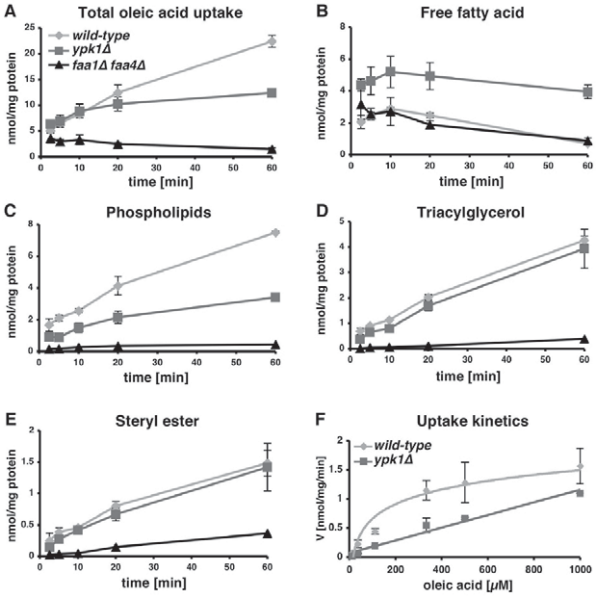

Fig. 3. Ypk1 is required for the saturable component of the fatty-acid-uptake path. (**A-E**) Ypk1 is required for efficient fatty-acid uptake. Wild-type and mutant cells of the indicated genotype were cultivated as described for Fig. 2 and uptake of [H^3]oleic acid was analyzed. Graphs display total uptake of the radiolabelled fatty acid (A), cell-associated free fatty acids (B), fatty acids incorporated into phospholipids (C), triacylglycerols (D) and steryl esters (E). (**F**) Kinetic analysis of fatty-acid uptake in wild-type and *ypk1Δ* mutant cells. Cells were cultivated in oleic-acid-supplemented media, concentrated and the initial velocity of fatty-acid uptake at different concentrations of oleic acid was examined as described for Fig. 2. Lipids were extracted, separated by TLC, and total levels of [H^3]oleic acid taken up and incorporated by the cells were quantified by radio-scanning.

Uptake and incorporation of fatty acids into lipids was dependent on both energy and temperature, because it was inhibited in cells treated with fluoride and azide, or when cells were placed on ice (Fig. 2E,G). Energy-depleted cells, however, accumulated three- to five-fold more free fatty acids compared with cells that were incubated on ice, indicating that an early step along the uptake pathway, possibly incorporation of the free fatty acid into the plasma membrane or its translocation across the plasma membrane, is energy independent but temperature dependent (Fig. 2F,H). Because the level of free fatty acids did not significantly increase over time in energy-depleted cells, this step along the putative uptake pathway seemed to be rapidly saturated in the absence of further metabolic conversion of the free fatty acid. Depletion of the transmembrane potential by prior incubation of the cells with the proton-gradient uncoupler FCCP resulted in a 20% reduction of oleic-acid uptake and/or incorporation and a slight (threefold) increase of free-fatty-acid levels (Fig. 2I,J).

Taken together, these results indicate that the biochemical assay monitors two discernible steps, a temperature-dependent uptake step and the subsequent energy-dependent metabolic incorporation of the fatty acid. This assay thus recapitulates key properties of fatty-acid uptake by fungal cells, which have been reported before (Faergeman et al., 1997; Knoll et al., 1995; Kohlwein and Paltauf, 1984; Trigatti et al., 1992).

Ypk1 is required for a facilitated and saturable component of the fatty-acid-uptake and/or -incorporation pathway

Examination of the rate of fatty-acid uptake and incorporation in the *ypk1Δ* mutant revealed a reduced uptake and/or incorporation of fatty acids when tested by the biochemical assay. Compared with wild-type cells, the *ypk1Δ* mutant displayed a 45% reduction of total incorporated oleic acid at the end of the 60-minute assay period (Fig. 3A), twofold elevated levels of free fatty acids (Fig. 3B) and a 55% reduction of incorporation of the radiolabelled oleic acid into phospholipids (Fig. 3C). Incorporation into the neutral lipids, steryl esters and triacylglycerol, however, was not significantly reduced in the *ypk1Δ* mutant (Fig. 3D,E). The *faa1Δ*

faa4Δ double mutant, by contrast, had a complete block in fatty-acid uptake and/or incorporation and displayed no elevated levels of free fatty acids as observed in *ypk1Δ*.

These observations indicate that uptake and/or incorporation of fatty acids into membrane lipids is impaired in the *ypk1Δ* mutant. The available levels of fatty acids, however, are still sufficient to satisfy neutral-lipid synthesis. The observed elevation of free-fatty-acid levels in *ypk1Δ* might indicate that a step prior to CoA activation becomes rate limiting in this mutant.

To define the fatty-acid-uptake defect of the *ypk1Δ* mutant more precisely, we examined the velocity of fatty-acid uptake and incorporation at different concentrations of oleic acid in wild-type and *ypk1Δ*-mutant cells. This analysis revealed that fatty-acid uptake and incorporation in wild-type cells is a saturable process with an apparent Michaelis constant (K_m) of 0.33 µM and a velocity at maximum concentrations of substrate (V_{max}) of 2.1 nmol oleic acid/minute/mg of protein (corresponding to 5.48 nmol/minute/10^8 cells) (Fig. 3F). In view of the different growth conditions employed, these kinetic values for wild-type cells are comparable to those previously reported for *S. cerevisiae* wild-type cells, i.e. 6.54 nmol/minute/10^8 cells (Faergeman et al., 1997; Knoll et al., 1995; Trigatti et al., 1992).

In the *ypk1Δ* mutant, the velocity of fatty-acid uptake and/or incorporation was strongly reduced and the kinetic data could not be fitted to a hyperbolic curve, but fitted a linear curve with a correlation coefficient of 0.9 (Fig. 3F). These data indicate that, in the absence of Ypk1, fatty acids enter cells by a passive process only and hence that Ypk1 is important for the facilitated component of the uptake pathway.

Ypk1 affects uptake of the fluorescent fatty-acid analogue Bodipy-C12

In the fatty-acid-uptake assays reported above, the fatty acid that is taken up is metabolized and rapidly incorporated into complex lipids. Under these conditions, fatty-acid uptake is thus intimately linked to its metabolic conversion. Because this metabolic coupling cannot be blocked without impairing cell viability, we examined uptake of an alternative transport substrate, the fluorescent fatty-acid analogue Bodipy-C12 (also known as BODIPY-3823), which has previously been employed to examine fatty-acid uptake in mammalian cells and yeast (Schaffer and Lodish, 1994; Faergeman et al., 1997).

Analysis of Bodipy-C12 uptake in wild-type, *ypk1Δ* and *faa1Δ faa4Δ* double-mutant cells by flow cytometry revealed a pronounced reduction in the cell-associated fluorescence in *ypk1Δ* and *faa1Δ faa4Δ* double-mutant cells (Fig. 4A). Compared with wild type, the average cell-associated fluorescence was reduced by 65% in *ypk1Δ* cells and by 77% in the *faa1Δ faa4Δ* double mutant (Fig. 4B).

Examination of the subcellular distribution of Bodipy-C12 in wild-type cells by fluorescence microscopy revealed intense staining of the cytosol and of internal membranes, particularly the ER membrane, whereas the vacuolar lumen was typically devoid of fluorescence (Fig. 4C). Uptake of this fluorescent fatty-acid analogue was temperature dependent, because it was blocked if cells were incubated on ice. It was also dependent on Faa1 or Faa4, because the double mutant showed no detectable intracellular fluorescence compared with wild type. The *ypk1Δ* mutant, by contrast, displayed strongly reduced cytosolic and membrane-associated fluorescence. Only punctuate intracellular structures were stained in the *ypk1Δ* mutant, and these punctuate structures correspond to lipid droplets, as shown by colocalization with a red fluorescent protein (RFP)-tagged version of the lipid droplet protein Erg6 (Erg6-RFP, Fig. 4D). Bodipy-C12 has been observed to label lipid droplets in mammalian cells before (Schaffer and Lodish, 1994). Staining of neutral-lipid-containing lipid droplets is consistent with the observation that neutral-lipid synthesis is not impaired in the *ypk1Δ* mutant. The results obtained with the fluorescent fatty-acid analogue Bodipy-C12 thus support the notion that the protein kinase Ypk1 is required for efficient uptake rather than downstream metabolic conversion of imported fatty acids.

The protein-kinase activity of Ypk1 is important for efficient fatty-acid uptake and/or incorporation

To examine whether the protein-kinase activity of Ypk1 itself is required for import of fatty acids, we examined growth and fatty-

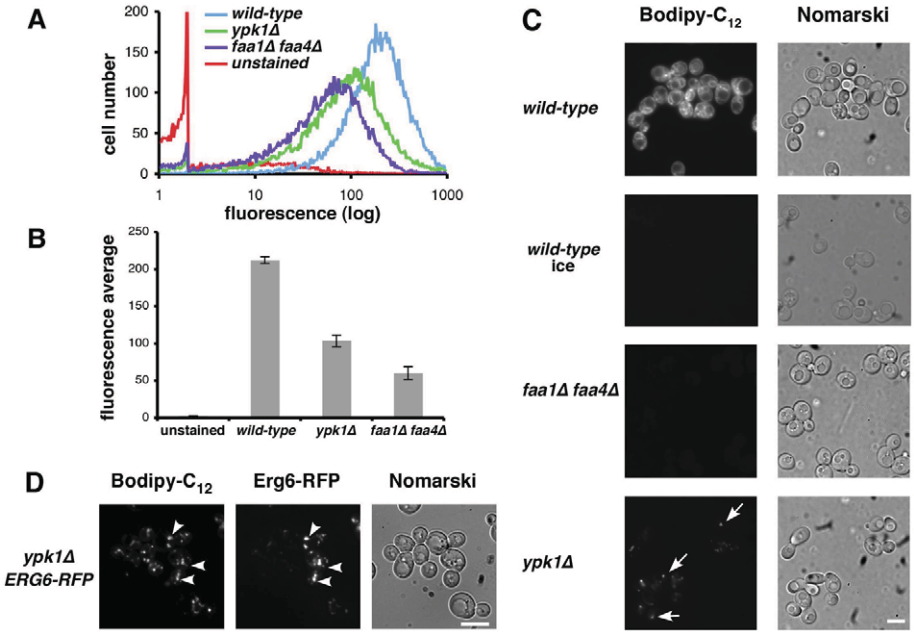

Fig. 4. Ypk1 is required for efficient uptake of the fluorescent fatty-acid analogue Bodipy-C12. (**A**) Uptake of Bodipy-C12. Cells of the indicated genotype were cultivated in oleic-acid-containing media, concentrated and incubated with Bodipy-C12 for 30 minutes at 24°C, washed with PBS containing BSA (50 µM), diluted, and cell-associated fluorescence was quantified by flow cytometry. (**B**) Graph of average cell-associated fluorescence. (**C**) Subcellular distribution of Bodipy-C12. Cells were incubated with Bodipy-C12 as described for panel A and examined by fluorescence microscopy. Punctuate structures stained in *ypk1Δ* cells are indicated by arrows. (**D**) Colocalization of Bodipy-C12 with the lipid-droplet marker Erg6-RFP. *ypk1Δ* mutant cells expressing Erg6-RFP were incubated with Bodipy-C12 as described for panel A and cells were analyzed by fluorescence microscopy. Colocalization of Bodipy-C12 with lipid droplets is indicated by arrowheads. Scale bars: 5 µm.

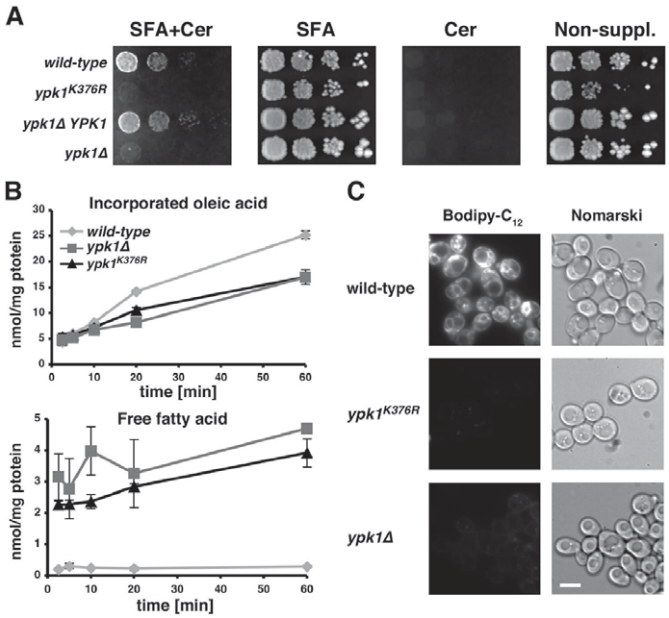

Fig. 5. The protein-kinase activity of Ypk1 is required for efficient fatty-acid uptake. (**A**) A kinase-dead allele of *YPK1*, *ypk1*^{K376R}, does not grow under fatty-acid auxotrophic conditions. Cells of the indicated genotype were serially diluted tenfold and spotted onto YPD media containing saturated fatty acids (SFA) with or without cerulenin (Cer), or lacking lipid supplementation (Non-suppl.). Plates were incubated for 4 days at 30°C. (**B**) Oleic-acid uptake requires the kinase function of *YPK1*. Wild-type and *ypk1*^{K376R} mutant cells were cultivated in fatty-acid-containing media and uptake of [H^3]oleic acid was monitored. The top graph shows the amount of incorporated fatty acids; the lower graph shows levels of cell associated free fatty acids. (**C**) Uptake of Bodipy-C12 requires the kinase function of *YPK1*. Cells of the indicated genotype were incubated with Bodipy-C12 for 30 minutes at 24°C, washed with PBS containing BSA (50 µM) and examined by fluorescence microscopy. Scale bar: 5 µm.

acid uptake of cells expressing a point-mutant allele of Ypk1 in which a lysine residue in the conserved catalytic domain of the kinase is changed to arginine, *ypk*^{K376R}, as their sole source of Ypk1 (Sun et al., 2000). These cells failed to grow under fatty-acid auxotrophic conditions (Fig. 5A). They displayed a reduction in fatty-acid uptake compared with cells expressing a wild-type version of the kinase, and accumulated free fatty acids (Fig. 5B). *ypk*^{K376R} cells showed little intracellular fluorescence when incubated with Bodipy-C12 and the fluorescence that was visible was mostly localized to small punctuate structures (Fig. 5C). These data show that cells expressing the kinase-dead allele of Ypk1 display the same phenotypes as cells that completely lack Ypk1 activity, indicating that the protein-kinase activity of Ypk1 is required for efficient uptake and/or incorporation of fatty acids.

The role of Ypk1 in fatty-acid uptake is functionally conserved in the human orthologue, Sgk1

To examine whether the role of Ypk1 in fatty-acid uptake and/or incorporation is functionally conserved, we examined whether the yeast *ypk1Δ* mutant is complemented by expression of the mammalian orthologue, Sgk1 (Sun et al., 2000). Cells expressing Sgk1 instead of Ypk1 grow under fatty-acid auxotrophic conditions (Fig. 6A), take up oleic acid at wild-type levels (Fig. 6B), and efficiently internalize the fluid-phase marker Lucifer Yellow and transport it to the vacuole (Fig. 6C), indicating that the function of Ypk1 in fatty-acid uptake and membrane internalization is complemented by expression of the human orthologue and hence is functionally conserved.

Ypk1 affects free fatty-acid levels in a BSA back-extractable compartment

The fatty-acid-uptake assays employed so far could not distinguish between a membrane-translocation step of the free fatty acid and its subsequent metabolic conversion and incorporation into lipids. To determine whether Ypk1 is important for uptake of the fatty acid or for its downstream utilization, we took advantage of the observation that energy depletion affects downstream utilization but not cell association of the free fatty acid because cells treated with sodium azide and fluoride displayed a sevenfold elevated level of free fatty acids compared with energized cells (Fig. 2F). On the basis of these observations, we reasoned that the elevated levels of free fatty acids observed in energy-depleted cells and in the *ypk1Δ* mutant might be due to incorporation of the radiolabelled fatty acid into the plasma membrane, a step that might be thermosensitive and blocked at 4°C (Fig. 2H). To test this hypothesis, we first examined whether, in energy-depleted cells, the levels of free fatty acids that are back-extractable with delipidated bovine serum albumin (BSA) are higher than in

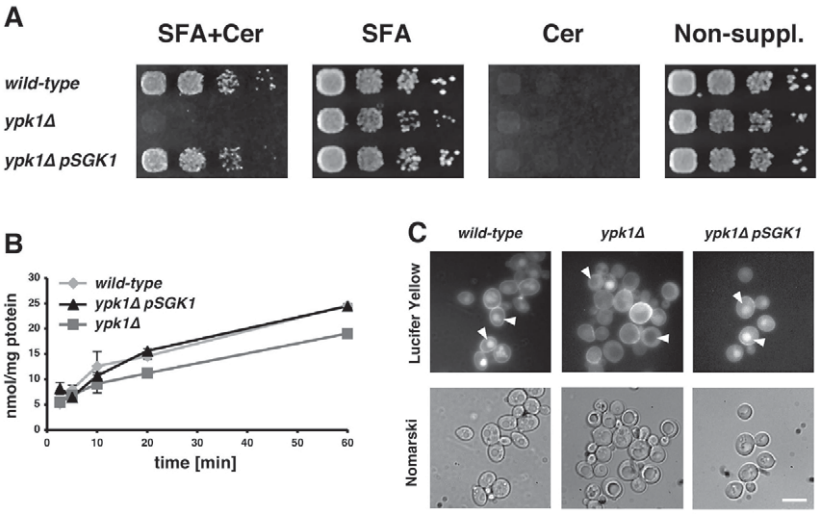

Fig. 6. The fatty-acid-uptake defect of *ypk1Δ* mutant cells is rescued by human Sgk1. (**A**) The growth defect of *ypk1Δ* cells under fatty-acid auxotrophic conditions is rescued by Sgk1. Wild-type cells, *ypk1Δ* mutant cells, and *ypk1Δ* mutant cells expressing a plasmid-borne copy of Sgk1 (pSGK1) were serially diluted tenfold and spotted onto YPD media containing saturated fatty acids (SFA) and cerulenin (Cer), SFA only, cerulenin only, or neither cerulenin nor SFA (Non-suppl.). Plates were incubated for 4 days at 30°C. (**B**) Sgk1 enhances oleic-acid uptake of *ypk1Δ* cells. Cells of the indicated genotype were cultivated in fatty-acid-containing media and uptake of [H^3]oleic acid was monitored. (**C**) Sgk1 rescues the defect in fluid-phase endocytosis of *ypk1Δ* mutant cells. Cells of the indicated genotype were incubated with Lucifer Yellow for 60 minutes and examined by fluorescence microscopy. Uptake and transport to the vacuole of the fluid-phase marker is indicated by arrowheads. Scale bar: 5 µm.

energized cells or in cells incubated at 4°C. This analysis revealed that energy-depleted cells had 18-fold elevated levels of back-extractable fatty acids compared with energized cells or in cells incubated at 4°C, indicating that these fatty acids remain accessible from the outside and thus have not yet crossed the plasma membrane (Fig. 7). This accumulation of free fatty acids in a BSA-accessible compartment was thermosensitive, because cells incubated on ice had only low levels of back-extractable fatty acids. Remarkably, *ypk1Δ* and *ypk1*^{K376R} mutant cells displayed an approximately 20-fold elevated level of back-extractable fatty acids compared with wild-type cells, indicating that, in the absence of Ypk1 activity, free fatty acids accumulate in a BSA-accessible compartment. Energy depletion of the *ypk1Δ* mutant did not further increase the levels of back-extractable fatty acids compared with the *ypk1Δ* mutant alone, indicating that the energy- and Ypk1-requiring steps cannot be uncoupled through the back-extraction assay employed here and that they might affect the same process.

The *faa1Δ faa4Δ* double mutant, by contrast, displayed fivefold reduced levels of back-extractable fatty acids compared with energy-depleted cells. Energy depletion of the *faa1Δ faa4Δ* double mutant, however, resulted in increased levels of BSA-extractable fatty acids. Because *ypk1Δ* and *faa1Δ faa4Δ* mutant strains display different phenotypes with regards to the accumulation of free and back-extractable fatty acids, the function of Ypk1 in fatty-acid uptake is probably not mediated by regulating the activity of Faa1 and/or Faa4. Consistent with such a proposition, we found that localization of Faa1 and Faa4 is not affected by deletion of *YPK1* (supplementary material Fig. S1).

From these results, we conclude that metabolic energy and the protein-kinase activity of Ypk1 are required for efficient uptake of fatty acids from the yeast plasma membrane, possibly through internalization of plasma-membrane domains enriched in free fatty acids.

Endocytosis and plasma-membrane recycling are required for efficient uptake of fatty acids

Given that Ypk1 activity is required for receptor and fluid-phase endocytosis, we finally examined whether its function in fatty-acid uptake is dependent on endocytosis. Therefore, we screened established mutants in endocytosis and membrane recycling for growth defects under fatty-acid auxotrophic conditions. This analysis revealed that mutants that affect actin remodelling and/or actin patch formation, such as *end3Δ*, *vrp1Δ* and *srv2Δ*, fail to grow in the presence of cerulenin and saturated fatty acids, and affect growth in the *ole1*^{ts} mutant background in the presence of unsaturated fatty acids (Fig. 8A,B). *END3* encodes an epsin homology (EH)-domain-containing protein required for the assembly of cortical actin structures, endocytosis and cell-wall

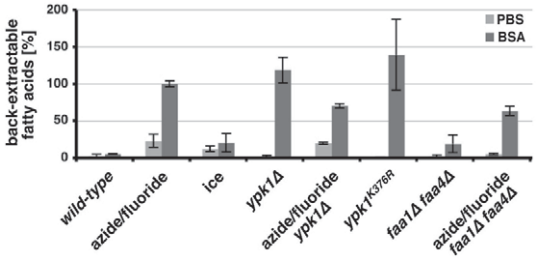

Fig. 7. *ypk1Δ* mutant cells accumulate free fatty acids in a BSA back-extractable compartment. Cells of the indicated genotype and wild-type cells treated with NaN_3 and NaF (20 mM each) were incubated with [H^3]oleic acid for 30 minutes, as described for fatty-acid uptake in Fig. 2 legend. Cells were then washed with 0.5% tergitol followed by one wash with PBS containing or lacking 1% delipidated BSA. Back-extracted free fatty acids were separated by TLC and quantified by radio-scanning.

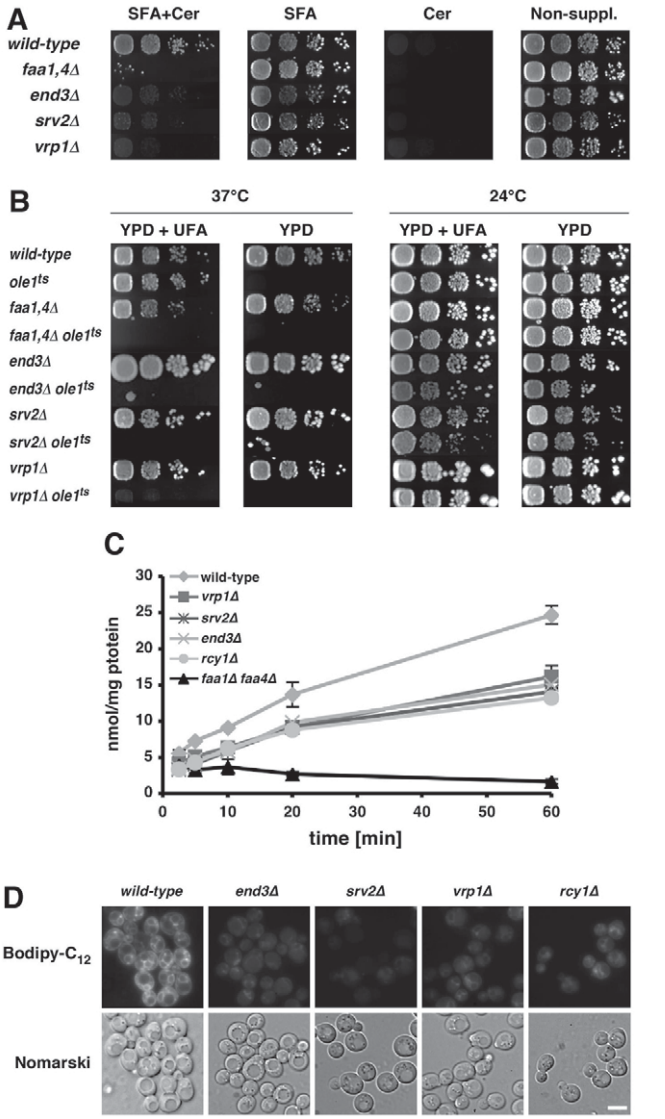

Fig. 8. Endocytosis and membrane recycling are required for efficient fatty-acid uptake. (**A**) Endocytosis mutants display a growth defect under fatty-acid auxotrophic conditions. Cells of the indicated genotypes were serially diluted tenfold and spotted onto YPD media containing palmitic and stearic acid as a saturated fatty acid (SFA) supplement (0.5 mM each) and cerulenin (10 μg/ml), SFA only, cerulenin only, or neither cerulenin nor SFA (Non-suppl.). (**B**) Endocytosis is required for growth of a conditional fatty-acid-desaturase (*ole1*^{ts}) mutant. Cells were serially diluted and spotted onto YPD media containing oleic acid as an unsaturated fatty acid (UFA) supplement, or on YPD without lipid supplementation. Plates were incubated at nonpermissive (37°C) or permissive (24°C) conditions. (**C**) Endocytosis and plasma-membrane recycling is required for efficient uptake and/or incorporation of oleic acid. Cells were cultivated in fatty-acid-containing media and uptake of [H^3]oleic acid was monitored. (**D**) Uptake of Bodipy-C12 requires endocytosis and plasma-membrane recycling. Cells were incubated with Bodipy-C12 for 30 minutes at 24°C, washed with PBS containing BSA (50 μM), and examined by fluorescence microscopy. Scale bar: 5 μm.

morphogenesis (Benedetti et al., 1994). *VRP1* encodes verprolin, a proline-rich actin-associated protein involved in cytoskeletal organization and cytokinesis [WASp-interacting protein (WIP) (Donnelly et al., 1993)]. And Srv2, a cyclase-associated protein, promotes cofilin-dependent actin turnover (Mattila et al., 2004). All three mutants thus link actin remodelling and endocytosis to uptake of exogenous fatty acids. In the biochemical uptake assay, all three mutants display reduced levels of oleic-acid uptake (Fig. 8C).

We also tested and identified deletions of *RCY1*, which encodes an F-box protein required for endocytosis and plasma-membrane recycling (Wiederkehr et al., 2000), as having a defect in oleic-acid uptake (Fig. 8C). The *rcy1Δ* mutant is detergent sensitive and therefore could not be assessed in the growth assay, which requires higher concentrations of detergents to solubilize saturated fatty acids (1% Brij 58) than is used in the biochemical uptake assay (0.1% Brij 58).

These internalization and recycling mutants also affect uptake of Bodipy-C12, indicating that they affect uptake of free fatty acids rather than their downstream metabolism (Fig. 8D).

Taken together, these results are consistent with a working model of fatty-acid uptake, in which fatty acids are first internalized in an energy- and Ypk1-dependent endocytotic step before they are activated to their CoA derivatives (Fig. 9).

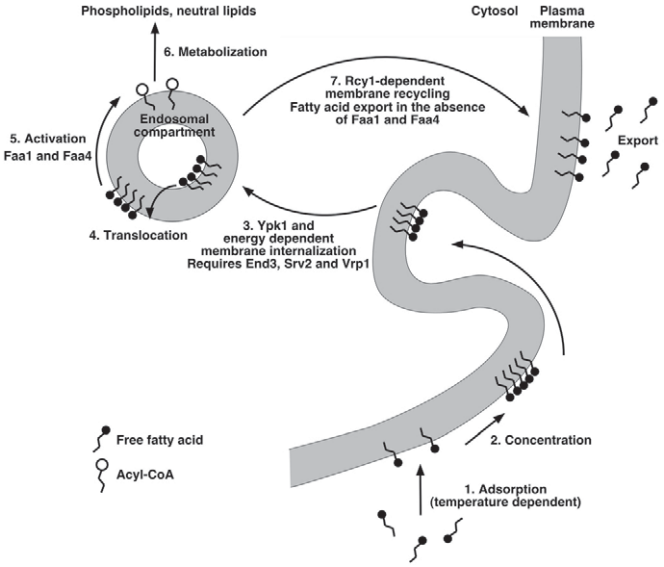

Fig. 9. Working model for a membrane-mediated fatty-acid-uptake pathway. The model postulates that fatty acids first adsorb to the plasma membrane, a step that is blocked at 4°C. They might then concentrate within the plane of the membrane and are internalized in a process that requires energy and a functional endocytic pathway, including Ypk1 function. The internalized membrane then matures to an endosomal compartment. Changes in internal pH and membrane remodelling might then trigger the transbilayer flip-flop of fatty acids. Fatty acids are then CoA-activated and further metabolized. Lack of CoA-dependent retrieval of the free fatty acids might result in export from the cell through membrane recycling.

Discussion

Here we identify Ypk1, the yeast orthologue of the human serum- and glucocorticoid-induced kinase Sgk1, as an important component for regulating fatty-acid uptake and show that efficient uptake of free fatty acids from the yeast plasma membrane depends on endocytosis and membrane recycling. This is thus the first report that links mutations in proteins that have no acyl-CoA-synthetase activity, as is conferred by the presence of Faa1, Faa4 and Fat1, to defects in fatty-acid uptake in yeast. Because the activity of Ypk1 is functionally conserved in the human orthologue Sgk1, endocytosis might constitute an important step in fatty-acid uptake in animal cells.

Ypk1 and Ypk2 are two related serine/threonine protein kinases with highest similarity to mammalian Sgk1 and Akt1, members of the family of AGC kinases (Casamayor et al., 1999). Deletion of *YPK1* results in a slow-growth phenotype and in rapamycin hypersensitivity, whereas loss of *YPK2* shows no apparent phenotype (Chen et al., 1993; Gelperin et al., 2002). A *ypk1Δ ypk2Δ* double mutant is not viable, indicating overlapping function in an essential pathway that can be functionally replaced by Sgk1, and partially by Akt1 (Casamayor et al., 1999). The fatty-acid-uptake defect that we report here is specific for *YPK1*, because a *ypk2Δ* mutant does not display either any growth defect under fatty-acid auxotrophic conditions or a reduced efficiency of oleic-acid uptake, nor does overexpression of Ypk2 ameliorate the uptake defect of a *ypk1Δ* mutant (our unpublished observations).

Ypk-deficient cells are defective in cell-integrity signalling via the Pkc1 effector MAP-kinase cascade that controls the organization of the actin cytoskeleton and transcription of cell-wall-biosynthesis genes. Upregulation of the Rho1 GTPase switch or the Pkc1 effector MAP-kinase pathway suppresses the growth and actin defects of Ypk-deficient cells. The target(s) of Ypk proteins in the cell-integrity pathway, however, is unknown. The finding that lethality owing to Ypk deficiency is suppressed by overexpression of the Rho1 GEFs (guanine-nucleotide-exchange factor) *ROM2* and *TUS1* suggests that the Ypk proteins act upstream of *RHO1* (Schmelzle et al., 2002). Overexpression of *ROM2* or *TUS1*, however, does not rescue the fatty-acid-uptake defect of *ypk1Δ* mutant cells, indicating that cell-integrity signalling does not control fatty-acid uptake (our unpublished observations).

Ypk1 is required for fluid-phase and for receptor-mediated endocytosis, but the precise role of Ypk1 in these processes is not completely understood (deHart et al., 2002). Given that *ypk1Δ* has profound effects on actin organization, which is crucial for endocytosis (for a review, see Moseley and Goode, 2006), it might be that the apparent role of Ypk1 in endocytosis depends on its function in actin organization (Roelants et al., 2002). Consistent with this proposition, we found that mutations in actin-organizing components such as End3, Vrp1 and Srv2 have a growth defect under fatty-acid auxotrophic conditions and display reduced uptake of oleic acid and Bodipy-C12 (Fig. 8). The fact that not all mutants that affect endocytosis exhibit growth defects under fatty-acid auxotrophic conditions or display fatty-acid-uptake defects is probably due to the fact that they either act too late, i.e. after membrane internalization, or do not block the internalization step tightly enough (our unpublished observations).

Ypk1 is itself phosphorylated and activated by Pkh kinases, homologues of mammalian 3′-phosphoinositide-dependent kinase 1 (PDK1) (Casamayor et al., 1999). Analysis of conditional Pkh mutants (*pkh1Δ pkh2*^{ts}) (Casamayor et al., 1999; Inagaki et al., 1999; Roelants et al., 2002), however, indicates that the function of Ypk1 in fatty-acid uptake is independent of the sphingoid-base-activated Pkh kinases (our unpublished observation). In support of this notion, we found that *lcb1-100*, a conditional mutant in sphingoid-base synthesis by serine palmitoyl transferase (Lcb1), does not block uptake of fatty acids. Similarly, treatment of wild-

type cells with an inhibitor of Lcb1, myriocin, does not block uptake of fatty acids (our unpublished observation). Sphingoid-base synthesis is required for the internalization step of endocytosis, because an *lcb1-100* strain is defective in receptor and fluid-phase endocytosis and the addition of sphingoid base suppresses this defect (Munn and Riezman, 1994; Zanolari et al., 2000). Ypk1 is localized in the cytosol and displays long-chain-base-dependent translocation to the plasma membrane (Sun et al., 2000). No comparable translocation of Ypk1-GFP to the plasma membrane is observed upon addition of fatty acids (our unpublished observations).

Pkh and Ypk kinases also affect eisosome assembly (Walther et al., 2007; Luo et al., 2008). Eisosomes are large protein complexes beneath the plasma membrane that are important for plasma-membrane organization and for endocytosis (Grossmann et al., 2007; Walther et al., 2006). Their major protein components, Pil1 and Lsp1, are phosphorylated by Pkh kinases and this sphingoid-base-dependent phosphorylation is crucial for proper eisosome assembly (Frohlich et al., 2009). Eisosomes are aberrantly enlarged in *ypk1Δ* mutant cells and this is likely to affect plasma-membrane organization and function, because the plasma membrane is laterally segregated into different domains and this organization is important for regulated protein turnover, and possibly for lipid internalization and turnover (Grossmann et al., 2007; Grossmann et al., 2008; Luo et al., 2008; Frohlich et al., 2009; Roelants et al., 2010). Pil1 and Lsp1 mutants, however, do not affect fatty-acid uptake, indicating that proper eisosome assembly is not important for fatty-acid uptake (our unpublished observation). Similarly, deletion of an integral membrane protein of the plasma membrane that seems to coordinate the lateral organization of the plasma membrane with sphingolipid production and eisosome assembly, Nce102, does not affect fatty-acid uptake (Frohlich et al., 2009; Grossmann et al., 2008) (our unpublished observation). These observations indicate that plasma-membrane organization is less crucial for efficient fatty-acid uptake in yeast, than, for example, in adipocytes, in which depletion of plasma-membrane cholesterol reversibly inhibits oleate uptake (Covey et al., 2007). Moreover, uptake of long-chain fatty acids in animal cells depends on proper localization of FAT to plasma-membrane lipid rafts (Ehehalt et al., 2006; Pohl et al., 2005).

A driving force for fatty-acid uptake is the metabolic demand for fatty acids. Both gain- and loss-of-function experiments indicate that fatty-acid uptake in animal cells can be modulated by activation at both the plasma membrane and internal sites, by intracellular fatty-acid-binding proteins and by enzymes in synthetic or degradative metabolic pathways (Mashek and Coleman, 2006; Richards et al., 2006). Overexpression of an ER-localized acyl-CoA synthase, for example, leads to increased fatty-acid uptake (Milger et al., 2006). Converting fatty acids to acyl-CoAs and downstream metabolic intermediates increases cellular fatty-acid uptake, possibly by limiting efflux of the free fatty acid and/or by removing the free fatty acid from the equilibrium between extracellular and intracellular free-fatty-acid concentrations (Milger et al., 2006). Consistent with such a view, a yeast *faa1Δ faa4Δ* double mutant has recently been shown to export fatty acids into the culture medium (Michinaka et al., 2003; Scharnewski et al., 2008). This mode of fatty-acid export is not compatible with vectorial acylation, because ATP and CoA required for the synthesis of acyl-CoA would need to be present in the extracellular space (Scharnewski et al., 2008). Thus, intracellular conversion of the free fatty acid to its acyl-CoA derivative might be required to trap the fatty acid in a cytosolic compartment rather than for the actual membrane-translocation step.

The observation that cells that cannot CoA-activate free fatty acids export fatty acids into the culture medium could also explain why the *faa1Δ faa4Δ* double mutant does not accumulate free fatty acids in an import assay or show elevated levels of fatty acids that are BSA extractable. In these cells the plasma membrane might be saturated already with endogenously synthesized fatty acids and hence unable to adsorb exogenously provided fatty acids. Consistent with such a proposition, energy depletion of these cells, which will also deplete the pool of fatty acids that are exported, restores the capacity of the plasma membrane to adsorb exogenously provided fatty acids.

Deletion of *YPK1* results in slow growth and rapamycin hypersensitivity (Chen et al., 1993; Gelperin et al., 2002). Ypk-deficient cells exhibit translational initiation arrest and, in wild-type cells, Ypk1 is rapidly degraded upon nitrogen starvation (Gelperin et al., 2002). These findings suggest that Ypk is a component of a nutrient-sensing pathway that can regulate translation initiation. A genetic screen revealed that a truncated version of *YPK2* suppresses the lethality of a strain with *TORC2* (target of rapamycin complex 2) deletion, and TORC2 can phosphorylate Ypk1 in vitro, indicating that TORC2 acts as an upstream regulator of Ypk proteins (Kamada et al., 2005). Ypk1 and TORC2 act in the same pathway, because overexpression of the Rho1-GEF-encoding gene *TUS1* suppresses Ypk and *tor2* mutants (Schmelzle et al., 2002). These data thus indicate that Ypk integrates cell-integrity signalling from TORC2 with a heat-responsive sphingoid-base signalling through the Pkh kinases (for reviews, see Jacinto and Lorberg, 2008; Dickson, 2008). The TORC2 complex has recently been shown to directly phosphorylate Ypk2 to regulate growth and ceramide synthesis in *S. cerevisiae* (Aronova et al., 2008). In *Caenorhabditis elegans*, by contrast, one physiological role of TORC2 is to regulate neutral-lipid accumulation and this function is mediated by Sgk1 (Jones et al., 2009; Soukas et al., 2009). Yeast mutants in TORC2, however, do not affect fatty-acid uptake, indicating that the reduced uptake of fatty acids observed in *ypk1Δ* mutant cells does not depend on its activation by TORC2 (our unpublished observation).

Ypk1 is required for the mediated and/or facilitated component of the uptake kinetics (Fig. 3F). In the absence of Ypk1, fatty acids are still taken up, but at greatly reduced efficiency. This reduced uptake efficiency is physiologically important because it probably accounts for the milder growth defect of cells under fatty-acid auxotrophic conditions compared with those that are completely blocked in fatty-acid utilization, i.e. *faa1Δ faa4Δ* (Fig. 1B). The reduced uptake efficiency in *ypk1Δ* mutant cells correlates with a reduced rate of fluid-phase endocytosis, assessed by monitoring uptake of Lucifer Yellow, as well as a reduced rate of membrane internalization, assessed by uptake of the lipophilic dye FM4-64 (our unpublished observation). Given that loss of Ypk1 function does not completely block endocytosis, we think that the residual membrane internalization accounts for the uptake of fatty acids observed in the biochemical uptake assays, i.e. the passive component of the uptake kinetics. On the basis of these observations, we postulate that membrane internalization from the plasma membrane is important for efficient uptake of fatty acids (Fig. 9). Membrane-mediated internalization of fatty acids could account for higher uptake capacity and/or reduced energetic demand compared with a strictly protein-dependent uptake pathway. Consistent with such a membrane-mediated uptake pathway, we find that mutants that affect the reorganization of the actin cytoskeleton, which is required for membrane internalization, i.e.

end3Δ, *srv2Δ* and *vrp1Δ*, and mutants that affect recycling between the early endosome and the plasma membrane, such as *rcy1Δ*, are impaired in fatty-acid uptake (Fig. 8).

A possible connection between endocytosis and fatty-acid uptake is indicated by studies in other systems, such as *Dictyostelium* and human microvascular endothelial cells. In the amoeba, a soluble form of the long-chain fatty acyl-CoA synthase (LC-FACS1) is localized on endosomal membranes and mutants in LC-FACS have a strong defect in intracellular accumulation of the fluorescent fatty-acid analogue Bodipy-C12. These observations have been taken to suggest that LC-FACS mediates the retrieval of fatty acids from the lumen of endosomes into the cytoplasm (von Lohneysen et al., 2003). Whether endocytosis is generally required for fatty-acid uptake in this organism, however, has not been investigated. In microvascular endothelial cells, by contrast, clathrin-coated vesicles and caveolae are important for a high-capacity, low-affinity component of the fatty-acid-uptake process (Ring et al., 2002).

Elevated levels of circulating fatty acids are sufficient to induce insulin resistance in mice and pharmacological inhibition of intracellular fatty-acid-binding protein renders mice resistant against diet-induced obesity and reduces the inflammatory response of lipid-loaded macrophages (Boden et al., 2005; Furuhashi et al., 2007). These examples illustrate that uptake and cellular utilization of fatty acids is key to the metabolic and inflammatory response against a lipid load and they emphasize the requirement to understand the process at a molecular level. The identification of a conserved protein kinase as a potential regulator of fatty-acid uptake now provides the opportunity to examine the role of this kinase in energy homeostasis in more detail.

Materials and Methods

Yeast strains and growth conditions

Yeast strains and their genotypes are listed in supplementary material Table S1. The haploid gene-deletion collection in the BY4742 background was obtained from EUROSCARF [see http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html; (Winzeler et al., 1999)]. Strains were cultivated in YPD-rich media [1% Bacto yeast extract, 2% Bacto peptone (USBiological, Swampscott, MA), 2% glucose] or minimal media [0.67% yeast nitrogen base without amino acids (USBiological), 2% glucose, 0.73 g/l amino acids]. Fatty-acid-supplemented media contained 1% Brij 58 (Fluka, Buchs, Switzerland), 0.5 mM palmitic acid and 0.5 mM stearic acid (Sigma). Fatty-acid de novo synthesis was inhibited by addition of cerulenin (10 µg/ml; Alexis Biochemicals, Lausen, Switzerland) to fatty-acid-supplemented plates (Omura, 1981). Selection for the kanMX4 marker was on media containing 200 µg/ml G418 (Gibco BRL, Life Technologies). For the depletion of the transmembrane potential, cells were incubated with 20 µM FCCP [carbonyl cyanide 4-(trifluoromethoxy)phenyl hydrazone] for 30 minutes in media buffered to pH 5.5. The efficiency of depletion was assessed by staining cells with methylene blue. More than 80% of cells were stained blue by this protocol.

Fatty-acid-uptake assay

Cells were first cultured in media containing 0.1% Brij 58 and 1 mM oleic acid overnight at 24°C. Cells in the logarithmic growth phase were then concentrated to 20 optical density (OD)/ml in fresh fatty-acid-supplemented medium and pre-incubated for 30 minutes at either 24°C or 37°C. The uptake assay was started by the addition of 20 µCi/ml [9-10-^{3}H] oleic acid (10 mCi/ml; American Radiolabeled Chemicals, St Louis, MO). Equal aliquots of cells were removed after 2.5, 5, 10, 20 and 60 minutes of incubation at the respective temperature. Cells were metabolically poisoned by the addition of NaN_3 and NaF (20 mM each) and samples were placed on ice. Cells were then washed three times with ice-cold 0.5% tergitol, broken with glass beads, and lipids were extracted with chloroform/methanol (1:1; vol/vol) and equal aliquots were brought to dryness. Lipids were separated on TLC plates (Merck, Darmstadt, Germany), developed in petroleum ether/diethylether/acetic acid (70:30:2; per vol) and quantified by scanning with a Berthold Tracemaster 20 Automatic TLC-Linear Analyzer (Berthold Technologies, Bad Wildbach, Germany). TLC plates were then exposed to a tritium-sensitive screen and visualized using a phosphorimager (Bio-Rad Laboratories, Hercules, CA). For determination of protein concentration, 3 OD units of cells from the time 0 sample were extracted by NaOH, proteins were TCA precipitated, and quantified using a Lowry assay and BSA as standard (Horvath and Riezman, 1994).

For the kinetic experiments, cells were incubated with different concentrations of oleic acid for 2.5 and 5 minutes at 24°C, cells were poisoned, and uptake and incorporation of the label into lipids was quantified as described above. Data was evaluated using LMMPro (Alfisol, Coventry, CT).

For back-extraction of accessible fatty acids by delipidated BSA, cells were washed with tergitol as described above and washed once more with 1% BSA in PBS. Lipids were then extracted from the cell pellet and the BSA fraction, separated by TLC, and quantified by radio-scanning.

Flow-cytometry analysis

For the analysis of Bodipy-C12 uptake, cells were cultivated in oleic-acid- and Brij-58-supplemented media, diluted to 0.5 OD/ml and incubated with 50 µM of Bodipy-C12 (4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid; Invitrogen, Carlsbad, CA, catalogue #D-3823) for 30 minutes at either 24°C or 37°C. Cells were washed twice with fatty-acid-free BSA (50 µM in PBS). Extracellular fluorescence was quenched by incubating the cells with 0.33 mM Trypan Blue (Li et al., 2005); cells were diluted to 2×10^5 cells/ml and analyzed by fluorescent cell sorting using a CyFlow Space (Partec, Münster, Germany). All recordings were made using identical instrument settings to allow for a quantitative comparison of the cell-associated fluorescent intensity.

Fluorescence microscopy

Cells were stained with Bodipy-C12 followed by an incubation with Trypan Blue, as described above, mounted on a glass slide and examined by fluorescence microscopy using a Zeiss Axioplan 2 (Carl Zeiss, Oberkochen, Germany) equipped with an AxioCam CCD camera and AxioVision 3.1 software. All recordings were made using identical exposure settings to allow for a semi-quantitative readout of the fluorescent intensity. Lipid droplets were visualized using Erg6-RFP as a marker for this organelle. Uptake of the fluid-phase-marker Lucifer Yellow was performed as described (Wiederkehr et al., 2000).

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