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Detection of a conformational change in Gγ upon binding Gβ in living cells

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Abstract **Interaction induced changes in the conformation of proteins are frequently the molecular basis for the modulation of their activities. Although proteins perform their functions in cells, surrounded by many potential interaction partners, the studies of their conformational changes have been mainly restricted to in vitro studies. Ste4p (Gβ) and Ste18p (Gγ) are the subunits of a heterotrimeric G-protein in the yeast *Saccharomyces cerevisiae*. A split-ubiquitin based conformational sensor was used to detect a major structural rearrangement in Ste18p upon binding to Ste4p. Based on these in vivo results and the solved structure of the mammalian Gβγ, we propose that Gγ of yeast adopts an equally extended structure, which is only induced upon association with Gβ.** **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Protein folding; Split-ubiquitin; Trimeric G-protein; Protein interaction; Ste4p–Ste18p; Gβγ dimer

1. Introduction

The physiological functions and properties of proteins are often affected by the binding to other proteins or ligands. Frequently, the molecular basis for this modulation in activity is a change in the conformation of the interacting proteins [1]. Due to the limitations of existing techniques, the development of novel methods to identify and measure the conformational changes of proteins within the living cell are indispensable to an understanding of the biological processes [2,3].

The Gα, Gβ and Gγ subunits of G-proteins assemble into trimeric complexes that are found in all eukaryotes [4]. A trimeric G-protein of the yeast *Saccharomyces cerevisiae* consists of Gpa1p (Gα), Ste4p (Gβ), and Ste18p (Gγ), and induces the kinase cascade of the mating pathway after mating hormone induced dissociation into Gpa1p and the Ste4p–Ste18p dimer [5–7]. The solved structure of the bovine Gβγ was used as a template to model the Ste4p–Ste18p complex [8–10]. Very similar to its mammalian counterpart, Ste18p is predicted to fold into a very extended conformation without internal contacts between its two helices. The N-terminal helix of the Gγ forms a coiled coil structure together with the N-terminal helix of Gβ. The C-terminal helix and additional residues of Gγ form extensive contacts to six of the seven blades of the WD40 propeller structure of Gβ [9]. To test the prediction of an extended structure of Ste18p and to find out whether this structure is only induced upon binding to Ste4p, we analyzed the conformation of Ste18p in living cells by applying a recently introduced variation of the split-ubiquitin (split-Ub) technique [11].

The split-Ub method is based on the ability of N_{ub} and C_{ub}, the N- and C-terminal halves of ubiquitin, to assemble into a quasi-native Ub. Ub specific proteases recognize the reconstituted Ub, though not its halves, and cleave the Ub moiety off a reporter protein which has been linked to the C-terminus of C_{ub}. The release of the reporter serves as readout indicating the reconstitution of Ub. The assay is designed in a way that prevents efficient association of N_{ub} and C_{ub} by themselves, but permits such association if the two Ub halves are separately linked to proteins which interact in vivo [12]. Attaching N_{ub} and C_{ub} to the N- and C-termini of the same polypeptide makes possible the measurement of intramolecular N_{ub} and C_{ub} reassociation by quantifying the ratio of cleaved to uncleaved fusion protein. This ratio is defined by the affinity of N_{ub} to C_{ub}, and by the nature of the polypeptide separating N_{ub} from C_{ub} [11]. A newly constructed set of N_{ub} mutants spans a wide range of affinities for C_{ub} and extends the application of this technique to protein structures with different N- and C-terminal arrangements. The isoleucine residues at positions 3 and 13 of N_{ub} (N_{ii}) were hereby replaced by valine (N_{xv}; N_{vx}), alanine (N_{xa}; N_{ax}), and glycine (N_{xg}; N_{gx}), where x is either i, v, a or g. Using this assay we here report on a conformational change of Ste18p upon binding Ste4p in vivo.

2. Materials and methods

2.1. Construction of fusion proteins

Fragments containing the open reading frame (ORF) of *STE18* lacking either the first 57 (*STE18*$_{91}$), 75 (*STE18*$_{85}$), 90 (*STE18*$_{80}$), or 108 (*STE18*$_{74}$) nucleotides and lacking the last 15 nucleotides were obtained by PCR using yeast genomic DNA, the P*wo* polymerase (Roche-Biochemicals, Penzberg, Germany) and oligonucleotide primers complementary to the 5′ and 3′ ends of the desired ORF, respectively (Metabion, Martinsried, Germany). All 5′ primers contained an additional *Bam*HI site, the 3′ primer an additional *Sal*I site to allow for the in-frame fusion with the N_{ub} and C_{ub} moieties [11]. The border between N_{ub} and the different STE18 constructs reads GGG ATC CCC XXX, where XXX is CAG as the first codon for *STE18*$_{91}$, AAG for *STE18*$_{85}$ and *STE18*$_{80}$, and GAA for *STE18*$_{74}$. The corresponding C_{ub}-*RURA3* constructs were obtained by inserting the *Eag*I-*Sal*I cut P_{CUP1}-N_{ub} ORF in front of the C_{ub}-RURA3 module on a pRS313 vector [13]. N_{ia}-*STE18*$_{91}$-Dha was derived from the corresponding P_{CUP1}-N_{ub}-*STE18*$_{91}$-C_{ub}-Dha constructs by cloning the

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Abbreviations: Ub, ubiquitin; N_{ub} and C_{ub}, N- and C-terminal half of Ub; ORF, open reading frame; Dha, DHFR extended by the HA epitope

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*Eag*I-*Sal*I fragment in front of the ORF of DHFR extended by the HA epitope (Dha). $STE18_{91}$ containing the natural stop codon and an additional start codon at the 5′ end was obtained by PCR using an oligonucleotide complementary to the 3′ region starting 61 bp downstream of the ORF and an oligonucleotide complementary to the 5′ region of the ORF. The HA-*STE4* construct was obtained by PCR using an oligonucleotide complementary to a 3′ region starting 60 bp downstream of the ORF and an oligonucleotide complementary to the 5′ end of the ORF. The introduced *Sal*I and *Kpn*I sites were used to clone the PCR fragment downstream and in-frame of the P_{GAL1}-HA module in a pRS416 vector. The 5′ sequence of the newly generated ORF reads: ATG TCG ACC TAC CCA TAC GAT GTT CCA GAT TAC GCT GGC TCG ACC **ATG**. The sequence of the HA epitope is underlined. The first codon of STE4 is printed in bold letters.

2.2. Immunoblotting

S. cerevisiae cells expressing the different fusion proteins were grown at 30°C to an OD_{600} of ~0.8 in 10 ml of SG lacking tryptophan and uracil. Cell extraction for immunoblotting was performed essentially as previously described [14]. Bound antibody was visualized with horseradish peroxidase coupled rabbit anti-mouse antibodies (Bio-Rad, Hercules, CA, USA), using the chemiluminescence detection system (Pierce, Rockford, IL, USA) and quantified with the lumi-imager system (Boehringer, Mannheim, Germany).

2.3. Yeast strains, functionality assay

S. cerevisiae strains were JD53 (*MATα his3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52*), JD55 (*MATα his3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52 ubr1Δ::HIS3*), KMY940 (*MATa his3-11,15 leu2-3,112 ade2-1 trp1-1 ura3-1 can1-100 ste18::LEU2*), YEL2 (*MATa his3-11,15 leu2-3,112 ade2-1 trp1-1 ura3-1 can1-100 ste4::URA3*). Yeast rich and synthetic minimal media with 2% dextrose (SD) or 2% galactose (SG) followed standard recipes.

In the functionality assay, the cells were tested for α-factor sensitivity by a halo assay. Filter disks containing 2.4 μg of α-factor were mounted onto media lacking tryptophan or uracil to select for the presence of the plasmids expressing the constructs and 2% galactose to express HA-Ste4p. Cells were grown at 30°C for 1 day.

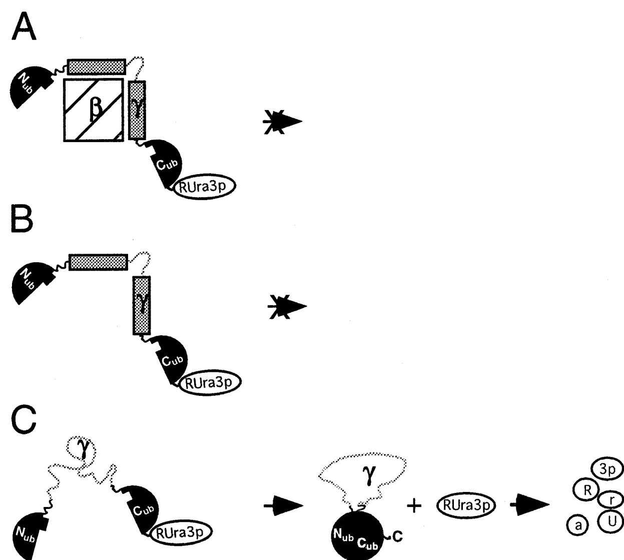

Fig. 1. The three potential outcomes of the split-Ub experiment. A: Ste18p is bound to Ste4p. The structure of Ste18p keeps N_{ub} and C_{ub} at a distance that inhibits their efficient reassociation. The RUra3p reporter is not cleaved off and the cells grow on SD−ura. B: Ste18p maintains its conformation in the absence of Ste4p. The cells can grow on SD−ura. C: Ste18p unfolds or adopts a very different conformation in the absence of Ste4p. The coupled N_{ub} and C_{ub} can reassociate, the RUra3p reporter is cleaved off and degraded. The cells cannot grow on SD−ura.

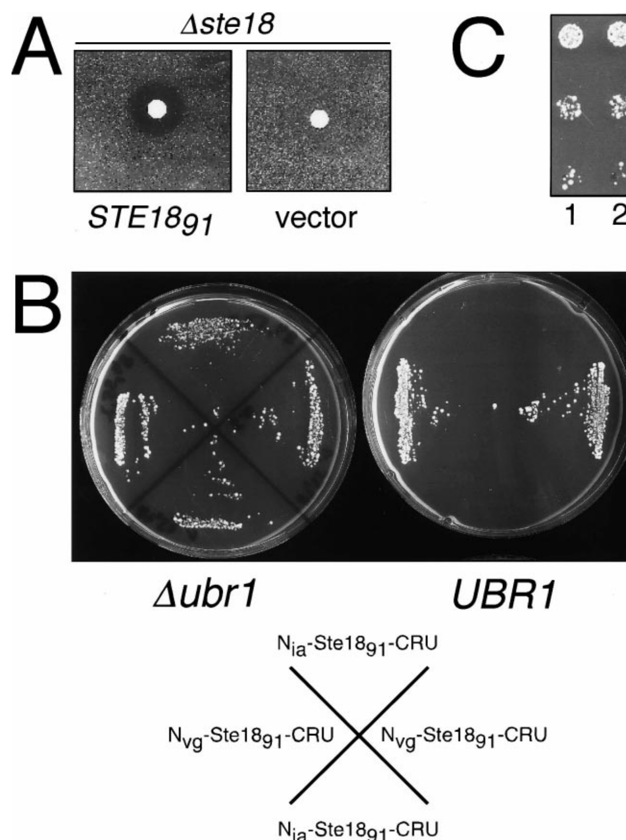

Fig. 2. A: $Ste18_{91}p$, an N-terminally truncated form of Ste18p that was used for the experiments, is functional. The halo of non-growing cells around a filter disk soaked with α-factor documents the functionality of the protein. The cells containing an empty vector instead are unaffected by the mating hormone. B: Cells lacking a functional N end rule and expressing N_{ia}-$Ste18_{91}$-C_{ub}-RUra3 or expressing N_{vg}-$Ste18_{91}$-C_{ub}-RUra3p grow on plates lacking uracil. In contrast wild-type cells that express N_{ia}-$Ste18_{91}$-C_{ub}-RUra3 do not grow on SD−ura−trp whereas the cells expressing N_{vg}-$Ste18_{91}$-C_{ub}-RUra3p still grow. Two different transformants are shown. Cells were grown for 2.5 days at 30°C. C: Cells that contain N_{vg}-$Ste18_{91}$-C_{ub}-RUra3 together with an empty vector (lane 1) or N_{ia}-$Ste18_{91}$-Dha (lane 2) were spotted (2×10^3, 2×10^2 and 2×10^1 cells) on plates lacking uracil and tryptophan.

3. Results and discussion

3.1. Split-Ub based approach

In the proposed structure of the Ste4p–Ste18p complex the N- and the C-termini of Ste18p are spatially separated. This should prevent the association of the N_{ub} and C_{ub} when attached to the N- and the C-termini of the same Ste18p polypeptide. As a result, a RUra3p reporter coupled to the C-terminus of C_{ub} is not cleaved and will enable yeast *ura3* cells to grow on plates lacking uracil (Fig. 1A). Two possibilities can be envisioned for the free Ste18p. If the structure of the free protein is indistinguishable from its bound form, the RUra3p reporter will remain linked to C_{ub} and the cells will retain the original phenotype (Fig. 1B). If the conformation of Ste18p is more flexible in its free than in its bound form it will less hinder the N_{ub}–C_{ub} reassociation and the RUra3p reporter will be cleaved off far more efficiently (Fig. 1C). The enzymes of the N end rule pathway rapidly degrade the released RUra3p rendering the cells uracil auxotrophic [13,15]. The

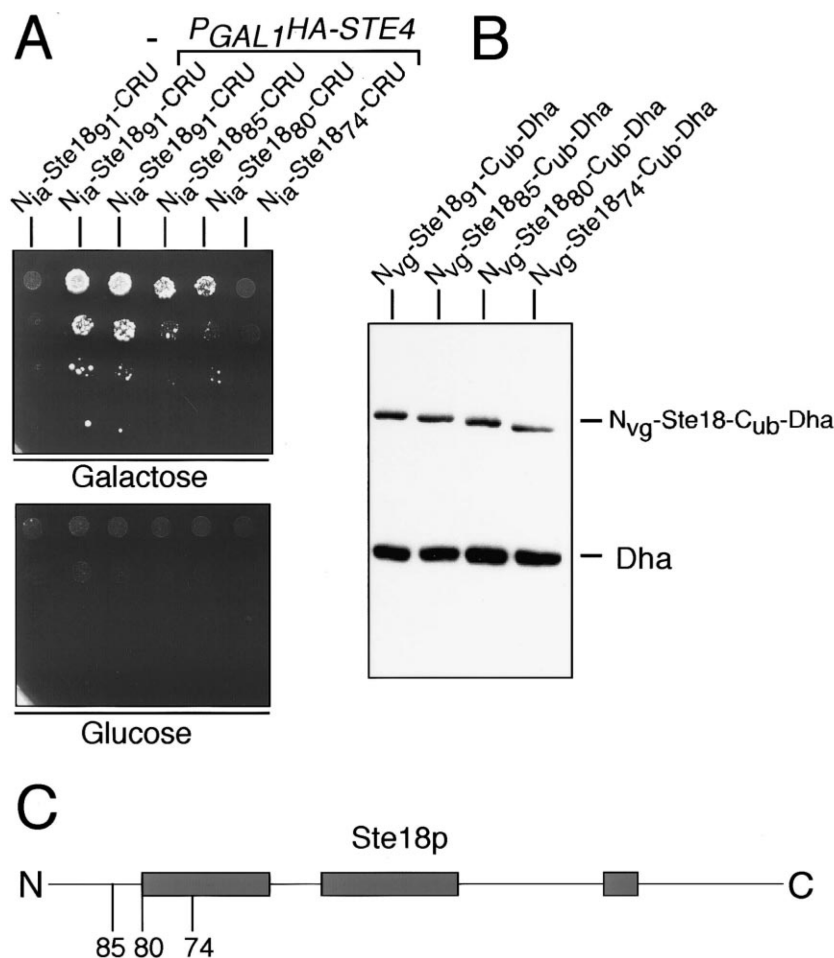

Fig. 3. Ste4p induces an altered conformation in Ste18p. A: Cells containing N_{ia}-*STE18$_{91}$*-C_{ub}-RURA3 and an empty vector or P_{GAL1}*HA-STE4* (shown are two independent transformants) and cells containing N-terminally truncated derivatives of N_{ia}-*STE18$_{91}$*-C_{ub}-RURA3 and P_{GAL1}*HA-STE4* were spotted (10^5, 10^3, 10^2, 10^1) on plates lacking uracil, tryptophan, and containing galactose to induce or glucose to repress the expression of HA-Ste4p. Plates contained 20 μM copper ions to moderately express the *STE18* constructs. Cells were grown for 3 days at 30°C. B: Cells expressing the different N_{vg}-Ste18$_{XY}$-C_{ub}-Dha fusions were grown in glucose medium and extracted for immunoblot analysis. Proteins were detected with anti-HA antibody after SDS–12.5% PAGE. C: Schematic drawing of the *STE18* constructs. Boxes indicate the position of the α-helices according to Sondek et al. [9]. Numbers indicate the lengths of the constructs in amino acids. The numbers include the last three C-terminal residues of Ste18p, which are removed proteolytically during the isoprenylation of the protein.

phenotype of cells that express N_{ub}-Ste18-C_{ub} in the absence of Ste4p will thus reflect the conformation of the uncomplexed Gγ.

3.2. *Ste18p undergoes a change in conformation upon binding to Ste4p*

The first 19 residues of Ste18p are unique to Gγ of the yeast. The structure of this N-terminal stretch could therefore not be predicted and its sequence was deleted to create Ste18$_{91}$p. α-Factor induced growth inhibition of cells expressing Ste18$_{91}$p instead of the wild-type protein documented an undiminished functionality of Ste18$_{91}$p and thereby indirectly its binding to Ste4p (Fig. 2A, and data not shown) [16]. A STE18$_{91}$ construct that lacked the last five C-terminal residues including the motif for isoprenylation was placed between N_{ub} and C_{ub}-RURA3. Our previous work has shown that the affinity between N_{ub} and C_{ub} is critical for reflecting the conformation of a protein in a N_{ub}–C_{ub} fusion [11]. A N_{ub} with a too strong affinity for C_{ub} will override the effect of the conformation of the inserted Ste18p whereas a N_{ub} with a too low affinity for C_{ub} will not sense any alterations in the conformation. Since we predicted that the conformation of the uncomplexed Ste18p would favor rather than impede the N_{ub}–C_{ub} reassociation we first had to choose a N_{ub} that just inhibits the growth of the N_{ub}-Ste18$_{91}$-C_{ub}-RUra3p transformed cells. N_{ii} and N_{ia} in a N_{ub}-STE18$_{91}$-C_{ub}-RURA3 construct both inhibit the SD−ura growth of cells, which do not overexpress Ste4p (Fig. 2B, and data not shown). We chose N_{ia} for our further studies since N_{ia} has a weaker affinity for C_{ub} and should therefore react more sensitively to alterations in the conformation of Ste18p. We performed two control experiments to show that the uracil auxotrophy of the cells reflects the efficient N_{ia}–C_{ub} reassociation within the N_{ia}-Ste18$_{91}$-C_{ub}-RUra3p and not a rapid degradation of the uncleaved fusion protein [16,17]. N_{vg} displays a lower affinity for C_{ub} that results in the accumulation of a larger fraction of uncleaved fusion protein than observed with the otherwise identical N_{ia} construct. The growth of cells on SD−ura proves that the uncleaved N_{vg}-Ste18$_{91}$-C_{ub}-RUra3p is sufficiently stable (Fig. 2B). This interpretation was further corroborated by

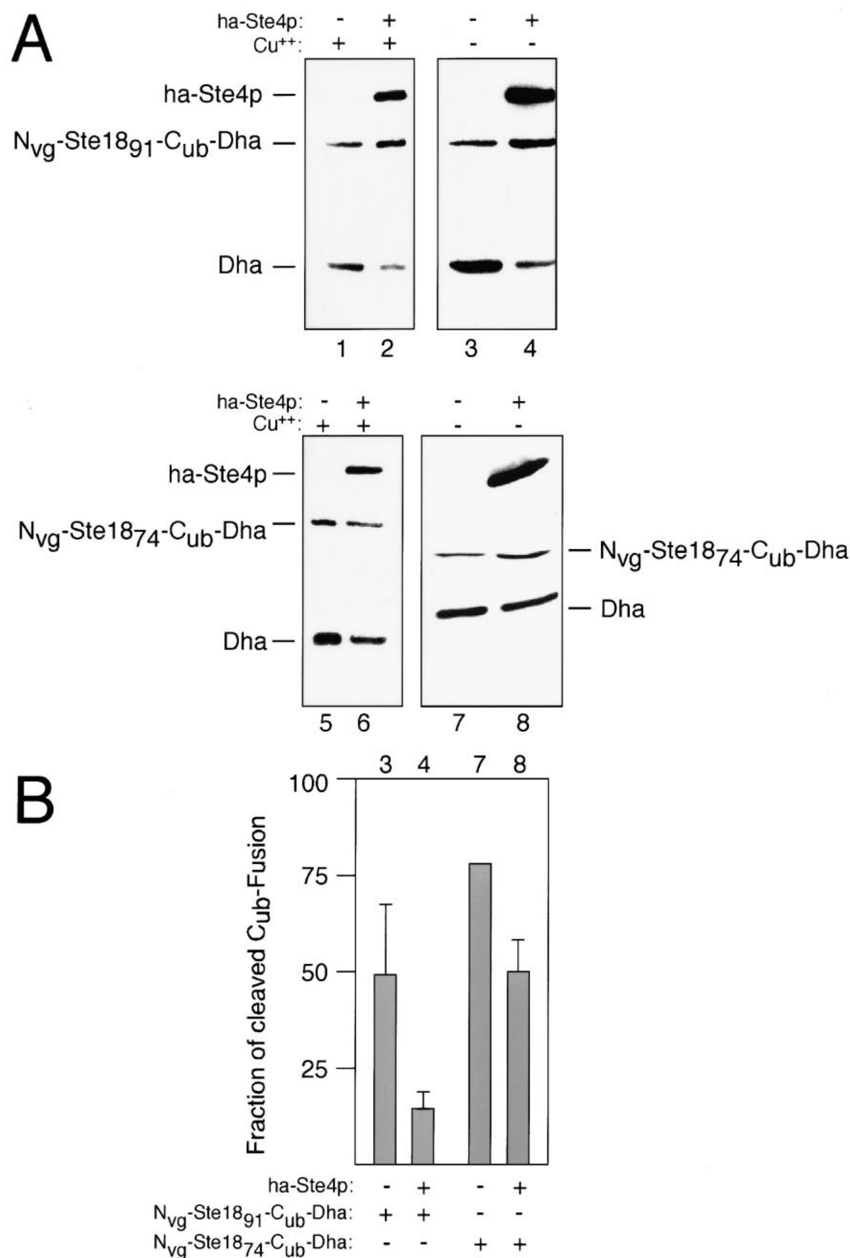

Fig. 4. The ratio of uncleaved to cleaved N_{vg}-Ste18_{91}-C_{ub}-Dha is influenced by coexpression of HA-Ste4p. A: Cells containing N_{vg}-*STE18*$_{91}$-C_{ub}-Dha (lanes 1–4) or N_{vg}-*STE18*$_{74}$-C_{ub}-Dha (lanes 5–8) and *HA-STE4* (lanes 2, 4, 6, 8) or an empty vector (lanes 1, 3, 5, 7) were grown in galactose to express HA-Ste4p. One hour prior to protein extraction 100 μM copper sulfate was added to one set of samples to increase the expression of the STE18 constructs (lanes 1, 2, 5, 6). Samples without addition of copper are shown in lanes 3, 4, 7, and 8. Proteins were detected by HA antibody after SDS–12.5% PAGE and transfer onto nitrocellulose. B: The ratio of uncleaved to cleaved fusion protein was calculated by quantitative chemiluminescence. Shown are the average of the values from five experiments with cells expressing N_{vg}-Ste18_{91}-C_{ub}-Dha from the uninduced P_{CUP1} promoter (lanes 3, 4), and the average of the values from three experiments with cells expressing N_{vg}-Ste18_{74}-C_{ub}-Dha from the uninduced P_{CUP1} promoter (lanes 7, 8).

the growth of the Δ*ubr1* cells expressing N_{ia}-Ste18_{91}-C_{ub}-RUra3p on SD−ura (Fig. 2B). This strain lacks a functional N end rule and will not degrade the cleaved RUra3p [13,15]. We conclude that the efficient cleavage at the C-terminus of C_{ub} followed by the rapid degradation of the released RUra3p causes uracil auxotrophy of the corresponding isogenic wild-type cells. An intramolecular or an intermolecular N_{ia}–C_{ub} reassociation could induce this efficient cleavage. The latter reaction might reflect the propensity of the free Ste18p to form defined complexes or aggregates. To distinguish between these two alternatives we coexpressed N_{vg}-Ste18_{91}-C_{ub}-RUra3p together with a N_{ia}-STE18_{91} construct which was C-terminally extended by Dha to facilitate the detection of the protein by immunoblotting (N_{ia}-Ste18_{91}-Dha). If the free Ste18p forms dimers or multimers, the coexpression of N_{ia}-Ste18_{91}-Dha should increase the cleavage of N_{vg}-Ste18_{91}-C_{ub}-RUra3p and thereby inhibit the SD−ura growth of the cells. This was not observed. The cells coexpressing N_{vg}-Ste18_{91}-C_{ub}-RUra3p and N_{ia}-Ste18_{91}-Dha still grow nearly as well on SD−ura as the cells expressing N_{vg}-Ste18_{91}-C_{ub}-RUra3p

alone, although N_{ia}-Ste18$_{91}$-Dha was clearly detected in extracts of the cells by the anti-HA antibody (Fig. 2C, and data not shown).

To measure the influence of Ste4p on the conformation of Ste18p, we coexpressed N_{ia}-Ste18$_{91}$-C_{ub}-RUra3p together with HA-tagged Ste4p (HA-Ste4p) in cells lacking the chromosomal *STE18* gene. The cells were spotted in different dilutions onto plates without uracil and containing either glucose or galactose as the carbon source. Since the expression of HA-STE4 was controlled by the P_{GAL1} promoter, the intracellular HA-Ste4p concentration was high on galactose medium but below the limits of detection on glucose medium (Fig. 4A, and data not shown). Transformants containing these constructs were uracil auxotrophs on glucose medium, but did grow without uracil on medium containing galactose (Fig. 3A). This effect was due to the presence of the HA-STE4 expressing plasmid since cells containing an empty vector instead were unable to grow without uracil independent of the nature of the carbon source (Fig. 3A). The outcome of this analysis supports the model that upon binding to Ste4p, Ste18p undergoes a substantial conformational change that interferes with the efficient interaction between the coupled N_{ub} and C_{ub} (Figs. 1A and 3A). To further confirm the predicted structure of the Ste4p bound Ste18p, we performed the identical assays with STE18 constructs in which an increasing portion of the N-terminus was removed from Ste18$_{91}$p. The deletion of another six or 11 residues in the STE18$_{85}$ and STE18$_{80}$ constructs already diminished the effect of the expression of HA-Ste4p on the SG−ura growth of those cells (Fig. 3A). Removing a further five residues form the N-terminus (Ste18$_{74}$), and thereby invading the predicted N-terminal helix which constitutes a part of the central binding interface, completely inhibits the SG−ura growth of the construct transformed cells (Fig. 3A). To compare the expression levels of the different fusion proteins, we replaced the RUra3p reporter with the Dha moiety and exchanged the N_{ia} against the N_{vg} to create the corresponding N_{vg}-Ste18-C_{ub}-Dha constructs. Since the released Dha bears a stabilizing N-terminus, we could simultaneously detect the cleaved and the uncleaved fusion proteins. The immunoanalysis revealed comparable expression levels for all fusion proteins (Fig. 3B). We therefore conclude that the N-terminal deletions of Ste18p reduce the binding to Ste4p (Fig. 3A).

We used the Dha reporter constructs to perform a more quantitative analysis by protein extraction and immunoblotting. N_{vg}-Ste18$_{91}$-C_{ub}-Dha or N_{vg}-Ste18$_{74}$-C_{ub}-Dha was either expressed alone or together with HA-Ste4p (Fig. 4). Expression of the N_{vg}-Ste18-C_{ub}-Dha constructs from the P_{CUP1} promoter was held at a relatively low level or was induced by addition of copper ions to 100 μM 1 h prior to protein extraction. The ratio of uncleaved to cleaved fusion protein was calculated after denaturing gel electrophoresis and immunoblotting with the anti-HA antibody. This value was compared to the ratio of uncleaved to cleaved N_{vg}-Ste18-C_{ub}-Dha from protein extracts of cells lacking HA-Ste4p. The expression of HA-Ste4p increases the fraction of uncleaved N_{vg}-Ste18$_{91}$-C_{ub}-Dha by a factor of two under inducing conditions (+Cu^{2+}) and a factor of 3.6 under non-inducing conditions (Fig. 4A,B). These experiments confirm the results of the growth assays that Ste18p undergoes a measurable change in conformation upon binding to Ste4p. No increase in the uncleaved fraction was observed for the N_{vg}-Ste18$_{74}$-C_{ub}Dha under inducing conditions (+Cu^{2+}) (Fig. 4A). However, a 1.6-fold increase in the ratio of uncleaved to cleaved fusion protein was detected when the expression of N_{vg}-Ste18$_{74}$-C_{ub}-Dha was not induced by the addition of copper before extraction (Fig. 4A,C). This relatively small effect might reflect a weak binding activity of Ste18$_{74}$p to Ste4p, which is not detected by the less sensitive growth assay (Fig. 3A). This interpretation is supported by experiments showing that similarly truncated γ-subunits display a still detectable albeit much reduced affinity for their cognate β-subunits [18,19].

We can only speculate about the reasons for employing such a significant conformational transformation in Gγ upon binding Gβ. Since the unfolded Gγ seems to exist only briefly before and during the maturation of Gβγ, it is an unlikely, even though not disproved possibility that the unfolded state of Ste18p fulfills additional roles besides serving as a precursor for Gβγ [20]. Alternatively, the lack of structure might be a mechanism to protect against unproductive or unwanted protein associations. By inducing the fold of Ste18p and thereby creating the binding interface for other proteins, Ste4p ensures that any interactions with Ste18p will only occur within the Ste18p–Ste4p complex. The subset of interactions that is detectable between other components of the signalling cascade and Gβγ and not with its separated subunits might fall into this category [21–23].

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