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A fluorescence assay for peptide translocation into mitochondria

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Abstract

Translocation of the presequence is an early event in import of preproteins across the mitochondrial inner membrane by the TIM23 complex. Import of signal peptides, whose sequences mimic mitochondrial import presequences, was measured using a novel, qualitative, fluorescence assay in about an hour. This peptide assay was used in conjunction with classical protein import analyses and electrophysiological approaches to examine the mechanisms underlying the functional effects of depleting two TIM23 complex components. Tim23p forms, at least in part, the pore of this complex while Tim44p forms part of the translocation motor. Depletion of Tim23p eliminates TIM23 channel activity, which interferes with both peptide and preprotein translocation. In contrast, depletion of Tim44p disrupts preprotein but not peptide translocation, and has no effect on TIM23 channel activity. Two conclusions were made. First, this fluorescence peptide assay was validated as two different mutants were accurately identified. Hence, this assay could provide a rapid means of screening mutants to identify those that fail an initial step in import, i.e. translocation of the presequence. Second, translocation of signal peptides required normal channel activity and disruption of the PAM complex did not modify TIM23 channel activity nor prevent presequence translocation.

Keywords

Mitochondria; Patch clamp; Protein import; Tim44; Tim23; Fluorescence Assay

1. INTRODUCTION

The vast majority of mitochondrial proteins are encoded in the nucleus, synthesized in the cytosol, and imported into mitochondria [1]. Numerous multi-subunit complexes mediate the import process and include the TOM1 and SAM complexes in the outer membrane and the TIM22 and TIM23 complexes in the inner membrane (for reviews see [2–5]).

The TIM23 translocase is responsible for import of matrix-bound preproteins and insertion of single pass proteins into the inner membrane. This complex contains several subunits which form a pore and a ‘motor’ that work together to enable preprotein translocation. Tim23p is part of the translocation pore [6–9] while Tim44p forms part of the presequence translocase-associated motor or PAM complex [10–17]. The channel activity at the core of the TIM23

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Import of radio-labeled preprotein was measured by classical means.

complex was linked to this translocase by patch clamping mitoplasts and proteoliposomes, and through bilayer studies with recombinant Tim23p [7,18,19].

A recent report by Krayl et al. described a fluorescence assay that accurately reflected protein import when compared with assays using radio-isotopes or westerns, and was considerably faster than either method [20]. Here, we describe an even more rapid fluorescence assay for peptide import that relies on microscopy and, unlike the other assays, does not require electrophoresis. While qualitative, this assay can determine peptide import competence, which allows a further dissection of the import pathway. Wildtype and mutant mitochondria that are or are not deficient in peptide and protein import were used to test the validity of this assay. Finally, these assays were used in conjunction with patch clamping to further characterize the effects of depletion of Tim23p and Tim44p on the TIM23 channel activities.

2. MATERIALS AND METHODS

Isolation of mitochondria and preparation of proteoliposomes

Tim23(Gal 10) and Tim44(Gal 10) are strains of *S. cerevisiae* in which the expression of *Tim23* and *Tim44* genes, respectively, is controlled by a Gal 10 promoter [21]. Mitochondria were isolated from both strains after growth in media with or without galactose for 24 hours as previously described [7]. Inner membranes were further purified as previously described [22] and cross contamination with outer membranes was typically less than 5%. Inner membranes were reconstituted into proteoliposomes by dehydration-rehydration using soybean L-α –phosphatidylcholine (Sigma Type IV-S) as previously described [7,23,24].

Patch clamping techniques

Patch-clamp studies of TIM23 channels were carried out on proteoliposomes containing purified inner membranes [7,22,25]. The solution was typically symmetrical 150 mM KCl, 5 mM HEPES, pH 7.4 at ~23° C as previously described [7,22,25]. Voltage clamp was established with the inside-out excised configuration and voltages are reported as bath potentials. Filtration was 2 kHz with 5 kHz sampling. Permeability ratios were calculated from the reversal potential in the presence of a 150:30 mM KCl gradient [7]. Peptides were introduced and removed by perfusion of the 0.5 mL bath. Flicker rates were determined as the number of transition events/sec from the open to lower conductance states with a 50% threshold of the predominant event (~250 pS) during ~30 seconds of current traces.

Peptide and Protein import

Import of radio-labeled preprotein Su9-(1-69)-DHFR into isolated mitochondria was previously described [26,27]. Peptides (yCoxIV$_{(1-13)}$, y CoxIV$_{(1-22)}$, SynB2) were labeled with Alexa Fluor®488 Protein Labeling Kit (Molecular Probes) and free dye was removed by dialysis through a 1 kDa cellulose acetate membrane. Import of Alexa Fluor-labeled peptides into isolated (12.5 μg, 1mg/mL) mitochondria was carried out in import buffer (0.6M Sorbitol, 25 mM KCl, 10 mM MgCl2, 2 mM KPO_4, 0.5 mM EDTA, 2 mM ATP, 2 mM NADH, 50 mM HEPES-KOH, pH 7.4) by incubation at 30°C for 10 min. 1 μM CCCP was included for negative controls. Mitochondria were pelleted by centrifugation at 14000 x g for 10 min, washed 3 times, and resuspended in import buffer. Fluorescence and DIC images were captured through a plan-Apo 60X lens on a Nikon Eclipse TE300 microscope by a Spot RT Monochrome CCD camera (Diagnostics Instr.).

3. RESULTS AND DISCUSSION

The mechanisms underlying the transport of full-length preproteins are more complex than that of short peptides. Here, we describe a novel fluorescence assay that rapidly determines

peptide import competence. Wildtype mitochondria and two mutants that are differentially deficient in peptide and protein import were used to test the validity of this assay. The single channel behavior and peptide sensitivity of the TIM23 channel activities of these mitochondria were then determined with patch clamp techniques to further dissect the import pathway.

Synthetic signal peptides have sequences that mimic presequences, competitively inhibit protein import into mitochondria [28,29] and modify TIM23 channel activity at similar concentrations [7,24,30,31]. Two signal peptides referred to as $yCoxIV_{(1-13)}$ and $yCoxIV_{(1-22)}$ (based on the first 13 or 22 amino acids of yeast cytochrome oxidase subunit IV*)* were labeled with Alexa Fluor®488 (Molec. Probes, Eugene, OR) to develop a tool for assessing peptide import. The charge and amphipathic alpha-helical structure of the peptide SynB2 is similar to that of signal peptides. However, this peptide does not modify TIM23 channel activity and this sequence does not target preproteins to mitochondria [24,32]. SynB2 was labeled in parallel and used as a negative control peptide. Previous studies comparing standard preproteins with those labeled with fluorescein have shown that the fluorescein moiety attached to preproteins may not influence the mitochondrial translocation properties [20].

Wildtype mitochondria became fluorescent when incubated under typical preprotein-import conditions (See methods) with the Alexa Fluor-labeled signal peptides $yCoxIV_{(1-22)}$ (Figure 1A) and $yCoxIV_{(1-13)}$ (not shown). Like preproteins, import of labeled peptides required energy. Mitochondria treated in parallel with the uncoupler CCCP and Alexa Fluor-labeled signal peptides were not fluorescent peptides (Figure 1B). In addition, mitochondria were not fluorescent when incubated with the negative control peptide Alexa Fluor-SynB2 (Figure 1C), consistent with import failure. Hence, this assay detected fluorescence accumulation of mitochondria with signal but not control peptides in an energy-dependent fashion, which is strikingly similar to preprotein import. The validity of this fluorescence assay was further tested using two mutant strains; one mutant [Tim44(Gal 10)] can import peptides but not preproteins while the second mutant [Tim23(Gal 10)] could import neither peptides nor preproteins.

Galactose withdrawal from Tim23(Gal 10) and Tim44(Gal 10) yeast [21] suppressed expression of Tim23p and Tim44p, respectively, as shown in the western blots of Figure 2A. However, depletion of Tim44p or Tim23p for 24 hr did not suppress expression of the other core components of the TIM23 translocase (Figure 2A). Tim23p forms at least part of the channel pore while Tim44p is essential to PAM, which forms the motor for translocation [6–17]. Mitochondria of both strains grown without galactose were incompetent in classical assays for radio-labeled preprotein translocation as significantly less DHFR was imported compared to +gal controls as shown in Figure 2B–E and as previously reported [21]. The ability of both mutants to import peptides was then determined using Alexa Fluor-labeled signal peptides.

Mitochondria of Tim23(Gal 10) and Tim44(Gal 10) strains grown with galactose (+gal) were fluorescently-labeled when incubated with Alexa Fluor-$yCoxIV_{(1-22)}$ (Figure 3A) or Alexa Fluor-$yCoxIV_{(1-13)}$ (not shown) under import conditions. However, fluorescence did not accumulate when CCCP (Fig. 3C) was included in the incubation mixture or if Alexa Fluor-SynB2 replaced the signal peptides (Fig. 3D). Hence, mitochondria from both strains grown with galactose behaved like wildtype. However, mitochondria depleted of Tim23p [Tim23(Gal 10)-gal] were not fluorescent when incubated with Alexa Fluor signal peptides (Fig. 3B upper panel). Hence, loss of Tim23p renders mitochondria incompetent to import both preproteins and signal peptides, consistent with Tim23p's role in the translocation pore. In contrast, mitochondria of the Tim44(Gal 10) strain grown with or without galactose were fluorescent if incubated with Alexa Fluor-$yCoxIV_{(1-22)}$ (Fig. 3B lower panel) or -$yCoxIV_{(1-13)}$ (not shown). These findings are consistent with the results of Milisav et al (2001), who elegantly determined that the presequence could be translocated after Tim44 depletion [21].

In summary, we describe here a qualitative fluorescence assay that determines peptide import competence in isolated yeast mitochondria. This assay relies on fluorescence microscopy to detect import, in lieu of electrophoresis of radio-labeled preproteins in classical assays, so that the assay can be completed in about 1 hour, instead of two or more days. Like the classical import assays, import of fluorescent-peptides required energized conditions and was eliminated by inclusion of uncouplers in the mixture. Consistent with classical import assays, the negative control peptide SynB2 was not imported. Finally, this assay was further validated using two protein import mutants. Consistent with the results of Milisav et al (2001) [21], this assay found that Tim23p is, but Tim44p is not, essential for import of signal peptides. The mechanisms underlying the import defects of these two mutants were then further evaluated by patch-clamping TIM23 channels.

TIM23 channel activity was linked to protein import in patch clamp studies with Tim23p-antibodies and mutants [7]. The activity of TIM23 channels with or without Tim44p is the same as wildtype in terms of all the single channel parameters examined including conductance, voltage dependence, and selectivity (Table 1 and Figure 4). However, striking differences are seen in the membrane activity after depletion of Tim23p compared to the controls (Figure 4B). Galactose removal typically suppressed Tim23p levels by >91% (Fig. 2A). Importantly, the frequency of detecting TIM23 channels increased with the amount of Tim23p present (Fig. 4C) regardless of whether the cells were grown with or without galactose. Hence, all TIM23 channels detected after Tim23p depletion could be accounted for by residual *Tim23* expression, i.e. leakiness of the gal promoter. This finding again illustrates the essential role of Tim23p in pore formation reported by others [7,19]. Furthermore, no novel channel activities were detected suggesting Tim17p or Tim50p do not form pores in the absence of Tim23p (see [33]). These findings indicate that the absence of a translocation pore underlies the failure of Tim23p-depleted mitochondria to import both preproteins and peptides.

Wildtype TIM23 channels rapidly flicker (downward deflections in current traces at positive voltages) between conductance levels upon addition of the signal peptides $yCoxIV_{(1-13)}$ or $yCoxIV_{(1-22)}$ (Figure 4D) [3,18,30]. This flickering is reversible and associated with an increase in current noise [3]. Like peptide import (Fig. 3), the sensitivity of TIM23 channels to signal peptides is not modified by depletion of Tim44p. The current traces of Tim44-depleted channels (−gal) are the same as wildtype and control (+gal) in the presence and absence of peptides (Figure 4D). A four-fold increase in the number of transition events (or flickering) is observed in the presence of $yCoxIV_{(1-13)}$ compared to the absence (control) or the presence of SynB2 for both normal and Tim44p-depleted channels (Fig. 4E). Hence, Tim44p is not essential for the peptide sensitivity of TIM23 channels, which is consistent with the labeling of mitochondria by Alexa Fluor-yCox-IV (Fig. 3A, B lower panels). These findings indicate that depletion of Tim44p likely causes loss of an intact PAM complex which is essential for preprotein import, but does not modify TIM23 channel activity nor peptide import.

Two modular forms of the TIM23 translocases are proposed to exist. One form contains the PAM complex and translocates preproteins into the matrix. The second form contains Tim21p and inserts single pass proteins into the inner membrane. Accumulation of the second form may be facilitated by depletion of PAM complex components like Tim44p [34]. In this last case, our findings (Fig. 3, 4) would suggest that switching between these two forms (by growing the Tim44(Gal 10) strain ±gal) does not modify the TIM23 channel activity or the ability of the complex to translocate peptides.

In summary, a novel, qualitative fluorescence assay is described that rapidly determines competence to translocate signal peptides. This assay was validated using two mutants known to be differentially deficient in preprotein and presequence import. This assay has some similarities with the approach recently applied to preproteins [20], but does not require

electrophoresis. Together, these assays could provide rapid screening of peptide and protein import mutants. While preprotein import requires both Tim23p and Tim44p, peptide import requires the pore protein Tim23p but not the motor protein Tim44p. Normal TIM23 channel activity requires Tim23p and is not affected by depletion of Tim44p.

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Abbreviations

CCCP

carbonyl cyanide m-chlorophenyl hydrazone

DHFR
dihydrofolate reductase

DIC
Differential interference contrast

PAM
Presequence translocase-associated motor

TIM
Translocase of the Inner Membrane

TOM
Translocase of the Outer Membrane

VDAC
Voltage dependent anion-selective channel

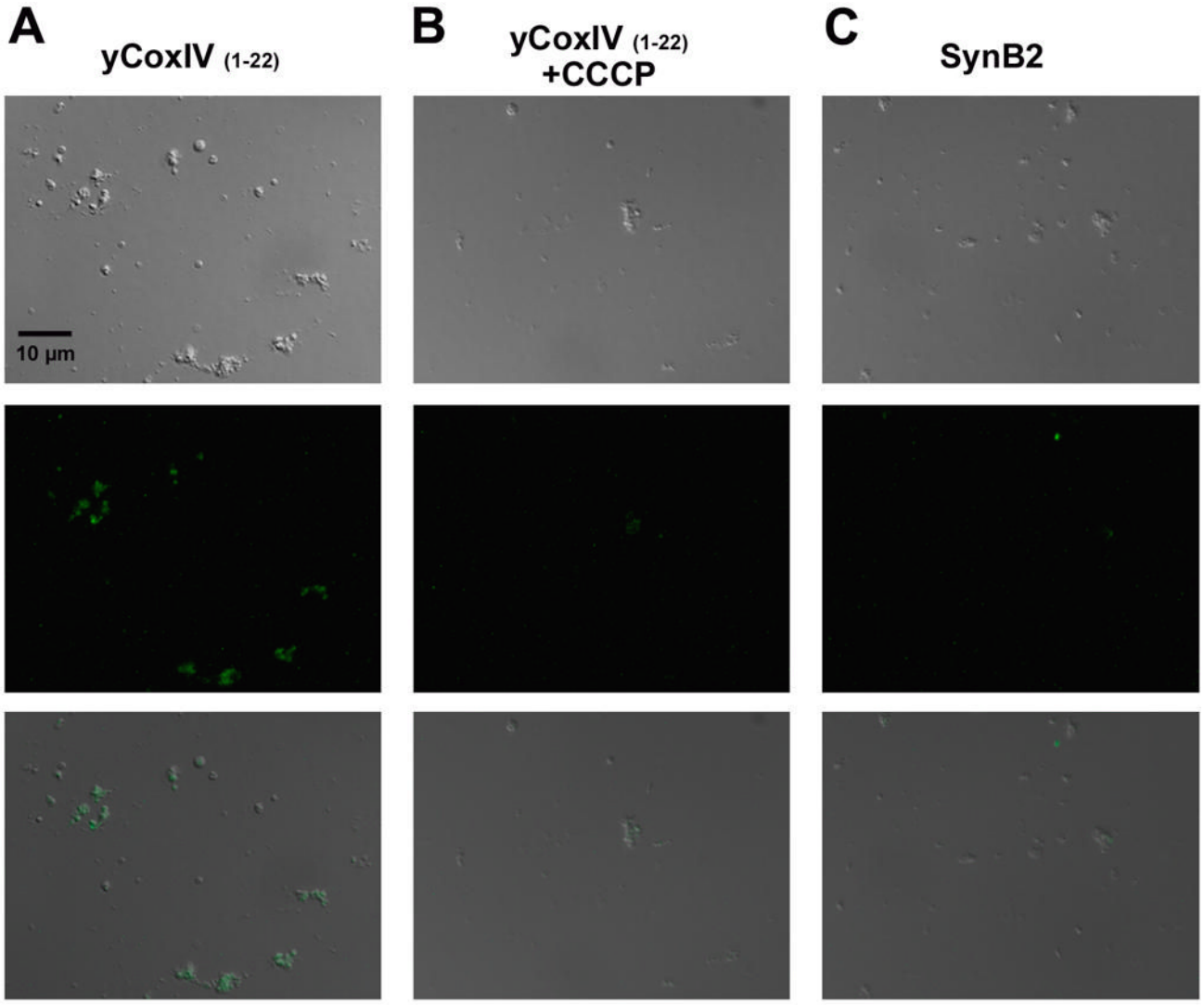

Figure 1. Fluorescent signal peptides label wildtype mitochondria

DIC (upper), fluorescence (middle) and overlay (lower) images are shown of wildtype mitochondria incubated with Alexa Fluor-yCox-IV$_{(1-22)}$ under energized (**A**) and de-energized (+1 μM CCCP) (**B**) conditions for 10 minutes. Images are shown of mitochondria incubated with control peptide Alexa Fluor-SynB2 under energized conditions(**C**). Scale bar in panel A corresponds to 10 microns for all panels.

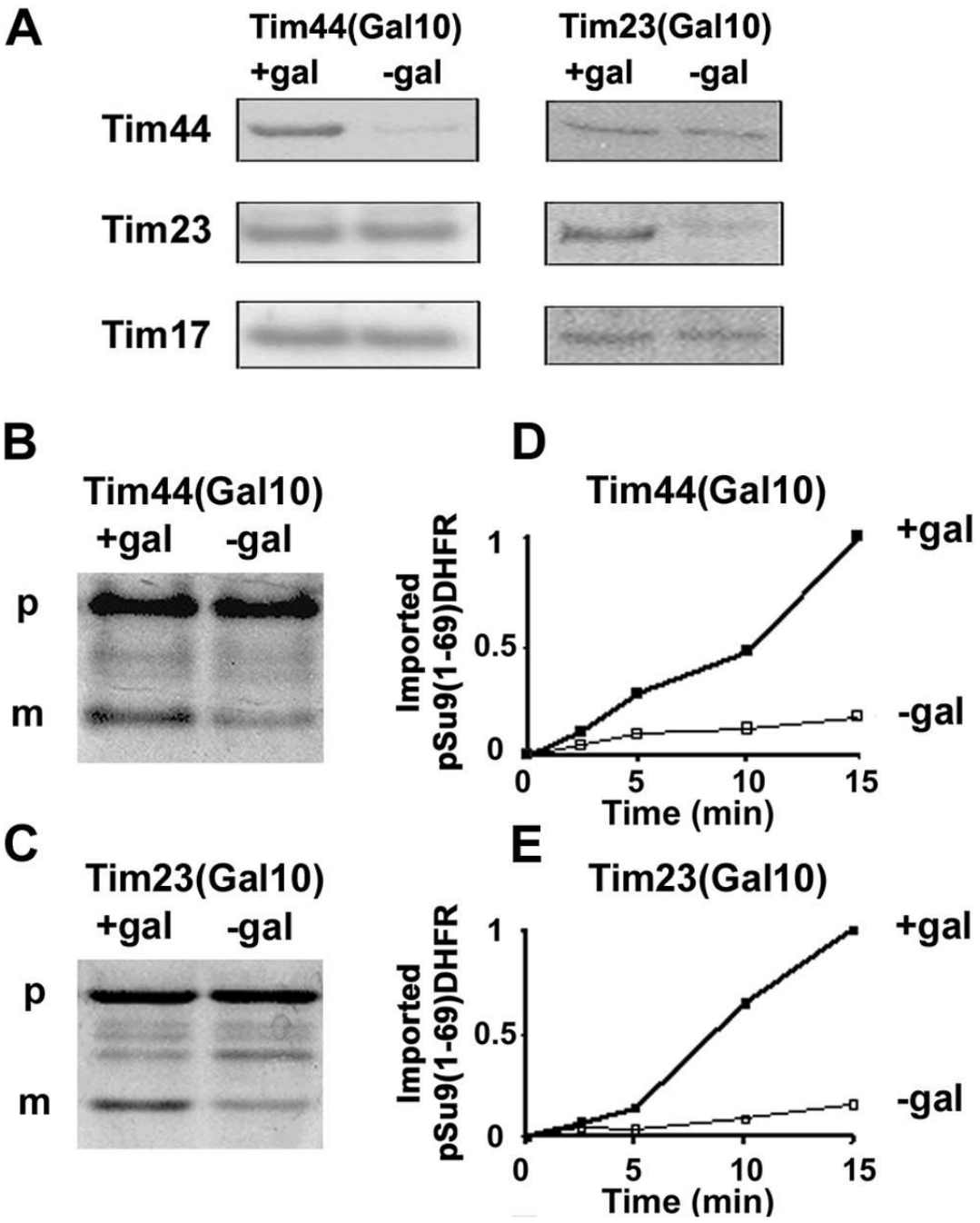

Figure 2. Depletion of Tim44p or Tim23p disrupts protein import

A. Western blots are shown for Tim17p, Tim23p, and Tim44p of inner membranes isolated from Tim23(Gal 10) and Tim44(Gal 10) yeast after 24 hours of growth with (+gal) or without (−gal) galactose. **B, C.** Autoradiographs: Radio-labeled preprotein Su9(1-69)DHFR was incubated for 5 minutes with mitochondria of Tim44(Gal 10) (**B**) and Tim23(Gal 10) (**C**) strains grown with or without galactose. Preprotein (p) and mature protein (m) were separated by SDS-PAGE and detected by autoradiography. Mature protein was quantified by densitometry. Plots show the amount of pSu9(1–69)DHFR imported as a function of time into mitochondria from Tim44(Gal 10) (**D**) and Tim23(Gal 10) (**E**) strains normalized to the amount imported after 15 minutes by mitochondria of that strain grown with galactose.

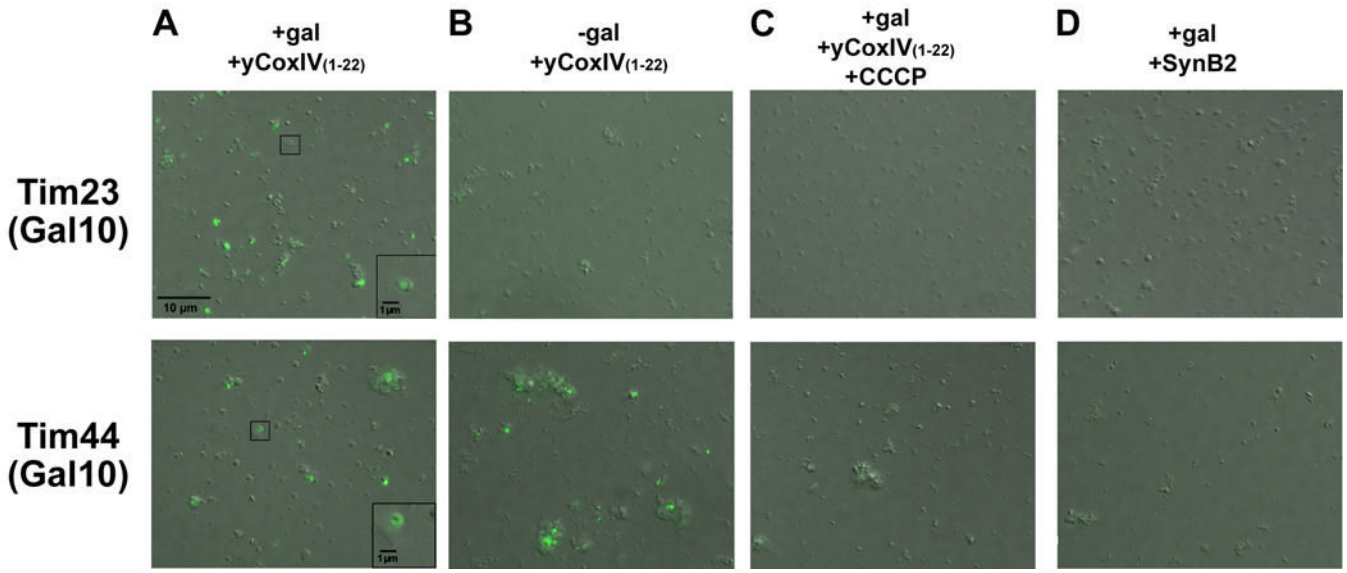

Figure 3. Fluorescent signal peptides label mitochondria lacking Tim44p but not Tim23p

Overlays of DIC and fluorescence images are shown of mitochondria from Tim23(Gal 10) (top) and Tim44(Gal 10) (bottom) strains after a 10 minute incubation under different conditions and 3 washes to remove peptides that were not imported. Mitochondria from yeast grown with (**A**) or without (**B**) galactose were incubated with Alexa Fluor-yCox-IV_{1-22} under energized conditions. Mitochondria from yeast grown with galactose were incubated with Alexa Fluor-yCox-IV_{1-22} plus CCCP (1 μM) (**C**) or with the control peptide Alexa Fluor-SynB2 under energized conditions (**D**). Scale bar in panel A corresponds to 10 microns for all panels. Squares in lower right corner of left panels show enlarged regions with 1 micron scale bar.

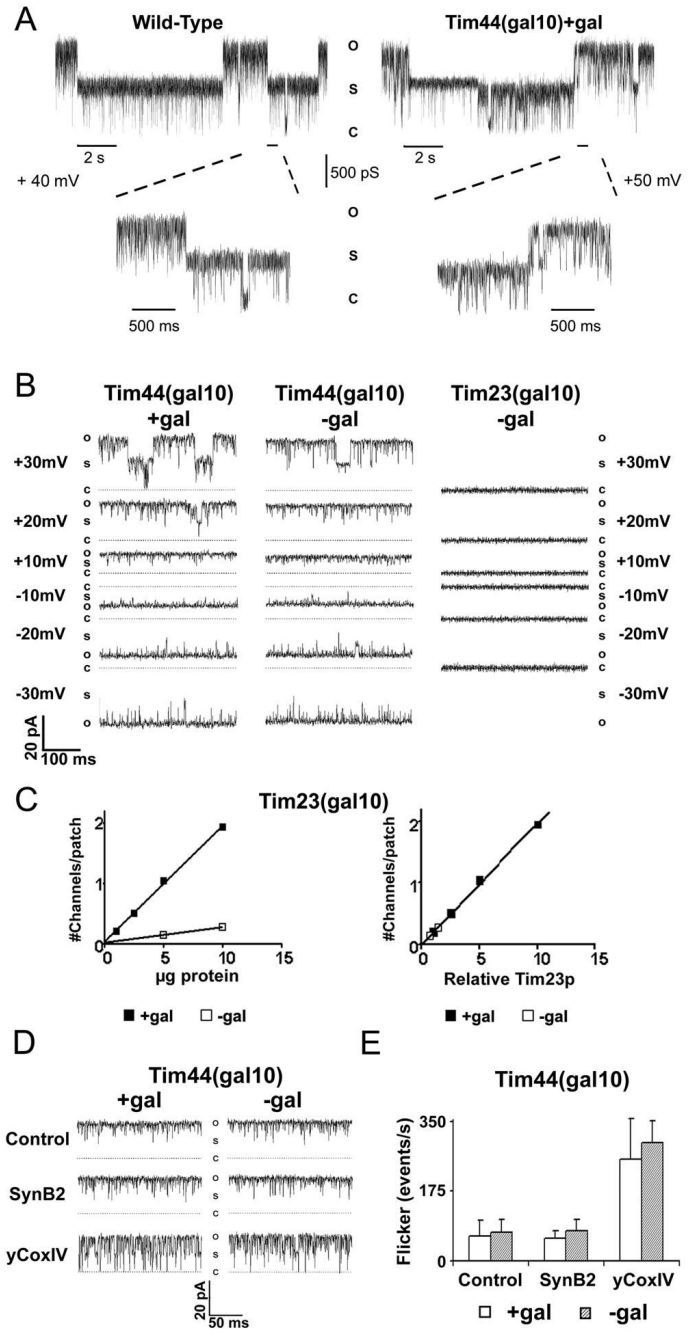

Figure 4. Electrophysiological behavior of TIM23 channels after depletion of Tim44p or Tim23p

A, B, D. Typical current traces are shown of single TIM23 channels recorded from proteoliposomes containing inner membranes of mitochondria of indicated strains grown with (+gal) and without (−gal) galactose. O, S, C corresponds to the open (1000 pS), sub- (500 pS), and closed states, respectively. **C.** Inner membranes from Tim23(Gal 10) yeast were reconstituted with different protein concentrations. Plots show the frequency of detecting normal TIM23 channels as a function of total protein (left) and relative amount of Tim23p (right), which was determined by semi-quantitative western blots using densitometry. 12 independent patches/point are shown. **D**. Current traces are shown at +20 mV before (Control) and after sequential perfusion of the bath with 20 μM SynB2 and then 20 μM yCox-IV$_{(1-13)}$.

E. Histograms of flicker rates (number of transition events/second) are shown in the absence (Control) and the presence of SynB2 or yCox-IV$_{(1-13)}$ for the TIM23 channels from Tim44 (Gal10) yeast.

Table 1

Comparison of the electrophysiological properties of TIM23 channels from control and mutant yeast strains.

	Wildtype[a]	**+gal**[b]	**Tim44(Gal 10) −gal**	**Tim23(Gal 10) −gal**
Peak conductance (pS)	1160 ± 140 n=10	1002 ± 60 n=5	1008 ± 52 n=10	0 n=45
Transition size (pS)	490 ± 43 n=10	498 ± 31 n=5	500 ± 26 n=10	n.a.
Mean open time (ms) (+20mV)	10.6± 4.3 n=13	12.0 ± 5.0 n = 19	13.1 ± 4.6 n = 5	n.a.
Voltage dependent	Yes	Yes	Yes	n.a.
Gating charge	−4.2 ± 0.7 n=20	−3.1 ± 0.4 n=19	−3.4 ± 0.5 n = 4	n.a.
V_0 (mV)[c]	50 ± 10 n=20	55 ± 10 n=19	59 ± 12 n = 5	n.a.
Permeability K^+/Cl^-	5.0 ± 0.3 n=20	5.2 ± 1.4 n=11	5.9 ± 1.3 n = 5	n.a.
Peptide sensitivity	↑Flicker	↑Flicker	↑Flicker	n.a.

[a] Data from Muro et al.(2003)[30].

[b] Data compiled from both Gal10 strains grown in the presence of galactose

[c] V_0 voltage at which the channel spends half of the time open (Open probability, P_O, is 0.5)

n is the number of independent patches, n.a. not applicable