BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

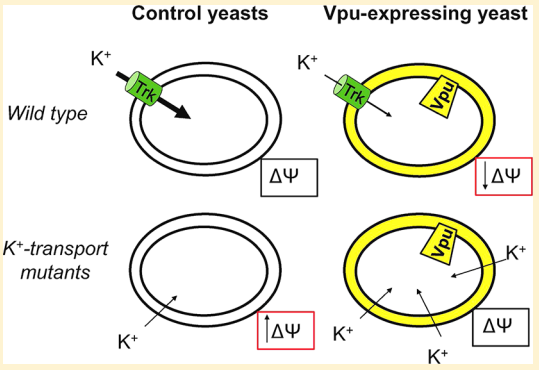
pubs.acs.org/biochemistry

HIV-1 Vpu Protein Mediates the Transport of Potassium in *Saccharomyces cerevisiae*

Laura Herrero, Noemí Monroy, and María Eugenia González*

Unidad de Expresión Viral, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Carretera de Majadahonda-Pozuelo Km 2, 28220 Majadahonda, Madrid, Spain

ABSTRACT: Human immunodeficiency virus type 1 (HIV-1) Vpu is an integral membrane protein that belongs to the viroporin family. Viroporins interact with cell membranes, triggering membrane permeabilization and promoting release of viral particles. In vitro electrophysiological methods have revealed changes in membrane ion currents when Vpu is present; however, in vivo the molecular mechanism of Vpu at the plasma membrane is still uncertain. We used the yeast *Saccharomyces cerevisiae* as a genetic model system to analyze how Vpu ion channel impacts cellular homeostasis. Inducible expression of Vpu impaired cell growth, suggesting that this viral protein is toxic to yeast cultures. This toxicity decreased with extracellular acidic pH. Also, Vpu toxicity diminished as the extracellular K^+ concentration was increased. However, expression of the Vpu protein suppresses the growth defect of K^+ uptake-deficient yeast (Δ*trk*1,2). The phenotype rescue of these highly hyperpolarized cells was almost total when they were grown in medium supplemented with high concentrations of KCl (100 mM) at pH 7.0 but was significantly reduced when the extracellular K^+ concentration or pH was decreased. These results indicate that Vpu has the ability to modify K^+ transport in both yeast strains. Here, we show also that Vpu confers tolerance to the aminoglycoside antibiotic hygromycin B in Δ*trk*1,2 yeast. Our results suggest that Vpu interferes with cell growth of wild-type yeast but improves proliferation of the hyperpolarized *trk*1,2 mutant by inducing plasma membrane depolarization. Furthermore, evaluation of the ion channel activity of the Vpu protein in Δ*trk*1,2 yeast could aid in the development of a high-throughput screening assay for molecules that target the retroviral protein.



The yeast *Saccharomyces cerevisiae* has been widely recognized as a model system for ion homeostasis studies of eukaryotic cells. Control of expression levels with inducible promoters and use of host strains with orthologous gene deletions facilitate functional studies of heterologous proteins in yeast. In addition, alkali metal cation transport and homeostasis in yeast have been characterized in great detail. Fine analyses performed with M2 of influenza virus, Kcv of PBCV-1, and PEDV ORF3 have proven the usefulness of the *S. cerevisiae* model for characterizing ion channel activity of viroporins.[1−3]

The Vpu protein of human immunodeficiency virus type 1 (HIV-1) is an oligomeric type I integral membrane protein.[4,5] Vpu enhances the release of virus particles by a still uncertain mechanism.[6] At present, when trying to explain the role that Vpu plays at this stage of the virus replication cycle, we find two reported activities for the viral protein that stand out. The first is the enhanced release of virus particles after Vpu antagonism with the IFN inducible factor tetherin (BST2).[7] A second approach focuses on similarities between Vpu and other small hydrophobic viral proteins (viroporins) that have been suggested to promote viral particle release by plasma membrane permeabilization (as reviewed in refs 8 and 9). Vpu associates with the plasma membrane, permeabilizing it to nonpermeating molecules.[10,11] Evidence has been provided that Vpu oligomers form ion channels or pores in the plasma membrane.[12,13] Besides Vpu, several other viroporins, including influenza M2, HCV p7, PBCV-1 KcV, and Coronavirus E protein, among others, have membrane permeabilization activity and ion channel capacity.[14−17] However, these viroporin-formed ion channels differ in the number of assembled monomer units, subcellular localization, and membrane topology.[9] Early ex vivo and in vivo studies demonstrated that Vpu forms cation-selective channels in lipid bilayers and also permeabilizes the *Escherichia coli* plasma membrane to proline.[18] Further studies performed in *Xenopus* oocytes suggested that Vpu ion channels are selective for monovalent cations over anions.[19] Nuclear magnetic resonance spectroscopy and computer simulations pointed to a homopentamer structure with some resemblance to potassium channels and weak cation selectivity.[20−22] 5-(*N*,*N*-Hexamethylene) amiloride (HMA) blocks Vpu-induced cation currents in planar lipid bilayers and inhibits HIV-1 replication in macrophages.[23,24] A novel inhibitor, BIT225, also inhibits Vpu ion channel activity.[25] Notably, Vpu-mediated tetherin antagonism is not affected by this viroporin inhibitor,[26] because ion channel activity and tetherin antagonism activities localize at different positions in the Vpu transmembrane domain.[27]

Received: August 17, 2012
Revised: December 13, 2012
Published: December 14, 2012

 © 2012 American Chemical Society dx.doi.org/10.1021/bi3011175 | *Biochemistry* 2013, 52, 171−177

Vpu interacts with mammalian TASK-1, a weak inward K^+ rectifier, and this interaction leads to mutual functional destruction.[28] In lymphoid cells, Vpu inhibits TASK-1 conductance, enhancing the release of the virus particle. In addition, in the absence of Vpu, overexpression of the first transmembrane segment from TASK-1 causes a similar enhancing effect on the release of virus. Further studies have demonstrated that the efficiency of HIV-1 release is inversely correlated with membrane potential stability.[29]

Here we used *S. cerevisiae* as a genetic model to analyze the ion channel capacity of Vpu protein. By heterologously expressing Vpu, we were able to functionally complement phenotypes with defective K^+ uptake. Vpu complementation was dependent on extracellular pH and mediated by depolarization of the plasma membrane.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Microbial Strains. The *vpu* gene sequence from the BH10 clone of HIV-1[30] was cloned into yeast expression vector pEMBLyex.[31] Following standard procedures, the viral sequence was inserted at *Bam*HI and *Hin*dIII sites in the polylinker. The exogenous gene is controlled by the *CYCGAL1* promoter, which is tightly repressed by glucose and strongly induced by galactose. The *E. coli* DH5α strain[32] was used for the construction of the *E. coli*–yeast shuttle vector. Two *S. cerevisiae* strains were used, W303-1B[33] and the isogenic mutant WΔ3 (*trk1::LEU2*, *trk2::HIS3*),[34] which were generously provided by L. Carrasco (CBM, Universidad Autónoma de Madrid, Madrid, Spain) and A. Rodríguez Navarro (ETSI Agrónomos, Universidad Politécnica de Madrid, Madrid, Spain), respectively.

Yeast Media, Transformation, and Induction. Yeast cells were grown at 30 °C, with orbital shaking at 300 rpm, in standard YNB glucose medium supplemented with 20 mg/L L-tryptophan, 40 mg/L adenine (W303-1B strain) or 20 mg/L adenine (WΔ3 strain), and 20 mg/L L-histidine (only W303-1B). For production of competent yeast and transformation, we used the standard lithium acetate protocol.[35] Transformants of the W303-1B strain were maintained on selective medium (containing 20 mg/L L-leucine). To achieve *vpu* expression, yeast cells were cultured in YNB medium containing 2% galactose and required supplements (inducing medium). To avoid extraneous cations in the growth medium, we adjusted the pH using arginine.[36] When required, solid medium was prepared by the addition of 1.5% purified agar (Difco) to the broth.

Assay Conditions. All experiments were independently repeated at least three times, using at least three cultures derived from independent colonies, with consistent results. Growth curves represent average data from three experiments. Before any experiments, cells were grown in noninducing medium, adjusted to pH 6.5 with arginine, to the exponential phase. After being washed with Milli-Q water,[36] cells were diluted in fresh inducing medium, and the cell density was properly adjusted. The cell growth was estimated by measuring the optical density at 600 nm (OD_{600}) (Genesys 10 VIS Thermo Scientific spectrophotometer). Incubations were conducted at 30 °C, with shaking at 300 rpm. Mutant cells were grown in medium supplemented with 100 mM KCl to fulfill their K^+ requirements. Liquid cultures were started with exponentially growing cells that were diluted in inducing medium to an OD_{600} of 0.005 (corresponding to 1.5×10^5 cells/mL). In growth kinetic assays, after incubation for 24 h (T = 0 h), samples were collected and the OD_{600} was determined. Agar plate assays were performed with exponentially growing cells. After cells had been washed, cultures were diluted with fresh medium to an OD_{600} of 0.2 (glucose) or 0.4 (galactose). Drop tests were performed by spotting 3 or 4 μL of serial dilutions of the cell suspension onto agar plates. These tests used the same (noninducing or inducing) medium, adjusted to pH 7.0, for dilutions and also for the preparation of agar plates. Plates were incubated for the indicated time at 30 °C and then scanned.

Statistical Analysis. To compare growth differences between control and transfected cultures, scatter plots were created with data from replicated experiments. The growth phase was divided into two or three segments that were independently fit to linear regression lines, and slopes with a correlation coefficient were calculated using Microsoft Excel.

Protein Extraction and Western Blot. Exponentially growing cells were diluted in YNB (2% galactose) to an OD_{600} of 0.005 before being incubated. When cultures reached an OD_{600} of 1.0, 1 mL samples were collected for protein extraction using the following procedure. Cells were pelleted and then resuspended in 1 mL of lysis buffer [0.2 M NaOH (Merck), 0.1 M β-mercaptoethanol (Merck), and 0.1 mM PMSF (Roche)]. After incubation on ice for 5 min, 2 μL of 100% TCA was added and the cell lysate was incubated at 65 °C for 5 min and then incubated at 4 °C for 5 min. The nonsoluble fraction was collected by centrifugation and washed with acetone at −20 °C (Merck). The pellet was speed-vac dried, and then the protein extract was resuspended in 50 μL of PBS and sonicated. The total protein content of each extract was assessed using the protein assay kit (Bio-Rad). All cellular extracts were adjusted to equal protein concentrations (1 mg/mL) with PBS. Then, an equal volume of 2× sample buffer [0.32 M Tris-HCl (pH 6.8) (Merck), 26.6% glycerol (Merck), 4% SDS (Serva), 3% DTT (Sigma), and 0.066% bromophenol blue (Sigma)] was added to each extract. Finally, samples were heated to 100 °C for 5 min, before being loaded onto a 20% polyacrylamide gel for separating proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After gel running, Vpu and PGK proteins were detected by Western blotting. Proteins were transferred to nitrocellulose membranes (Trans-blot Transfer Medium Bio-Rad) by wet blotting. The efficiency of protein transference was checked by staining proteins on the membrane using Ponceau S. Blots were quickly destained in distilled water and then incubated with the Vpu polyclonal antibody[10] and the PGK monoclonal antibody (Molecular Probes) (diluted 1:5000). After incubation with a secondary peroxidase-conjugated goat (anti-rabbit/anti-mouse) IgG antibody (Pierce) (diluted 1:10000), labeled proteins were detected using ECL Western Blotting Detection Reagents (Amersham). Blots were exposed to X-ray films to visualize the chemiluminescence signal.

Hygromycin B Sensitivity. Changes in membrane potential in the yeast plasma membrane were tested by measuring the entry of the antibiotic hygromycin B (Roche). Cells were grown to exponential phase, washed with Milli-Q water, resuspended in inducing medium, adjusted to pH 7.0, and supplemented with 30 or 100 mM KCl. The final cell density was adjusted to an OD_{600} of 0.003. Increasing concentrations of hygromycin B were added to cells. After incubation for 45 or 48 h, the cellular density of each culture was determined by measuring the OD_{600}. Relative growth was

calculated as the ratio between growth in the presence and absence of added antibiotic and expressed as a percentage.

RESULTS

Inducible Expression of Vpu Protein in *S. cerevisiae*. Our initial rationale was that structural and functional similarities between yeast and human cell membranes[37,38] might help characterize in vivo how Vpu–plasma membrane interactions permeabilize cells. We used high-copy number plasmid pEMBLyex4 to express Vpu in *S. cerevisiae* W303. Phosphoglycerate kinase (PGK), one of the most abundant proteins in the cell, allowed us to monitor the relative content of the viral protein. As shown in Figure 1A, yeast growth in induction liquid medium yielded cells producing high levels of Vpu protein when bearing the *vpu* plasmid. Clones bearing the *vpu* plasmid or the empty plasmid (control cells) were grown in solid minimal medium containing glucose and also in induction medium containing galactose (Figure 1B). In glucose medium, both clones grew at the same levels. However, in induction medium, cells expressing the *vpu* gene showed impaired growth relative to that of control cells. The growth defect of Vpu-expressing cells disappeared after a longer incubation (data not shown); even so, the drop test documented growth differences of an ~2-fold dilution. This finding suggests that HIV-1 Vpu exhibits deleterious effects on yeast cells but that cultures can recover from such stress, probably using their adaptive plasticity in response to changes in the ionic fluxes.[39]

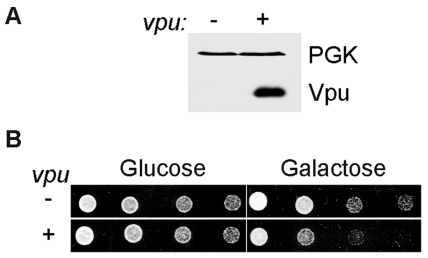


Figure 1. Growth impairment of cells expressing the *vpu* gene. Cells, transformed with control plasmid (−) or *vpu* plasmid (+), were inoculated into glucose- or galactose-containing medium. (A) Yeasts were grown in liquid medium with vigorous aeration. Expression levels of Vpu protein were analyzed in each culture by Western blot, using PGK or Vpu antibodies. (B) Drop test analysis with series of 2-fold dilutions. Before being digitally scanned, plates were incubated for 2 (glucose) or 3 (galactose) days.

Sensitivity to Extracellular Conditions. Some channels expressed in yeast are only functional over a narrow pH range.[40] We assessed the growth characteristics on liquid cultures of both clones bearing empty or *vpu* plasmids at various pH values (Figure 2). A time course comparison of both transformants at pH 5.4 revealed a nearly indistinguishable growth pattern, although growth of *vpu*-expressing cells was slightly delayed. Thus, growth differences were shown from the beginning of the growth (slopes S1). At more neutral pH values, growth differences were more evident. Thus, major differences in growth rates between both cultures were reached at pH 7.0. The highest rate of growth was reached by *vpu*-expressing cells just after control cells moved to the early stationary growth phase (pH 6.5 and 7.0). Western blot analysis, with PGK serving as a loading control, demonstrated that Vpu levels did not vary with the pH of the medium (data not shown). However, after longer adaptation periods as the

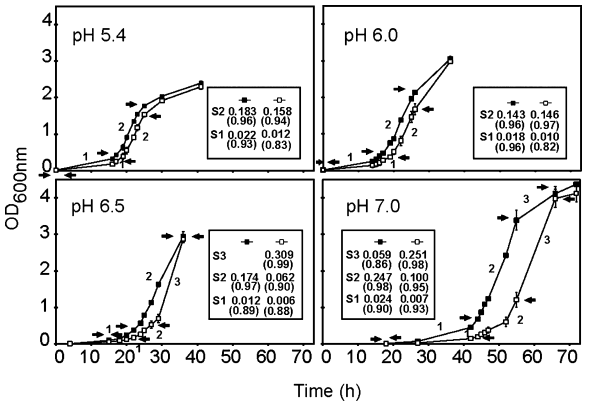


Figure 2. pH_{out} sensitivity of the Vpu activity in *S. cerevisiae*. Cells were grown in inducing medium adjusted with arginine to the corresponding pH. OD_{600} was measured at the indicated times: control (■) and Vpu (□). Growth curves represent the averages of three independent experiments with error bars indicating the standard deviation (mean ± SD). Linear regression lines were fit to the replicate data included in two or three segments indicated with arrows in each curve. S1–S3 indicate the corresponding values obtained for the slope (R^2) of the early (1), middle (2), and late (3) segments, respectively.

pH became more neutral, *vpu*-expressing cultures reached the same level of growth as the control. These results suggested that *vpu*-expressing cells were unable to adapt to neutral pH_{out} as efficiently as control cells, indicating that the deleterious effect of Vpu was pH-dependent.

Having established that Vpu-induced growth impairment is modulated by extracellular pH, we were interested in investigating how Vpu protein may affect transport systems at the plasma membrane. Potassium is the major inorganic cation in the cell cytoplasm. Although yeast cells can grow in media with a wide range of extracellular potassium concentrations (low millimolar to micromolar), intracellular stores are always maintained at ~200–300 mM by means of a mechanism of transport that may vary depending on the growth conditions.[41] We analyzed the effect of *vpu* when yeasts were cultured with varying extracellular potassium concentrations, by supplementing growth media with 10 or 100 mM KCl (Figure 3). Both *vpu*-expressing and control cultures reached faster growth rates with increasing potassium concentrations. However, under conditions with standard medium (7 mM K^+), the growth delay of *vpu*-expressing cells was significant; they hardly reached the log phase when the control culture underwent the transition from the log phase to the stationary phase. When growth medium was supplemented with 10 mM KCl, *vpu* expression delayed the early growth; however, the cells recovered rapidly, so that growth differences between both cultures were reduced. When growth medium was supplemented with 100 mM KCl, *vpu* expression slightly delayed the early growth; however, cells recover rapidly from it, and growth differences were almost indistinguishable between both transformants. These results suggest that the Vpu protein interferes with potassium uptake or its accumulation inside cells.

Channel Activity in K^+ Uptake-Deficient Cells. The conditional negative phenotype of Δ*trk*1,2 cells has been used to detect the function of heterologously expressed potassium channels by restoration of growth.[40] Therefore, the auxotrophic double-mutant strain, WΔ3, was used for phenotypic complementation studies, allowing us to specifically test for the K^+ channel activity of Vpu. In this strain, the deletion of

dx.doi.org/10.1021/bi3011175 | *Biochemistry* 2013, 52, 171–177

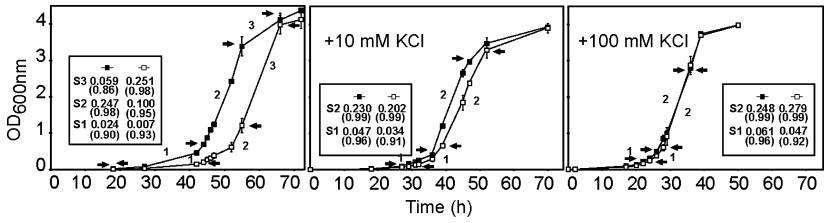


Figure 3. Extracellular K^+ concentration dependency of growth impairment by Vpu protein. Cells, transformed with control or *vpu* plasmid, were grown in inducing medium adjusted to pH 7.0 and containing different K^+ concentrations. OD_{600} is plotted vs time (in hours): control (■) and Vpu (□). Growth curves represent the averages of three independent experiments with error bars indicating the standard deviation (mean ± SD). Linear regression lines were fit to data included in two or three segments indicated with arrows in each curve. S1−S3 indicate the corresponding values obtained for the slope (R^2) of the early (1), middle (2), and late (3) segments, respectively.

TRK1 and TRK2 transporters abolishes high-affinity uptake of extracellular potassium, resulting in impaired cellular growth in potassium-limiting medium. We transfected the K^+ uptake-deficient strain with control or *vpu*-containing plasmids. As shown in Figure 4, when cells were grown in the presence of

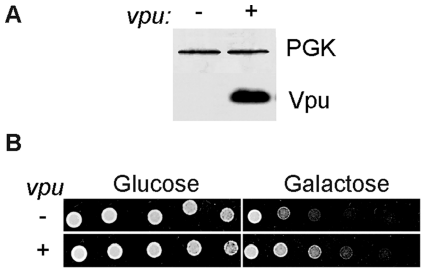


Figure 4. Complementation of the growth defect by *vpu* expression in a Δ*trk*1 Δ*trk*2 yeast mutant. Cells, transformed with control (−) or *vpu* plasmid (+), were inoculated into glucose or galactose medium, supplemented with 100 mM KCl. (A) Cells were grown in liquid medium with vigorous aeration. Western blot used PGK or Vpu antibodies. (B) Drop test analysis of serial 2-fold dilutions. Before being digitally scanned, plates were incubated for 2 (glucose) or 3 (galactose) days.

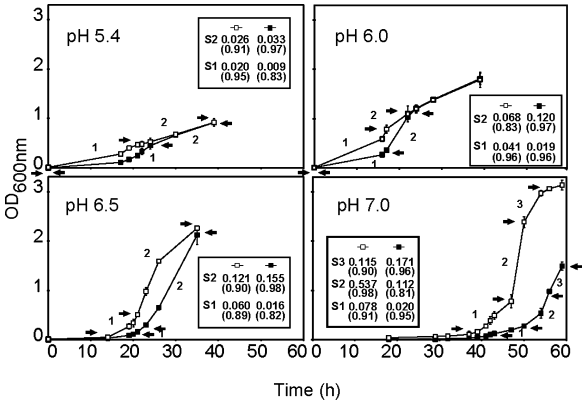


Figure 5. Acid sensitivity of Vpu channel activity. Δ*trk*1 Δ*trk*2 cells were grown in inducing media, supplemented with 100 mM KCl and adjusted with arginine to the appropriate pH values. OD_{600} was measured at the indicated times: control (■) and Vpu (□). Growth curves represent the averages of three independent experiments with error bars indicating the standard deviation (mean ± SD). Linear regression lines were fit to data included in two or three segments indicated with arrows in each curve. S1−S3 indicate the corresponding values obtained for the slope (R^2) of the early (1), middle (2), and late (3) segments, respectively.

galactose, high levels of Vpu protein were synthetized by cells transfected with the plasmid containing the retroviral gene. Notably, these *vpu*-expressing cells grew to a greater extent than control cells (Figure 4B). Drop tests showed that both cultures differed by a >2-fold dilution. These results revealed that Vpu can rescue the growth phenotype of Δ*trk*1,2. Vpu complementation of the yeast Trk system corroborated its ion permeation ability. Unfortunately, it was not possible to prove the capacity of HMA to inhibit this Vpu-induced growth enhancement; the intrinsic stimulation of yeast growth by the DMSO solvent was too high to permit reliable detection of growth differences induced by the dissolved drug (data not shown).

Double-mutant yeasts exhibit hypersensitivity to low extracellular pH that can be suppressed by the addition of high concentrations of potassium to the medium.[42] We tested the impact of Vpu on the growth behavior of mutant cells at different extracellular pH values ranging from 5.4 to 7.0 in the presence of 100 mM KCl (Figure 5). When grown at pH 5.4, mutant cells could not reach the log phase, but a slight advantage of *vpu*-expressing culture was detected for early growth compared with the control mutant. As the pH increased, the Vpu-induced growth advantage became more pronounced. At pH 7.0, the difference in OD_{600} between both cultures reached 8.7 ± 0.35-fold (50 h); in the absence of Vpu protein, the log phase was delayed but Vpu endowed mutant cells with growth characteristics similar to those of isogenic wild-type cells (Figure 3). Thus, these observations demonstrated that Vpu restored the growth of K^+ uptake-deficient cells.

To gain further insight into the mechanism by which Vpu is involved in rescuing the mutant phenotype, potassium requirements for growth stimulation of Trk-deficient cells were tested (Figure 6). Growth of mutant cells depended on the presence of high concentrations of extracellular K^+, but Vpu endowed mutant cells with a growth advantage, even at a low extracellular potassium concentration (10 mM KCl). We observed that K^+ uptake mutants exhibited growth defects. The final OD_{600} and growth slope of mutants are smaller than isogenic wild-type values (Figure 3). However, at a nonlimiting potassium concentration (100 mM), Vpu protein shortened the lag phase and increased the growth yield of the mutant strain, which now exhibited growth kinetics similar to those of the wild-type strain. These results indicated that Vpu protein was able to catalyze low-affinity K^+ uptake.

Impact of Vpu on Low-Affinity K^+ Transport. Similar ionic properties of potassium and ammonium permit, at limiting potassium concentrations, the entry of ammonium via potassium channels. Thus, ammonium inhibits low-affinity K^+ uptake in Δ*trk*1,2 cells.[34] To avoid any competitive ammonium

dx.doi.org/10.1021/bi3011175 | *Biochemistry* 2013, 52, 171−177

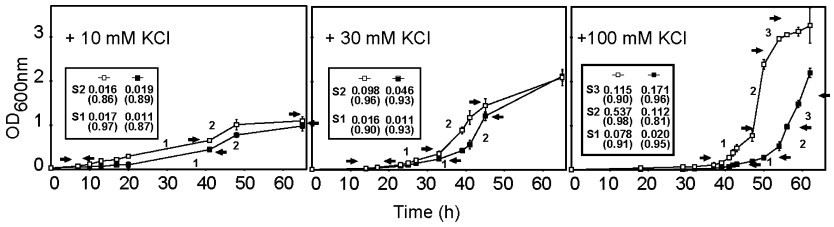


Figure 6. Modulation of Vpu phenotype complementation in *trk*1,2 null mutants by extracellular K^+ concentration. Δ*trk*1 Δ*trk*2 cells, transformed with control or *vpu* plasmids, were grown in inducing media adjusted to pH 7.0 and containing different K^+ concentrations. OD_{600} is plotted vs time (in hours): control (■) and Vpu (□). Growth curves represent the averages of three independent experiments with error bars indicating the standard deviation (mean ± SD). Linear regression lines were fit to data included in two or three segments indicated with arrows in each curve. S1−S3 indicate the corresponding values obtained for the slope (R^2) of the early (1), middle (2), and late (3) segments, respectively.

inhibition, we used arginine as the unique nitrogen source during the culture of transformed mutant cells.[36] K^+ uptake-deficient cells grew to a lesser extent in ammonium-free medium than in ammonium-containing medium (Figure 7).

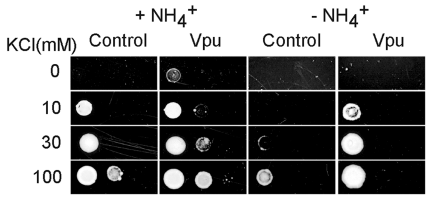


Figure 7. Influence of the type of nitrogen source on the release from potassium requirements of mutants by the Vpu channel. Δ*trk*1 Δ*trk*2 cells, transformed with control or *vpu* plasmids, were suspended in standard medium (with NH_4^+) or modified medium (without NH_4^+); both media were adjusted to pH 7.0. Drop test analysis with a series of 5-fold dilutions. Before being digitally scanned, plates were incubated for 5 (with NH_4^+) or 8 (without NH_4^+) days.

Even though Vpu ameliorated potassium requirements in both media, differences in K^+ requirements were more appreciable in the absence of ammonium. This dramatic increase in tolerance to low K^+ concentrations indicates a possible competition between potassium and ammonium during Vpu-facilitated uptake.

The knockout of both Trk potassium transporters has been reported to result in a high level of membrane hyperpolarization leading to increased sensitivity to hygromycin B.[34] We used the sensitivity toward this cationic antibiotic as an indirect indicator of plasma membrane potential of Δ*trk*1,2 transformants (Figure 8). Under low-K^+ conditions (30 mM KCl), vpu-expressing cells exhibited tolerance to 10 μg/mL hygromycin B in comparison with control cells. However, under near-physiological K^+ conditions (100 mM), differences in hygromycin B tolerance between both transformants were significantly reduced. Thus, given that resistance to the positively charged aminoglycoside is closely correlated with a depolarization of the cellular membrane potential,[43] the presence of Vpu protein should decrease the membrane potential of K^+ uptake-deficient cells.

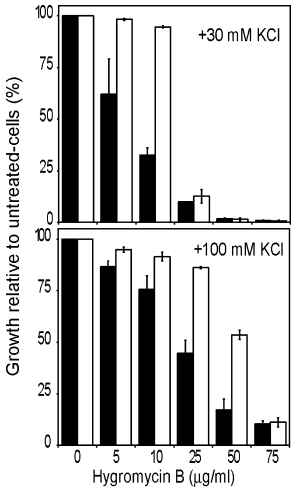


Figure 8. Influence of Vpu protein on the hygromycin B sensitivity of *trk*1,2 null mutants. Control cells (black) and Vpu cells (white) were grown in the presence of increasing hygromycin B concentrations (micrograms per milliliter). Drug sensitivity was tested using induction medium supplemented with 30 or 100 mM KCl. OD_{600} was determined after incubation for 45 and 48 h. Data are represented as means ± SD of each pair of measurements.

■ DISCUSSION

Vpu induces changes in the composition and functioning of the plasma membrane that may lead to the enhancement of the release of the virus particle from the plasma membrane.[29,44] This viroporin enhances even the release of unrelated viruses, such as Sindbis virus, Ebola virus, etc.[45,46] A debate about the dual pore/ion channel functioning of Vpu viroporin during virus replication cycle is ongoing.[12,13] We previously demonstrated that HIV-1 Vpu enhances the permeability of the plasma membrane in prokaryotic and mammalian cells.[10] Here, transfected yeasts produce high levels of Vpu that result in the impairment of growth similar to that reported for the influenza M2 protein.[2] Notably, the influenza viroporin affected the transmembrane proton flux in the wild-type strain. Instead, we found that the HIV-1 viroporin modifies potassium transport in both wild-type and K^+ uptake-deficient strains. The defective growth phenotype of *trk*1,2 null mutant cells is fully rescued by expressing the Vpu protein, in agreement with observations reported with other viroporin channels such as Kcv of PBCV-1 and PEDV ORF3.[1,3]

Vpu protein complements the potassium uptake-deficient phenotype in an extracellular pH-dependent manner. This behavior is similar to that reported for mouse inward K^+ rectifying channel, Kir2.1.[47] In yeast, Trk1 and Trk2 are involved in the response of the membrane potential to changes in the external pH, increasing cell permeability to H^+.[34] This may be the reason why the pH dependence of Vpu activity was stronger in the defective mutant than in the wild-type strain. The K^+ uptake complementation by Vpu is more evident in the absence of ammonium. This suggests that Vpu may function as

dx.doi.org/10.1021/bi3011175 | *Biochemistry* 2013, 52, 171−177

a nonspecific cation channel with inward transport of K^+ and also ammonium ions.

The knockout of both Trk potassium transporters has been reported to result in a high level of plasma membrane hyperpolarization.[34] At an extracellular pH of 7 and a high potassium concentration, Vpu-expressing mutants grow even better than nonexpressing wild-type cells under K^+ restriction conditions, but nonexpressing mutants do not. The Vpu-induced restoration of growth phenotype in mutant cells suggested a restoration of membrane potential as a result of an increased level of inward transport of extracellular K^+. Wild-type yeasts are sensitive to hygromycin B and become resistant to the drug by defects in the plasma membrane H^+-ATPase, which result in membrane depolarization.[43] Thus, the hyperpolarized Δ*tkr*1 Δ*tkr*2 strain is hypersensitive to hygromycin B.[34] Notably, our results show that Vpu confers hygromycin B resistance to the hypersensitive Δ*tkr*1 Δ*tkr*2 strain. This evidence suggests a depolarizing role for the Vpu protein in eukaryotic cells. Our results suggest that Vpu interferes with the signaling regulation of potassium transport.[48] It is possible that depolarization by Vpu protein destabilizes the Trk system at the plasma membrane. Thus, under low-K^+ conditions, Vpu would interfere with potassium homeostasis by accelerating the turnover of essential transporters.

Taken together, these data support a model in which Vpu depolarizes the plasma membrane by forming channels with weak selectivity for K^+. The ion channel activity of Vpu should affect functioning of cellular channels involved with ion homeostasis and eventual membrane disturbance. In fact, recent studies in human cells showed that vesicle release was promoted as a result of silencing K2P channels.[49] Similarly, the Vpu channel may depolarize the budding membrane, making particle release an energetically favorable process, as previously proposed. Thus, our results are compatible with suggested changes in the composition and functioning of the plasma membrane that may lead to the Vpu-induced enhancement of the release of the virus particle from the plasma membrane.[29,44]

Contrary to most studies, which have used the transmembrane domain of Vpu or a fusion protein including the full sequence, here we used genuine Vpu protein expressed in a cellular system. Although other models could just show a slight cation preference of Vpu channels, here we specifically demonstrate that Vpu modifies the transport of K^+ through plasma membrane. Because of its good phenotype complementation, *trk*1,2 null mutants of *S. cerevisiae* represent a promising model for screening of inhibitors of channel-forming Vpu protein and potentially also other viroporins.

AUTHOR INFORMATION

Corresponding Author

*Phone: (34) 91 822 3698. Fax: (34) 91 509 7966. E-mail: megonzalez@isciii.es.

Funding

This study was supported by Grants PI PI05/00013 and PI08/0912 from Fondo de Investigación Sanitaria. L.H. and N.M. were holders of Predoctoral Fellowships from Instituto de Salud Carlos III.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We acknowledge Dr. Luis Carrasco (Universidad Autónoma de Madrid) for discussions and critical reading of the manuscript.

ABBREVIATIONS

PBCV-1, *Paramecium bursaria* chlorella virus 1; PEDV, porcine epidemic diarrhea virus; HCV, hepatitis C virus; YNB, yeast nitrogen base; PGK, phosphoglycerate kinase; K2P, two-pore domain potassium.

REFERENCES

(1) Gazzarrini, S., Kang, M., Abenavoli, A., Romani, G., Olivari, C., Gaslini, D., Ferrara, G., Van Etten, J. L., Kreim, M., Kast, S. M., Thiel, G., and Moroni, A. (2009) Chlorella virus ATCV-1 encodes a functional potassium channel of 82 amino acids. *Biochem. J. 420*, 295−303.

(2) Kurtz, S., Luo, G., Hahnenberger, K. M., Brooks, C., Gecha, O., Ingalls, K., Numata, K., and Krystal, M. (1995) Growth impairment resulting from expression of influenza virus M2 protein in *Saccharomyces cerevisiae*: Identification of a novel inhibitor of influenza virus. *Antimicrob. Agents Chemother. 39*, 2204−2209.

(3) Wang, K., Lu, W., Chen, J., Xie, S., Shi, H., Hsu, H., Yu, W., Xu, K., Bian, C., Fischer, W. B., Schwarz, W., Feng, L., and Sun, B. (2012) PEDV ORF3 encodes an ion channel protein and regulates virus production. *FEBS Lett. 586*, 384−391.

(4) Cohen, E. A., Terwilliger, E. F., Sodroski, J. G., and Haseltine, W. A. (1988) Identification of a protein encoded by the vpu gene of HIV-1. *Nature 334*, 532−534.

(5) Maldarelli, F., Chen, M. Y., Willey, R. L., and Strebel, K. (1993) Human immunodeficiency virus type 1 Vpu protein is an oligomeric type I integral membrane protein. *J. Virol. 67*, 5056−5061.

(6) Klimkait, T., Strebel, K., Hoggan, M. D., Martin, M. A., and Orenstein, J. M. (1990) The human immunodeficiency virus type 1-specific protein vpu is required for efficient virus maturation and release. *J. Virol. 64*, 621−629.

(7) Neil, S. J., Zang, T., and Bieniasz, P. D. (2008) Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature 451*, 425−430.

(8) Gonzalez, M. E., and Carrasco, L. (2003) Viroporins. *FEBS Lett. 552*, 28−34.

(9) Nieva, J. L., Madan, V., and Carrasco, L. (2012) Viroporins: Structure and biological functions. *Nat. Rev. Microbiol. 10*, 563−574.

(10) Gonzalez, M. E., and Carrasco, L. (1998) The human immunodeficiency virus type 1 Vpu protein enhances membrane permeability. *Biochemistry 37*, 13710−13719.

(11) Madan, V., Castello, A., and Carrasco, L. (2008) Viroporins from RNA viruses induce caspase-dependent apoptosis. *Cell. Microbiol. 10*, 437−451.

(12) Fischer, W. B., and Hsu, H. J. (2011) Viral channel forming proteins: Modeling the target. *Biochim. Biophys. Acta 1808*, 561−571.

(13) Lamb, R. A., and Pinto, L. H. (1997) Do Vpu and Vpr of human immunodeficiency virus type 1 and NB of influenza B virus have ion channel activities in the viral life cycles? *Virology 229*, 1−11.

(14) Pinto, L. H., Holsinger, L. J., and Lamb, R. A. (1992) Influenza virus M2 protein has ion channel activity. *Cell 69*, 517−528.

(15) Griffin, S. D., Beales, L. P., Clarke, D. S., Worsfold, O., Evans, S. D., Jaeger, J., Harris, M. P., and Rowlands, D. J. (2003) The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, Amantadine. *FEBS Lett. 535*, 34−38.

(16) Plugge, B., Gazzarrini, S., Nelson, M., Cerana, R., Van Etten, J. L., Derst, C., DiFrancesco, D., Moroni, A., and Thiel, G. (2000) A potassium channel protein encoded by chlorella virus PBCV-1. *Science 287*, 1641−1644.

(17) Wilson, L., McKinlay, C., Gage, P., and Ewart, G. (2004) SARS coronavirus E protein forms cation-selective ion channels. *Virology 330*, 322−331.

(18) Ewart, G. D., Sutherland, T., Gage, P. W., and Cox, G. B. (1996) The Vpu protein of human immunodeficiency virus type 1 forms cation-selective ion channels. *J. Virol. 70*, 7108−7115.
(19) Schubert, U., Ferrer-Montiel, A. V., Oblatt-Montal, M., Henklein, P., Strebel, K., and Montal, M. (1996) Identification of an ion channel activity of the Vpu transmembrane domain and its involvement in the regulation of virus release from HIV-1-infected cells. *FEBS Lett. 398*, 12−18.
(20) Grice, A. L., Kerr, I. D., and Sansom, M. S. (1997) Ion channels formed by HIV-1 Vpu: A modelling and simulation study. *FEBS Lett. 405*, 299−304.
(21) Kukol, A., and Arkin, I. T. (1999) vpu transmembrane peptide structure obtained by site-specific fourier transform infrared dichroism and global molecular dynamics searching. *Biophys. J. 77*, 1594−1601.
(22) Moore, P. B., Zhong, Q., Husslein, T., and Klein, M. L. (1998) Simulation of the HIV-1 Vpu transmembrane domain as a pentameric bundle. *FEBS Lett. 431*, 143−148.
(23) Ewart, G. D., Mills, K., Cox, G. B., and Gage, P. W. (2002) Amiloride derivatives block ion channel activity and enhancement of virus-like particle budding caused by HIV-1 protein Vpu. *Eur. Biophys. J. 31*, 26−35.
(24) Ewart, G. D., Nasr, N., Naif, H., Cox, G. B., Cunningham, A. L., and Gage, P. W. (2004) Potential new anti-human immunodeficiency virus type 1 compounds depress virus replication in cultured human macrophages. *Antimicrob. Agents Chemother. 48*, 2325−2330.
(25) Khoury, G., Ewart, G., Luscombe, C., Miller, M., and Wilkinson, J. (2010) Antiviral efficacy of the novel compound BIT225 against HIV-1 release from human macrophages. *Antimicrob. Agents Chemother. 54*, 835−845.
(26) Kuhl, B. D., Cheng, V., Donahue, D. A., Sloan, R. D., Liang, C., Wilkinson, J., and Wainberg, M. A. (2011) The HIV-1 Vpu Viroporin Inhibitor BIT225 Does Not Affect Vpu-Mediated Tetherin Antagonism. *PLoS One 6*, e27660.
(27) Bolduan, S., Votteler, J., Lodermeyer, V., Greiner, T., Koppensteiner, H., Schindler, M., Thiel, G., and Schubert, U. (2011) Ion channel activity of HIV-1 Vpu is dispensable for counteraction of CD317. *Virology 416*, 75−85.
(28) Hsu, K., Seharaseyon, J., Dong, P., Bour, S., and Marban, E. (2004) Mutual functional destruction of HIV-1 Vpu and host TASK-1 channel. *Mol. Cell 14*, 259−267.
(29) Hsu, K., Han, J., Shinlapawittayatorn, K., Deschenes, I., and Marban, E. (2010) Membrane potential depolarization as a triggering mechanism for Vpu-mediated HIV-1 release. *Biophys. J. 99*, 1718−1725.
(30) Hahn, B. H., Shaw, G. M., Arya, S. K., Popovic, M., Gallo, R. C., and Wong-Staal, F. (1984) Molecular cloning and characterization of the HTLV-III virus associated with AIDS. *Nature 312*, 166−169.
(31) Cesareni, G., and Murray, J. A. H. (1987) Plasmid vectors carrying the replication origin of filamentous single-stranded phages. In *Genetic engineering. Principles and methods* (Setlow, J. K., Ed.) pp 135−153, Plenum Press, New York.
(32) Sambrook, J., and Rusell, D. W. (2001) *Molecular cloning. A laboratory manual*, Cold Spring Harbor Laboratory Press, Plainview, NY.
(33) Thomas, B. J., and Rothstein, R. (1989) Elevated recombination rates in transcriptionally active DNA. *Cell 56*, 619−630.
(34) Madrid, R., Gomez, M. J., Ramos, J., and Rodriguez-Navarro, A. (1998) Ectopic potassium uptake in trk1 trk2 mutants of *Saccharomyces cerevisiae* correlates with a highly hyperpolarized membrane potential. *J. Biol. Chem. 273*, 14838−14844.
(35) Burke, D., Dawson, D., and Stearns, T. (2000) *Methods in yeast genetics. A Cold Spring Harbor laboratory course manual*, Cold Spring Harbor Laboratory Press, Plainview, NY.
(36) Rodriguez-Navarro, A., and Ramos, J. (1984) Dual system for potassium transport in *Saccharomyces cerevisiae*. *J. Bacteriol. 159*, 940−945.
(37) Bill, R. M. (2001) Yeast: A panacea for the structure-function analysis of membrane proteins? *Curr. Genet. 40*, 157−171.
(38) Sychrova, H. (2004) Yeast as a model organism to study transport and homeostasis of alkali metal cations. *Physiol. Res. 53* (Suppl. 1), S91−S98.
(39) Varela, J. C., and Mager, W. H. (1996) Response of *Saccharomyces cerevisiae* to changes in external osmolarity. *Microbiology 142* (Part 4), 721−731.
(40) Nakamura, R. L., and Gaber, R. F. (1998) Studying ion channels using yeast genetics. *Methods Enzymol. 293*, 89−104.
(41) Arino, J., Ramos, J., and Sychrova, H. (2010) Alkali metal cation transport and homeostasis in yeasts. *Microbiol. Mol. Biol. Rev. 74*, 95−120.
(42) Ko, C. H., Buckley, A. M., and Gaber, R. F. (1990) TRK2 is required for low affinity K^+ transport in *Saccharomyces cerevisiae*. *Genetics 125*, 305−312.
(43) Perlin, D. S., Brown, C. L., and Haber, J. E. (1988) Membrane potential defect in hygromycin B-resistant pma1 mutants of *Saccharomyces cerevisiae*. *J. Biol. Chem. 263*, 18118−18122.
(44) Ruiz, A., Guatelli, J. C., and Stephens, E. B. (2010) The Vpu protein: New concepts in virus release and CD4 down-modulation. *Curr. HIV Res. 8*, 240−252.
(45) Gonzalez, M. E., and Carrasco, L. (2001) Human immunodeficiency virus type 1 VPU protein affects Sindbis virus glycoprotein processing and enhances membrane permeabilization. *Virology 279*, 201−209.
(46) Neil, S. J., Sandrin, V., Sundquist, W. I., and Bieniasz, P. D. (2007) An Interferon-α-Induced Tethering Mechanism Inhibits HIV-1 and Ebola Virus Particle Release but Is Counteracted by the HIV-1 Vpu Protein. *Cell Host Microbe 2*, 193−203.
(47) Hasenbrink, G., Schwarzer, S., Kolacna, L., Ludwig, J., Sychrova, H., and Lichtenberg-Frate, H. (2005) Analysis of the mKir2.1 channel activity in potassium influx defective *Saccharomyces cerevisiae* strains determined as changes in growth characteristics. *FEBS Lett. 579*, 1723−1731.
(48) Perez-Valle, J., Jenkins, H., Merchan, S., Montiel, V., Ramos, J., Sharma, S., Serrano, R., and Yenush, L. (2007) Key role for intracellular K^+ and protein kinases Sat4/Hal4 and Hal5 in the plasma membrane stabilization of yeast nutrient transporters. *Mol. Cell. Biol. 27*, 5725−5736.
(49) Huang, D. T., Chi, N., Chen, S. C., Lee, T. Y., and Hsu, K. (2011) Background K(2P) channels KCNK3/9/15 limit the budding of cell membrane-derived vesicles. *Cell Biochem. Biophys. 61*, 585−594.