Imaging fluorescence resonance energy transfer between two green fluorescent proteins in living yeast

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Abstract We show that fluorescence resonance energy transfer between two mutants of the green fluorescent protein (GFP) can be monitored by imaging microscopy in living yeast. This work is based on the constitutive expression of a GFP-containing fusion protein and the inducible expression of the tobacco etch virus (TEV) protease. In the fusion protein, the P4.3 GFP mutant is linked to the YS65T GFP mutant by a spacer bearing the TEV protease-specific cleavage site.

Key words: Green fluorescent protein; Fluorescence resonance energy Transfer; *Saccharomyces cerevisiae*

1. Introduction

Many biological functions involve the formation of protein-protein complexes which may be studied by a large number of methods based on genetic, biochemical and biophysical concepts. Fluorescence resonance energy transfer (FRET) is a sensitive, non-destructive method that has been extensively used to monitor macromolecular interactions [1,2]. It is a non-radiative process in which an excited fluorophore (the donor) transfers its excited state energy to an appropriate acceptor. FRET only occurs if the donor emission spectrum significantly overlaps the acceptor absorption spectrum and when the two fluorophores are in molecular proximity (< 10 nm) [3,4].

Green fluorescent proteins (GFP) have been extensively used to study protein localization in a broad range of cells (for review see [5,6]). Recently, FRET between two GFP mutants was used as a dynamic Ca^{2+} sensor in mammalian cells [7,8] to measure caspase activity in HeLa cells by FACS analysis [9] and to localize the site of interaction of Bax and Bcl-2 in fixed mammalian cells [10].

The yeast *Saccharomyces cerevisiae* is an important model organism to study many biological phenomena. In this paper we describe FRET imaging between two GFP mutants in living yeast. The two GFP mutants were the donor GFP mutant P4.3, bearing the Y66H and Y145F mutations [11], and the acceptor GFP mutant S65T [12]. As FRET efficiency depends on the distance between the two fluorophores, we constructed a concatemer where the two GFP mutants are linked by a spacer containing a protease-specific recognition site. We used the tobacco etch virus (TEV) protease because it has been shown to be active and non-lethal when expressed in yeast [13]. This is the first demonstration of FRET between two GFP mutants in living yeast.

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Abbreviations: GFP, green fluorescent protein; FRET, fluorescence resonance energy transfer; TEV, tobacco etch virus

2. Materials and methods

2.1. In vitro mutagenesis

The plasmid pUC19 yEGFP3 (a generous gift from B.P. Cormack) contains an optimized coding sequence for the expression in *S. cerevisiae* of the GFP variant Mut3 [14]. It was used as a matrix to create a S65T mutant [12] named YS65T for 'yeast S65T' using the site-directed mutagenesis kit (Clontech). The resulting plasmid was named pUC YS65T. Using the same kit, the plasmid pGFP P4.3, containing the coding sequence of the P4.3 mutant [11], was created starting with the pGFP (Clontech) as a matrix.

2.2. Gene constructions

The cDNA of the P4.3 mutant was amplified by PCR using pGFP P4.3 as a matrix with the upstream primer 5′-GCGTCGACTATGAGTAAAGGAGAAGAAC-3′ to create a *Sal*I site and the downstream primer 5′-GGAATTCACCTTGAAAATACAAAGTTTCTTTGTATAGTTCATCCATGC-3′ to create an *Eco*RI site, eliminate the P4.3 stop codon and add the TEV protease target (ETLYFQG) coding sequence [15]. The *Sal*I/*Eco*RI restricted product was cloned in pBluescript II KS$^+$ (Stratagene). The resulting plasmid was named pKS P4.3t. The cDNA of the YS65T mutant was amplified using the pUC YS65T as a matrix with the upstream primer 5′-GGAATTCATGTCTAAAGGTGAAGAATT-3′ to create an *Eco*RI site and the downstream primer 5′-GACTAGTTATTTGTACAATTCATCCAT-3′ to create a *Spe*I site. The *Eco*RI/*Spe*I restricted product was cloned in the pKS P4.3t. The resulting plasmid was named pKS P4.3tYS65T. The pKS P4.3tYS65T *Sal*I-*Xba*I fragment containing the concatemer ORF was cloned between the *Xho*I and *Xba*I sites of pVT100-U [16]. The resulting plasmid was named pVT P4.3tYS65T. The pUC19 YS65T *Xba*I-*Hin*dIII fragment was cloned into pVT100-U. The resulting plasmid was named pVT YS65T. The 27 kDa TEV protease cDNA was amplified using pVT100-L TEV protease as a template [13] (a generous gift from B.D. Kohorn) with the upstream primer 5′-GGGGCGGCCGCCGGAGAAAGCTTGTTTAAG-3′ to create a *Not*I site and the downstream primer 5′-GGAATTCTATTGCGAGTACACCAAT TC-3′ to create a stop codon and an *Eco*RI site. The *Not*I/*Eco*RI restricted product was cloned in the pYeF1-T plasmid [17] in frame with the HA epitope. The resulting plasmid was named pYeF1-TEV. Polymerase fidelity was evaluated by checking the fluorescence spectra of GFP mutant proteins and by sequencing for the TEV protease coding sequence.

2.3. Protein expression and in vitro spectroscopy

P4.3tYS65T protein was produced in the *Escherichia coli* strain XL1 Blue by using pKS P4.3tYS65T. After induction as described by Mitra et al. [18], cells were lysed according to Inouye and Tsuji [19]. Debris was removed by centrifugation and the supernatant was loaded onto a Sephacryl S200 column (Pharmacia). Fractions containing the P4.3tYS65T protein were identified by spectrofluorimetry. The peak fraction of the preparation was used for proteolytic cleavage assay.

Spectra were recorded on a Perkin Elmer Cetus LS50B spectrofluorimeter. Recombinant TEV protease was obtained from Gibco-BRL. Cleavage experiments were done at 30°C according to Gibco-BRL instructions using 100 units of TEV protease.

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2.4. In vivo fluorescence microscopy

Strain LG811-5D (*MAT*a, *trp1Δ63*, *leu2Δ1*, *ura3-52*) came from our laboratory and was derived from the X2180-1B and FY833 strains.

To monitor GFP fluorescence by microscopy in growing yeast a special microscope slide was made (similar to those of Waddle et al. [20]), using a galactose medium (agarose 4%, galactose 2%, a yeast nitrogen base without amino acids from Difco, 6.7 g/l) supplemented with appropriate amino acids. Cells from 1-day fresh SD medium plate [21], transferred 1 night (30°C) to a glucose 0.1% SD medium plate ($SD_{0.1}$ medium) supplemented with appropriate amino acids, were placed in the center of this galactose special slide and immediately imaged. After incubation at 24°C in a dark humid chamber, the same cells were re-imaged.

Imaging was with a Leica DMRXA epifluorescence microscope (immersion objective Leica PL APO 100×) with a cooled CCD camera MicroMax (Princeton Instruments) controlled by the Metamorph 3.0 software (Universal Imaging). Images were captured with a 500 ms exposure time. Cells were viewed with two distinct filter sets: the Leica FITC filter set (excitation: 450–490 nm, dichroic mirror: 510 nm, emission: 515–560 nm) and the FRET filter set (excitation: 340–380 nm, dichroic mirror: 400 nm, emission: 515–560 nm from Omega Optical), with a Hg lamp (HB0 50W/AC, Osram).

2.5. FRET measurement

Fluorescence intensity data were collected in a 5×5 pixel square region per cell. One pixel corresponds to about 3600 nm^2. The background corresponding to the same area in a region without cells was subtracted. Cells in which fluorescence intensity led to saturation of the camera even for one pixel, were ignored. Nomenclature: A: acceptor chromophore: YS65T protein. D: donor chromophore: P4.3 protein. The superscript AD indicates the presence of acceptor and donor while the superscript A means the acceptor only. f: fluorescence intensity acquired through the FITC filter set (background subtracted). F: fluorescence intensity acquired through the FRET filter set (background subtracted).

For the FITC filter set, the fluorescence intensity obtained for the P4.3tYS65T protein is equal to the sum of the fluorescence intensity of the acceptor (f_A^{AD}) plus the fluorescence intensity of the donor (f_D^{AD}) plus the fluorescence intensity due to FRET

$$(f_{fret}^{AD}) \text{ i.e.}: f^{AD} = f_A^{AD} + f_D^{AD} + f_{fret}^{AD} \quad (1)$$

The P4.3 protein is excited with wavelengths shorter than 420 nm. The FITC filter set allows an excitation between 450–490 nm. In this condition f_D^{AD} is near zero. If the donor is not excited, FRET cannot occur, and f_{fret}^{AD} is negligible.Therefore Eq. 1 becomes:

$$f^{AD} = f_A^{AD} \quad (2)$$

As for the FITC filter set, the equation for the FRET filter set is:

$$F^{AD} = F_A^{AD} + F_D^{AD} + F_{fret}^{AD} \quad (3)$$

Considering the P4.3 mutant fluorescence emission spectrum [11], when there is no FRET, the observed donor emission with the FRET filter set (F_D^{AD}) represents less than 6% of the total emission of the donor. Thus, when FRET occurs and due to the P4.3 and the S65T mutants fluorescence properties [11], F_D^{AD} is negligible compared to $F_A^{AD} + F_{fret}^{AD}$.

Thus, Eq. 3 becomes

$$F^{AD} = F_A^{AD} + F_{fret}^{AD} \quad (4)$$

The fluorescence intensity obtained with the FITC filter set is only due to the acceptor (Eq. 2). If we consider only the fluorescence intensity due to the acceptor, the ratio of the fluorescence intensity obtained with the FRET filter set to the fluorescence intensity for the FITC filter set is the same both in the absence and in the presence of the donor. Therefore, $F_A^A/f_A^A = F_A^{AD}/f_A^{AD}$.

By replacing this in Eq. 4, we get

$$F_{fret}^{AD} = F^{AD} - (F_A^A/f_A^A)\, f_A^{AD} \quad (5)$$

2.6. Western blot analysis

Yeast cells were grown as described in section Section 2.4. After incubation, cells were retrieved from microscope slides and resuspended in water. Then, 1 ml of this cell suspension ($OD_{600nm} = 2.5$) was lysed as described [22]. Western blot analysis was done as described [23]. GFP was monitored with Clontech anti-GFP polyclonal antibodies (dilution 1:2000) and Pierce peroxidase-conjugated anti-rabbit IgG (dilution 1:2000). To study the TEV protease, the same membrane was stripped by incubation for 30 min at 60°C in 100 mM β-mercaptoethanol, 62.5 mM Tris-HCl pH 7.6, 2% SDS and a second Western blot was done with Boehringer 12CA5 anti-HA antibodies (dilution 1:2000) and Pierce peroxidase-conjugated anti-mouse IgG (dilution 1:2000).

3. Results

3.1. In vitro spectrofluorimetric analysis of P4.3tYS65T protein fluorescence

We initially verified in vitro that FRET could occur in the P4.3tYS65T protein and that the TEV protease is able to disrupt FRET by cleavage of the covalent linkage between the two fluorophores. The P4.3tYS65T protein was produced in *E. coli*, partially purified and its fluorescence was analyzed by spectrofluorimetry (Fig. 1). The emission spectrum of the intact P4.3tYS65T protein clearly shows that an excitation of the donor (P4.3) at 385 nm leads to an emission of the donor (445 nm emission band) and of the acceptor YS65T (511 nm emission band) (Fig. 1A). When recombinant TEV protease is added, the emission of the donor increases whereas the emission of the acceptor decreases. Therefore, FRET occurs in an intact P4.3tYS65T protein. By doing the same experiment without TEV protease (Fig. 1B), we confirmed that the TEV protease is responsible for the disappearance of FRET in Fig.

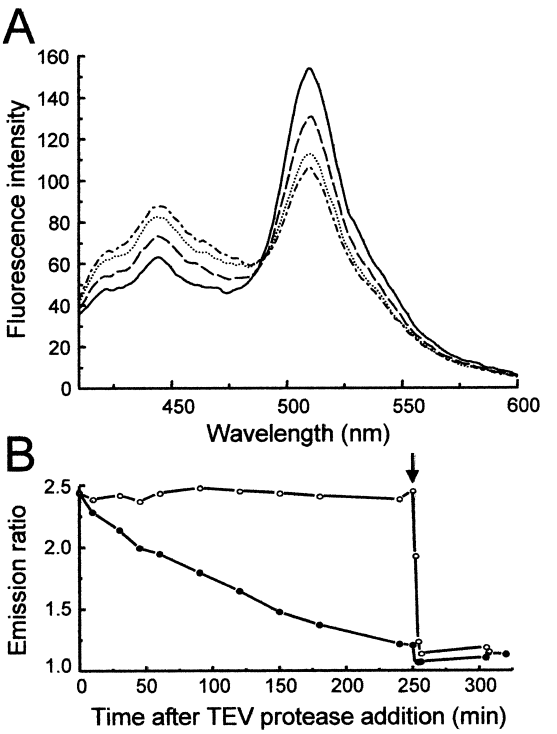

Fig. 1. In vitro, FRET between covalently linked GFP P4.3 and YS65T is abolished after cleavage by TEV protease. A: Emission spectra of the P4.3tYS65T protein when excited at 385 nm: before TEV protease addition (solid line), 90 min (dashed line), 180 min (dotted line), and 240 min (dot-dashed line) after addition of 100 units of TEV protease. B: Emission ratio (511 nm/445 nm), for an excitation at 385 nm, versus time. Open circles: without TEV protease. Filled circles: with 100 units of TEV protease. The arrow indicates the addition of 0.2 μg of trypsin.

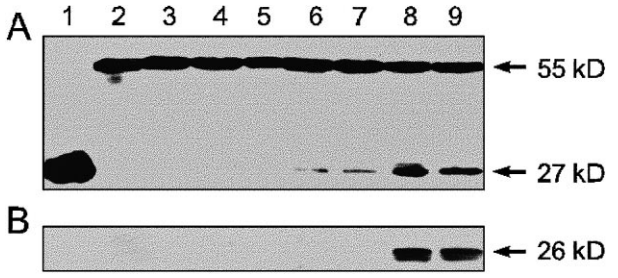

Fig. 2. Western blot analysis of TEV protease cleavage in yeast. Total protein extract from yeast transformed with: lane 1: pVT YS65T, lane 2: pVT P4.3tYS65T. Lane 3: pVT P4.3tYS65T+pYeF1-T grown on SD medium plate. Lane 4: pVT P4.3tYS65T+pYeF1-T grown on $SD_{0.1}$ medium plate. Lane 5: pVT P4.3tYS65T+pYeF1-T grown on galactose special microscope slide for 8 h. Lane 6: pVT P4.3tYS65T+pYeF1-TEV grown on SD medium plate. Lane 7: pVT P4.3tYS65T+pYeF1-TEV grown on $SD_{0.1}$ medium plate. Lane 8: pVT P4.3tYS65T+pYeF1-TEV grown on galactose slide for 2 h. Lane 9: pVT P4.3tYS65T+pYeF1-TEV grown on galactose slide for 4 h. A: Western blot with anti-GFP polyclonal antibody. B: Same membrane hybridized with anti-HA (12CA5) antibody.

1A. The addition of trypsin at the end of the kinetic (which cuts the link between the two GFPs without affecting their fluorescence properties [11]) totally abolished FRET. The remaining signal at 511 nm arise from the direct excitation of the acceptor. This is confirmed by the analysis of the excitation and emission spectra obtained for an equal amount of fluorescent acceptor only (data not shown). In conclusion, FRET occurs in an intact P4.3tYS65T protein and is abolished after cleaving the linker by the TEV protease or trypsin.

3.2. *P4.3tYS65T protein and TEV protease expressions in yeast*

To transfer this assay to living yeast, two vectors were constructed: the first, pVT P4.3tYS65T, allows the constitutive expression of the P4.3tYS65T protein; the second, pYeF1-TEV, allows the conditional expression of an HA-tagged TEV protease under the control of a galactose-inducible promoter [17]. A Western blot analysis was done to monitor the GFP status using the corresponding antibody (Fig. 2A). To assess the inducible TEV protease expression and because the molecular mass expected for the TEV protease is close to that of the GFP, the same membrane was de-hybridized and a second Western blot was done on the same membrane with anti-HA antibodies (Fig. 2B). Using GFP antibodies, a single signal corresponding to a protein with an apparent molecular mass of 55 kDa was revealed for yeast cells transformed with the pVT P4.3tYS65T alone (Fig. 2A, lane 2). This apparent molecular mass was consistent with that expected for the intact P4.3tYS65T protein. Control experiments were done to be sure that no significant P4.3tYS65T protein proteolysis occurred in our growth conditions without TEV protease expression (Fig. 2A, lanes 3–5). When yeast cells were co-transformed with pVT P4.3tYS65T and pYeF1-TEV (Fig. 2A, lanes 6–9), a band appeared with a relative molecular mass corresponding to GFP alone (Fig. 2A, lane 1). Its intensity was very weak when yeast cells were grown on glucose or on $SD_{0.1}$ medium (Fig. 2A, lanes 6 and 7), but strongly increased for cells grown for 2 h on the galactose special microscope slide (Fig. 2A, lane 8). There were no further detectable changes even when the yeast cells were incubated for 4 h (Fig. 2A, lane 9) or 8 h (data not shown) in the same conditions. TEV protease expression was detectable only for yeast cells transformed with pYeF1-TEV grown on galactose slide (Fig. 2B, lanes 8 and 9). Therefore, TEV protease can cleave the linker peptide between the two GFPs in the P4.3tYS65T protein in living yeast after 2 h of induction on a galactose slide. No more proteolysis occurred if the time of induction was extended. Nevertheless, a large proportion of the GFP concatemer remained intact (Fig. 2A, lanes 8 and 9). Note that the 27 kDa band is double (Fig. 2A, lane 8), probably due to the asymmetric cleavage of the linker by the TEV protease occurring between the Q and G of the ETLYFQG site [15].

3.3. *Determination of F_A^A/f_A^A ratio*

The linker between the two GFPs in the P4.3tYS65T protein was cleaved by the TEV protease after 2 h of induction on a galactose slide. There was no further proteolysis even if the incubation time was increased. Consequently, the changes in fluorescence intensity were quantitated in living yeast after 3 h of induction.

In Eq. 5, F^{AD} and f_A^{AD} can be directly measured with the FRET and FITC filter sets, respectively. However, for FRET quantification, the ratio F_A^A/f_A^A must be determined. In more than 550 yeast cells transformed with pVT YS65T grown on galactose slide, the ratio F_A^A/f_A^A was estimated to be equal to 6.3240×10^{-3} (S.D. $=0.8848\times10^{-3}$). We also studied the evo-

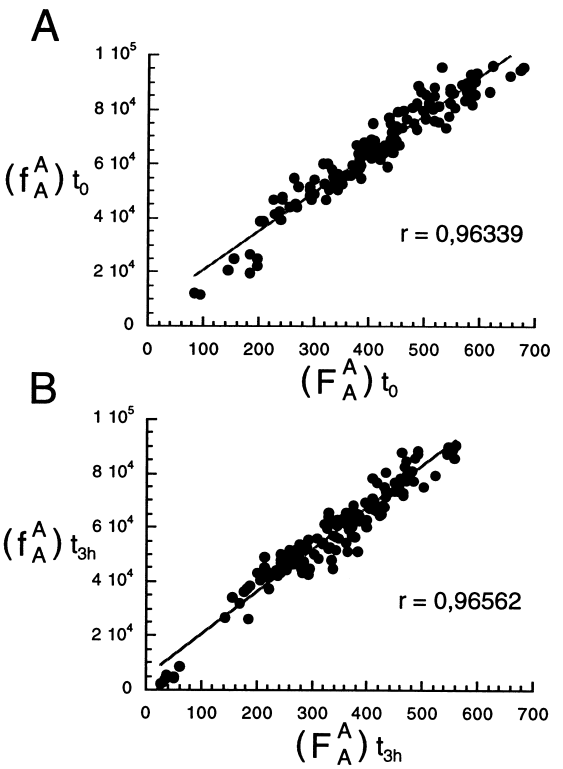

Fig. 3. Estimation and evolution of the ratio F_A^A/f_A^A. Yeast cells transformed with pVT YS65T from a 1-day fresh SD medium plate were transferred to a $SD_{0.1}$ medium plate. After one night at 30°C, cells were transferred to a galactose slide. A: Just after plating, 137 cells were viewed with the two filter sets. For each cell, $(f_A^A)t_0$ and $(F_A^A)t_0$ values were determined. B: The same cells were re-imaged with the two filter sets after 3 h of induction. Again, for each cell, $(f_A^A)t_{3h}$ and $(F_A^A)t_{3h}$ values were determined.

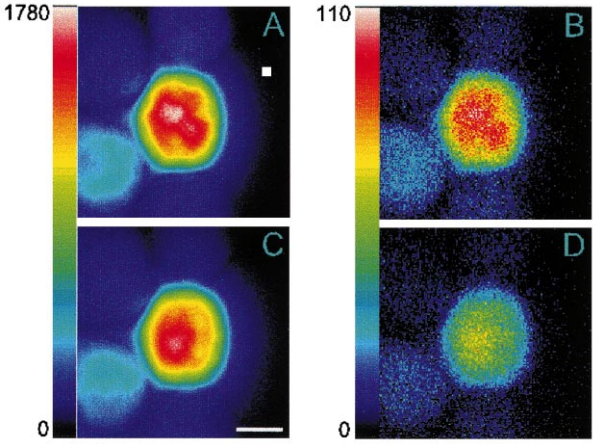

Fig. 4. Fluorescence of the P4.3tYS65T protein in a single yeast cell. A and C: Cell viewed with the FITC filter set. B and D: Same cell viewed with the FRET filter set. A and B: Images taken immediately after laying cells on a galactose special microscope slide. C and D: Same cell re-imaged 3 h after deposit. The pseudo-color calibration bar depicts low (black, bottom) to high (white, top) fluorescence intensity for each filter set. Scale bar: 2.5 μm.

lution of this ratio with time. Yeast cells transformed with pVT YS65T were imaged just after plating on galactose slide. Fig. 3A shows the values of $(F_A^A)t_0$ and $(f_A^A)t_0$ obtained for 137 cells. The ratio $(F_A^A/f_A^A)t_0$ was estimated to be equal to 6.3405×10^{-3} (S.D. = 0.6576×10^{-3}). The same cells were then re-imaged after 3 h of incubation (Fig. 3B). The ratio $(F_A^A/f_A^A)t_{3h}$ was estimated to be equal to 6.0753×10^{-3} (S.D. = 1.2930×10^{-3}). In addition, the cells were able to grow and divide during incubation (data not shown). Thus, the ratio F_A^A/f_A^A did not change significantly during the experiment, so Eq. 5 can be used to calculate FRET. Moreover, the intensities for F_A^A and f_A^A at t_0 were higher than those at t_{3h}. This decrease in intensity of about 15% is not due to the change in carbon metabolism but to the photobleaching of the chromophore during the first exposure (data not shown).

3.4. FRET measurements in living yeast

Fig. 4 shows in A and B a single yeast cell viewed just after plating on a galactose special microscope slide. In C and D, the same living yeast cell was re-imaged after 3 h of incubation. Between t_0 and t_{3h}, the fluorescence intensity strongly decreased with the FRET filter set (Fig. 4B,D respectively). Fig. 5 shows quantitative results obtained in a cell population (75 cells). Using Eq. 5, it is possible to calculate the value of $(F_{fret}^{AD})t_0$ and $(F_{fret}^{AD})t_{3h}$ for each cell analyzed. Fig. 5 shows the percentage of F_{fret}^{AD} loss between t_0 and t_{3h} for yeast co-transformed with pVT P4.3tYS65T and pYeF1-TEV (Fig. 5A) and with pVT P4.3tYS65T and pYeF1-T (Fig. 5B) as control. For yeast co-transformed with pVT P4.3tYS65T and pYeF1-TEV, the mean F_{fret}^{AD} loss is 57% (S.D. = 12) and for the control it is 34% (S.D. = 10).

4. Discussion

We demonstrate that FRET between two linked GFP mutants can be monitored by imaging microscopy in living yeast. The work is based on a fusion protein in which the P4.3 mutant is linked to the YS65T mutant by a 9-residue spacer bearing the TEV protease-specific cleavage site. In vitro experiments show that not only FRET can occur in an intact fusion protein but also that the specific cleavage site is accessible for the TEV protease. The in vitro addition of TEV protease almost totally disrupts FRET, as confirmed by trypsin experiments [11]. Our results are in agreement with similar previous results [11,18]. However, quantitative comparisons can only be tentative because the GFP mutants, linker, protease and experimental conditions were not strictly the same.

In yeast cells the fusion protein is not proteolysed in the absence of TEV protease gene induction, but there is a small leak of the galactose-inducible promoter in glucose medium, as shown in Western blot experiments. After induction of TEV protease expression, proteolysis occurs in living yeast but a large proportion of the GFP fusion protein remains intact. No more proteolysis occurs even if the induction time is increased. This might be explained by the balance between the new synthesis of P4.3tYS65T protein and the TEV protease activity, especially since the amount of TEV protease did not increase with induction time. This is in accordance with the fact that in cells the FRET signal is not entirely lost (see Fig. 5A). Despite this only partial proteolysis, imaging experiments are able to detect a loss of FRET signal in cells expressing both fusion protein and TEV protease.

In view of these imaging experiments, cells expressing the fusion protein alone show an apparent loss of FRET signal

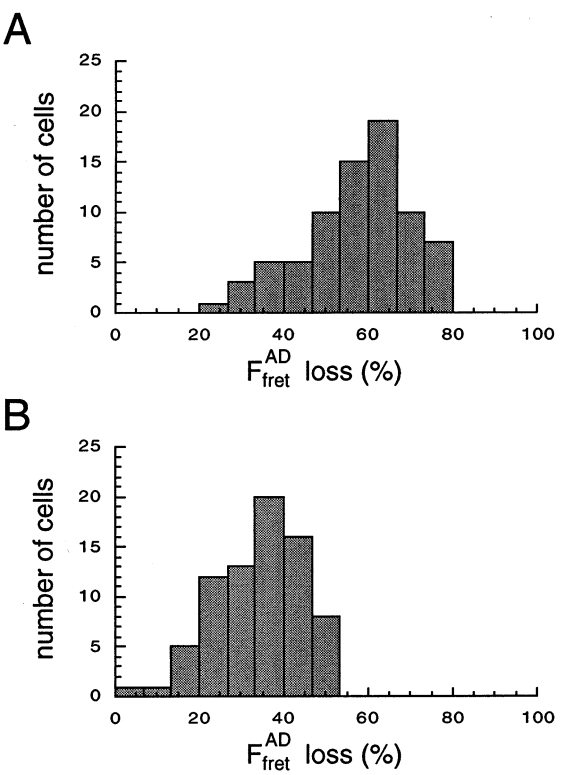

Fig. 5. Percentage of FRET loss in a population of yeast grown for 3 h on galactose special microscope slide. F_{fret}^{AD} loss (%) = $100-[100/(F_{fret}^{AD})t_0]\times(F_{fret}^{AD})t_{3h}$. A: Yeast co-transformed with pVT P4.3tYS65T and pYeF1-TEV. B: Yeast co-transformed with pVT P4.3tYS65T and pYeF1-T.

(Fig. 5B). Certain hypotheses for this can be made. For example, the signal due to the direct excitation of the acceptor (F_A^{AD}) may be overestimated in the FRET calculation after 3 h of induction. This hypothesis is in part supported by the fact that the P4.3 mutant is known to be more subject to photobleaching than the other GFP variants [6]. One way to improve imaging of GFP-based FRET is to develop a GFP donor mutant with faster folding and better resistance to photobleaching, such as the cyan GFP mutants described by Miyawaki et al. [7]. In addition more adapted filter sets, for example with a narrower excitation band, could be used.

With such improvements, FRET between two GFP mutants could be used to study the major conformational changes of any protein and in any cellular compartment of living cells. Another attractive goal for using GFP-based FRET is imaging the localization and dynamic of non-covalent protein-protein interactions in living cells. This is particularly pertinent in yeast where functional studies following the availability of the whole genome sequence have provided much data about protein-protein interactions through the two-hybrid approach.

Nevertheless, for GFP-based FRET imaging, three parameters must be considered. At least two different states of interaction leading to a variation in FRET efficiency are required. Indeed, a non-FRET state is necessary to appreciate the donor concentration. Finally, the most sensitive element to study intermolecular interactions is that in a formed complex: the two GFP mutants must be in a position allowing FRET to occur.

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