Tfs1p, a Member of the PEBP Family, Inhibits the Ira2p but Not the Ira1p Ras GTPase-Activating Protein in Saccharomyces cerevisiae

Hélène Chautard,1 Michel Jacquet,2 Françoise Schoentgen,1 Nicole Bureaud,1 and Hélène Bénédetti1*  

Centre de Biophysique Moléculaire, Centre National de la Recherche Scientifique, UPR 4301, University of Orleans and INSERM, 45071 Orleans Cedex 2,1 and Laboratoire Information Génétique et Développement, Institut de Génétique et Microbiologie, UMR CNRS-Université 8621, Université Paris-Sud, 91405 Orsay Cedex,2 France

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Ras proteins are guanine nucleotide-binding proteins that are highly conserved among eukaryotes. They are involved in signal transduction pathways and are tightly regulated by two sets of antagonistic proteins: GTPase-activating proteins (GAPs) inhibit Ras proteins, whereas guanine exchange factors activate them. In this work, we describe Tfs1p, the first physiological inhibitor of a Ras GAP, Ira2p, in Saccharomyces cerevisiae. TFS1 is a multicopy suppressor of the cdc25-1 mutation in yeast and corresponds to the so-called Ic CPY cytoplasmic inhibitor. Moreover, Tfs1p belongs to the phosphatidylethanolamine-binding protein (PEBP) family, one member of which is RKIP, a kinase and serine protease inhibitor and a metastasis inhibitor in prostate cancer. In this work, the results of (i) a two-hybrid screen of a yeast genomic library, (ii) glutathione S-transferase pulldown experiments, (iii) multicopy suppressor tests of cdc25-1 mutants, and (iv) stress resistance tests to evaluate the activation level of Ras demonstrate that Tfs1p interacts with and inhibits Ira2p. We further show that the conserved ligand-binding pocket of Tfs1—the hallmark of the PEBP family—is important for its inhibitory activity.

* Corresponding author. Mailing address: Centre de Biophysique Moléculaire, Centre National de la Recherche Scientifique, UPR 4301, rue Charles Sadron, 45071 Orleans Cedex 2, France. Phone: 00 33 2 38 25 55 84. Fax: 00 33 2 38 63 15 17. E-mail: benedetti@cnrs-orleans.fr.
TABLE 1. Yeast strains used in this study

| Strain | Genotype | Source |
|--------|----------|--------|
| BY4742 | MATa his3A1 leu2Δ0 his3A1 ura3Δ0 | Euroscarf |
| BY474ΔT | BY4742 tfs1::kan | This study |
| BY474Δ2T | BY4742 tfs1 ylr179c::kan | This study |
| LRB7 | MATa his4 leu2::ura3 cdc25-1 | L. Robinson |
| LRB2Δ2r2BD | LRB2Δ2r2BD | This study |
| LRB2ΔΔa2GAP | LRB2ΔΔa2GAP | This study |
| LRB2ΔΔa2CT | LRB2ΔΔa2CT | This study |
| LRB2ΔΔrim11 | LRB2ΔΔrim11 | This study |
| EGY48 | MATa trp1 his3 ura3 leu2::6 LexAop-LEU2 | MobiTec |

three sets of data demonstrating (i) that Tfs1p and Ira2p interact directly and specifically, (ii) that there is a direct link between the ability of Tfs1p to interact with Ira2p and its ability to be a multicopy suppressor of the cdc25-1 mutation, and (iii) that Tfs1p directly affects the activation level of the Ras/cAMP/PKA pathway. We also demonstrate that conserved residues (involved in the formation of the cavity in members of the PEBP family) are required for Tfs1p function.

TABLE 2. Plasmids used in this study

| Plasmid | Description | Source or reference |
|---------|-------------|---------------------|
| Ycp352  | 2μm URA3    | 22a                  |
| Ycp352T | Ycp352, TFS1 | See text            |
| Ycp352TP99L | Ycp352, TFS1(P99L) | See text |
| Ycp352TH111A | Ycp352, TFS1(H111A) | See text |
| Ycp352TR162A | Ycp352, TFS1(R162A) | See text |
| Ycp352Y | Ycp352, YLR179c | See text |
| pYX423 | 2μm LEU2 | R&D Systems |
| pYXira2TBD | pYXira2, GALI-IR42 TDB-HA | See text |
| pYXira1 | pYXira2, GALI-IR41 (1987–2358)-HA | See text |
| pKA | kanMX | 14 |
| pKAT | pKA, TFS1' | See text |
| pKAY | pKA, YLR179c' | See text |
| pAN | 'kanMX | 14 |
| pANT | pAN, TFS1 | See text |
| pEGKG | 2μm LEU2-d, URA3, GST-fusion expression vector | 28a |
| pEGKGr2TBD | pEGKGr2, IRA2 TDB | See text |
| pGEXira3-383 | Amp', plac, GST-Ira2 (1644–2026) | 30 |
| pGEXira7-704 | GST-Ira2 (1644–2347) | See text |
| pEG202 | 2μm HIS3, LexA DBD, NLS | MobiTec |
| pEG202T | pEG202, TFS1 | See text |
| pEG202TP99L | pEG202, TFS1(P99L) | See text |
| pEG202TH111A | pEG202, TFS1(H111A) | See text |
| pEG202TR162A | pEG202, TFS1(R162A) | See text |
| pEG202Y | pEG202, YLR179c | See text |
| pEG202ira2TBD | pEG202, IRA2 TDB | See text |
| pGJ4-5 | 2μm TRP1, transcriptional AD | MobiTec |
| pGJ4-5b | pGJ4-5, IRA2 (1982–2332) | See text |
| pGJ4-5c | pGJ4-5, IRA2 (1992–2444) | See text |
| pGJ4-5f | pGJ4-5, IRA2 (1920–2347) | See text |
| pGJ2ara2TBD | pGJ2ara2, IRA2 TDB | See text |
| pGJ2ara2TBDΔ | pGJ2ara2, IRA2 TDB Δ 2232–2347 | See text |
| pGJara1 | pGJara1, IRA2 (1987–2358) | See text |
| pGJara2 | pGJara2, TFS1 | See text |
| pGJara3 | pGJara3, YLR179c | See text |
| pGNG | 2μm URA3, LexAop-GFP | MobiTec |
| pET14-b | Amp', pT7-His6 | Novagen |
| pET14T | pET14-b, pT7-His6, TFS1 | See text |
| pCR2.1 TOPO | Amp', Kan', 3'-T, topoI activated | Invitrogen |
genes, respectively, with their own promoter sequences; each fragment was PCR amplified with the primer pair HB11 and HB12. As an EcoRI site is present in this fragment, it was cloned into the dephosphorylated XhoI site of pJG4-5 to give pJGIra1.

To construct pJGIra2TBD, the production of Ira2TBD is under the control of the Gal10 promoter. The PCR fragment encoding Ira2TBD was amplified with primers HB13 and HB14. It was inserted into the NcoI and HindIII sites of pYX243 such that it was in frame upstream of the HA tag.

The TFS1 and YLR179c open reading frames were amplified by PCR with the primer pair HB13 and HB2, and primer pair HB15 and HB16, respectively. After cleavage with EcoRI and XhoI, the PCR fragments were ligated into the EcoRI and XhoI sites of both pEG202 and pJG4-5. This generated pEG202T and pJG4-5. The fragments were inserted in frame downstream of LexA in pEG202 that had been cut with EcoRI and XhoI, yielding pEG202TP99L, pEG202TH111A, and pEG202TR162A, respectively. The fragments were then cut with SacI and PstI and cloned into Yep352 that had been cut with the same enzymes.

TABLE 3. Oligonucleotides used in this study

| Name | Sequence (5' to 3') |
|------|-------------------|
| HB1  | ------------------ |
| HB2  | ------------------ |
| HB3  | ------------------ |
| HB4  | ------------------ |
| HB5  | ------------------ |
| HB6  | ------------------ |
| HB7  | ------------------ |
| HB8  | ------------------ |
| HB9  | ------------------ |
| HB10 | ------------------ |
| HB11 | ------------------ |
| HB12 | ------------------ |
| HB13 | ------------------ |
| HB14 | ------------------ |
| HB15 | ------------------ |
| HB16 | ------------------ |
| HB17 | ------------------ |
| HB18 | ------------------ |
| HB19 | ------------------ |
| HB20 | ------------------ |
| HB21 | ------------------ |
| HB22 | ------------------ |
| HB23 | ------------------ |
| HB24 | ------------------ |
| HB25 | ------------------ |
| HB26 | ------------------ |
| HB27 | ------------------ |
| HB28 | ------------------ |
| HB29 | ------------------ |
| HB30 | ------------------ |
| HB31 | ------------------ |
| HB32 | ------------------ |
| HB33 | ------------------ |
| HB34 | ------------------ |
| HB35 | ------------------ |
| HB36 | ------------------ |
| HB37 | ------------------ |
| HB38 | ------------------ |

The **Tfs1p activity** in vivo

In pYXIra2TBD, the production of Ira2TBD is under the control of the Gal10 promoter. The PCR fragment encoding Ira2TBD was amplified with primers HB13 and HB2. It was cut with the Ndel and XhoI enzymes and inserted into the Ndel and EcoRI sites of pGEXIra2-383 after filling in the opened EcoRI and XhoI sites with the Klenow enzyme.

In pYXIra2TBD, the production of Ira2TBD is under the control of the Gal10 promoter. The PCR fragment encoding Ira2TBD was amplified with primers HB13 and HB2. It was cut with the Ndel and XhoI enzymes and inserted into the Ndel and EcoRI sites of pGEXIra2-383 after filling in the opened EcoRI and XhoI sites with the Klenow enzyme.
used to obtain a PCR fragment encoding the IRA1 fragment, which was then inserted between the NdeI and MluI sites of pYX243.

pKAT contains a 660-bp DNA fragment corresponding to the region immediately upstream of the TFS1 gene. This fragment was amplified with primers HB28 and HB29. After cutting with XmaI and BamHI, the resulting fragment was cloned into pKA, which allowed its excision with the KmI and BamHI enzymes and its subsequent cloning into the same sites of pKA. pANT contains a 770-bp DNA fragment corresponding to a DNA region localized immediately downstream of the TFS1 gene. This fragment was amplified with primers HB30 and HB6. It was cloned into pCR2.1 TOPO, which allowed its excision with Xmal and BamHI enzymes and its subsequent cloning into pKA opened with the same sites.

**Strain construction.** TFS1 was deleted from BY4742 by “split-marker” recombination (14) with pKAT and pANT. In the resulting disrupted strain, BY4742ΔTFS1 was replaced by the kanMX5 gene (51). TFS1 and YLR179C are adjacent; therefore, the two genes were disrupted simultaneously in BY4742 by the kanMX5 gene through the use of the pKAY and the pANT plasmids. The resulting strain was designated BY4742ΔTFS1. LRB27Δira2ΔTBD was constructed by replacing the IRA2 fragment encoding aa 2232 to 2347 of the TBD with kanMX5. The fragment of IRA2 encoding the GAP and TBSs was deleted by transforming a PCR-generated DNA fragment containing kanMX5 flanked by 50 bases homologous to a region located immediately upstream of the first amino acid of this region and directly downstream of the last amino acid of this region. Primers HB31 and HB32 were used to amplify this fragment. The same procedure was used to construct LRB27Δira2ΔGAP and LRB27Δira2ΔCT. For LRB27Δira2ΔGAP, the fragment of IRA2 encoding the GAP and TBDs was deleted with kanMX5. For this purpose, LRB27 cells were transformed with a PCR-generated DNA fragment containing kanMX5 flanked by 50 bases homologous to a region located immediately upstream of the first amino acid of this region (aa 1644) and directly downstream of the last amino acid of this region (aa 2347). Primers HB33 and HB32 were used to amplify this fragment. The same procedure was used to construct LRB27Δira2ΔGAP and LRB27Δira2ΔCT. For LRB27Δira2ΔGAP, the fragment of IRA2 encoding the GAP and TBDs was deleted with kanMX5. For this purpose, LRB27 cells were transformed with a PCR-generated DNA fragment containing kanMX5 flanked by 50 bases homologous to a region located immediately upstream of the first amino acid of this region and directly downstream of the last amino acid of this region (aa 1644) and directly downstream of the last amino acid of this region (aa 2347). Primers HB33 and HB32 were used to amplify this fragment. The same procedure was used to construct LRB27Δira2ΔGAP and LRB27Δira2ΔCT. For LRB27Δira2ΔGAP, the fragment of IRA2 encoding the GAP and TBDs was deleted with kanMX5. For this purpose, LRB27 cells were transformed with a PCR-generated DNA fragment containing kanMX5 flanked by 50 bases homologous to a region located immediately upstream of the first amino acid of this region (aa 1644) and directly downstream of the last amino acid of this region (aa 2347). Primers HB33 and HB32 were used to amplify this fragment. The same procedure was used to construct LRB27Δira2ΔGAP and LRB27Δira2ΔCT.

**Protein purification and antibody preparation.** 6His-Tfs1 was purified from the cell lysate of BL21(DE3) cells transformed with pET14T and induced for 120 min by IPTG (100 μM) essentially as described by Serre et al. (38). Cleared cell lysate was obtained by sonicating the cells in buffer B (50 mM sodium phosphate buffer [pH 8.0], 1 mM EDTA, 10% glycerol containing Complete protease inhibitors (Sigma), lysozyme (100 μg/ml), Dnase (5 μg/ml), and MgCl2 (10 mM) and then centrifuging for 2 h at 40000 g. This lysate was incubated with Talon TM cobalt affinity resin (Clontech) for 30 min at room temperature. The resin was washed several times with buffer B for 20 min, and the protein was eluted by two 20-min incubations in buffer B containing 400 mM imidazole. The eluate was dialyzed against 25 mM Tris-HCl (pH 7.5)–30 mM NaCl–7 mM β-mercaptoethanol and concentrated to 1 mg/ml.

Anti-Tfs1 antibodies were raised by injecting rabbits with purified 6His-Tfs1. The rabbits received four injections each containing 150 μg of protein mixed with Freund’s adjuvant over a period of 40 days. The antiserum was used at a 1/500 dilution.

YNL281W-6His was a kind gift of N. Coste. 6His-YLR179C purification and anti-YLR179C antibodies were described elsewhere.

**Protein analysis.** Except for the cell extracts used in the GST pulldown experiments, all cell extracts were prepared by a method adapted from Yaffe and Schatz (54). Cells were grown to an optical density at 600 nm of 1 and then centrifuged and resuspended in 500 μl of water containing Complete protease inhibitor cocktail (Roche) and phenylmethylsulfonyl fluoride (1 mM). We then added 150 μl of freshly prepared 2 M NaOH and 5% β-mercaptoethanol to the cells; after incubation for 10 min on ice, trichloroacetic acid (10% final concentration) was added. After a further 10-min incubation on ice, samples were centrifuged for 10 min at 10000 g. The pellet was then resuspended in 50 μl of SDS-PAGE sample buffer and boiled for 5 min. Western blot analyses were performed as described previously (50) and were probed with anti-HA rat antibodies (Roche), anti-lexA rabbit antibodies (a kind gift from R. Llobet), anti-Tfs1 or anti-YLR179C rabbit antibodies, anti-GST monoclonal antibodies (Roche), and anti-six His tag monoclonal antibodies (Invitrogen). Bound antibodies were detected using anti-rat, anti-mouse, or anti-rabbit secondary antibodies coupled to alkaline phosphatase (Promega). The presence of these secondary antibodies was revealed using BCIP (5-bromo-4-chloro-3-indolylphosphosphate) and Nitro Blue Tetrazolium as described by the manufacturer (Promega).

**RESULTS**

**Interaction of Tfs1p with Ira2p.** (i) Two-hybrid screening to identify proteins that interact with Tfs1p. The entire coding sequence of TFS1 was used as a bait (pEG202T) to screen an S. cerevisiae genomic library containing 4 × 108 independent clones (OriGene Technologies, Rockville, Md.). We screened a total of 1.2 × 109 prey clones and identified 217 positive-testing clones that were prototrophic for uracil, histidine, tryptophan, and leucine and fluorescent under UV light. We subsequently analyzed 80 of the 217 isolated positive-testing colonies. For each interaction tested, the bait (LexA-Tfs1 or LexA-Ylr179c) and the prey (B42-IA-Ira2TBD, B42-HA-Ira2TBD2232-2347, or B42-HA-Ira1) were sought by Western blotting using different antibodies. Anti-LexA antibodies were used for detection of baits and anti-HA antibodies were used for detection of prey.

**Heat shock and oxidative stress resistance.** Heat shock and H2O2 resistance assays were performed on BY4742, BY4742ΔTFS1, or BY4742ΔTFS1 cells transformed or not transformed with pYEp352 or pYEp532T and grown to saturation. Cells were first incubated at 37°C for 1 h. For the H2O2 resistance assays, they were then cooled and spotted in 10-fold serial dilutions onto YPD plates containing 0, 2, or 3 mM H2O2. For the heat shock assays, the cells were incubated for various times at 35°C before being spotted onto plates.

**GST pulldown.** For the in vivo GST pulldown, GST and GST-Ira2p TBD were produced by growing 50 ml of BY4742 cells transformed with pEGKGIra2 and pEGKGIra2TBD, respectively, to an optical density at 600 nm of 1 at 30°C in SDGα/Raf-Ura medium. Cell extracts were obtained by breaking the cells open with glass beads (Sigma) (0.5-mm diameter) in buffer A (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA, 10% glycerol containing Complete protease inhibitor cocktail (Roche), phenylmethylsulfonyl fluoride (1 mM), and lysozyme (5%). Cleared cell lysates (named “input”) were analyzed by Western blotting, and 300 μl was incubated with glutathione Sepharose beads (50 μl) (Amersham) for 1 h at 4°C. After three washes with buffer A containing 0.08% Triton X-100 and two washes with buffer A without Triton or glycerol, the beads were boiled for 5 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The eluted material (named “Beads” [see Fig. 2]) was separated from the beads and analyzed by Western blotting. For the in vitro GST pulldown, GST-Ira2p-704 was produced from SCS1 E. coli cells after overnight induction at 17°C in addition after induction of IPTG (isopropyl-β-D-thiogalactopyranoside). Cells were lysed in Tris 20 mM (pH 7.5)–NaCl (75 mM)–NP-40 (0.1%). 500 μl of cells lysate was incubated with glutathione beads (25 μl) in this buffer for 1 h at 4°C. After five washes with the same buffer and a last wash in this buffer containing 5% lysozyme, the beads were incubated with 6His-Tfs1 and YNL281W-6His (6 μg) in a total volume of 700 μl of the latter buffer for 1 h at 4°C. After three washes with this buffer and two washes without lysozyme and NP-40, the beads were incubated in the presence of 10 μM glutathione (50 μl) for 5 min at room temperature and the eluted material was analyzed by Western blotting.

**Two-hybrid assays.** We used the LexA-based version of the yeast two-hybrid system originally developed by Fields and Song (15). The components used for these assays (pEG202, pG4-5, and pGNG plasmids and the EG48 strain) were obtained from MoBiTec GmbH (Göttingen, Germany), and all assays were carried out as suggested by the manufacturer. The bait, pEG202T, was created as described above. The pG4-5 yeast genomic library was obtained from OriGene Technologies (Rockville, Md.) and introduced into the reporter strain EGY48.
false-positive test results and did not give positive test results after a second screening with the bait.

The minimum DNA fragment common to all the positive prey clones (between residues 1992 and 2347 of Ira2p) was cloned into pJG4-5 and appeared to interact with Tfs1p as a bait (Table 4). This domain of Ira2p was called TBD (Fig. 1).

The transcription of GFP and LEU2 was activated regardless of whether the sequences encoding TFS1 or the Ira2 TBD were in pEG202 or pJG4-5. Furthermore, none of the constructs activated GFP and LEU2 transcription with the empty vector, confirming that the interaction between Tfs1p and the Ira2 TBD was required for the expression of these genes.

(ii) Tfs1p coprecipitates with Ira2 TBD and Ira2 GAP TBD (Ira2p-704) tagged with GST in vivo and in vitro. Ira2p is a 3,079-residue integral membrane protein. Its N terminus is predicted to contain three transmembrane domains, and the rest of the protein is predicted to be cytoplasmic. The “GAP domain” is contained within a 383-residue fragment (aa 1644 to 2026) (11) (Fig. 1). The Ira2 TBD region identified here overlaps the C-terminal part of the Ira2p GAP domain and extends over 364 residues (Fig. 1).

To confirm the two-hybrid screen data, we carried out two GST pulldown experiments, one in vivo in yeast and the other one in vitro with purified proteins.

To perform the in vivo pulldown experiment, the DNA fragment encoding the Ira2 TBD was cloned into pEGKG to produce a GST-Ira2 TBD fusion protein. Sepharose glutathione beads were then used to affinity purify GST-Ira2 TBD or GST alone as a negative control. Tfs1p was specifically coprecipitated with GST-Ira2 TBD but not with GST alone by glutathione Sepharose beads (Fig. 2). Therefore, it can be concluded that Tfs1p and the Ira2 TBD interact in vivo in yeast cells.

To prove that no other yeast protein was needed for this interaction to take place, a GST pulldown experiment was carried out in vitro with purified recombinant GST-Ira2p-704 and Tfs1 proteins. First, Sepharose glutathione beads were used to affinity purify GST-Ira2p-704 from E. coli extracts. Then, these beads were incubated with 6His-Tfs1 or YNL281W-6His (as a negative control) purified from E. coli extracts. Figure 3 shows that 6His-Tfs1 but not YNL281W-6His specifically coprecipitated with GST-Ira2p-704. Indeed, 6His-Tfs1 didn’t bind to glutathione beads in the absence of GST-Ira2p-704 (data not shown).

The suppression of cdc25-1 by TFS1 requires the Ira2 TBD. cdc25-1 cells (LRB27) are unable to grow at the restrictive temperature of 37°C on glucose or at the semipermissive temperature of 30°C on nonfermentable carbon sources. The overproduction of Tfs1p in that strain allows it to grow at 37°C on glucose but not at 30°C on glycerol (33). The fact that the overexpression of TFS1 suppressed the Cdc25-1 allelic mutation of CDC25 more efficiently than the other allelic mutations suggested that Cdc25p and Tfs1p might interact directly. Our

FIG. 1. Ira2p domains and truncated Ira2 protein constructs. The putative membrane-spanning segments are indicated by gray-shaded boxes. The GAP domain is indicated by a filled box; the TBD is indicated by an open box. The overlap between the two domains is hatched. The first and last residues of Ira2p and the limits of the GAP and TBDs are indicated. f, b, and c designate the three IRA2 DNA fragments isolated from positive-testing prey plasmids. + indicates that an interaction was detected by the two-hybrid system; − indicates that no interaction was detected.

TABLE 4. Two-hybrid pairings tested for GFP fluorescence and Leu2 prototrophy

| Bait       | Ira2 TBD (Leu prototrophy and GFP activity) | Ira2TBDΔ (Leu prototrophy and GFP activity) | Ira1 (Leu prototrophy and GFP activity) |
|------------|---------------------------------------------|---------------------------------------------|-----------------------------------------|
| Tfs1       | Yes                                         | No                                          | No                                      |
| Tfs1: P999L| No                                          | ND                                         | ND                                      |
| Tfs1: H111A| No                                          | ND                                          | ND                                      |
| Tfs1: R162A| No                                          | ND                                          | ND                                      |
| YLR179c    | No                                          | ND                                          | No                                      |

* Yes stands for Leu prototrophy and GFP activity in the two-hybrid test. No stands for no Leu prototrophy or GFP activity in the two-hybrid test.

* ND, not determined.
finding that Tfs1p interacts with Ira2p rather than with Cdc25p suggests that this interaction is responsible for the suppression effect. To address this issue, we constructed an Ira2p mutant lacking the TBD and looked for the maintenance or loss of the TFS1 suppression of cdc25-1.

The deletion of the 115 C-terminal residues of Ira2 TBD resulted in an Ira2 fragment (Ira2TBDΔ2232-2347) that was unable to interact with Tfs1p in the two-hybrid system (Table 4) and in the GST pulldown assay (data not shown). The chromosomal IRA2 gene was mutated in a cdc25-1 strain such that the DNA region encoding residues 2232 to 2347 of Ira2p was replaced by the Kana marker (LRB27Δira2TBD). This marker insertion in the gene also resulted in the loss of the residues 2348 to 3079 of the Ira2 protein. As a control, another cdc25-1 strain was constructed in which the Kana marker was inserted downstream of the DNA region encoding TBD, resulting in the loss of residues 2360 to 3079 of the Ira2 protein (LRB27Δira2CT). These strains were unable to grow on glucose at 37°C (Fig. 4 and data not shown). Thus, these IRA2 deletions do not suppress the cdc25-1 mutation; therefore, the GAP activity of the resulting Ira2 protein is likely not affected.

In contrast, the deletion of the Ira2 GAP domain (LRB27Δira2GAP) restored growth at 37°C (Fig. 4).

We tested the effect of Tfs1p overproduction in the LRB27Δira2TBD and LRB27Δira2CT strains grown at 37°C on glucose. The IRA2 deletion affecting the TBD region but not that affecting the region in the C terminus of TBD prevented Tfs1p from exerting its suppressor effect on the cdc25-1 mutation (Fig. 4 and data not shown). This unambiguously demonstrates that the TFS1 dose-dependent suppressor effect requires the interaction between Tfs1p and Ira2 TBD and strongly suggests that Tfs1p inhibits Ira2 GAP activity.

To confirm this result we developed a competition assay to prevent the suppressive effect of TFS1 upon cdc25-1 thermosensitivity.

The Ira2 TBD was overproduced in cdc25-1 cells (LRB27). The DNA fragment encoding the Ira2 TBD was cloned into pYX243 behind the Gal10-inducible promoter and in frame with the HA epitope in the C terminus (pYXima2TBD). The overproduction of Ira2 TBD in the presence of galactose was checked by Western blot analysis using anti-HA antibodies (data not shown). We tested the growth of LRB27 cells harboring or not harboring Yep352T at different temperatures on galactose, and it appeared that 32°C was the best temperature at which to observe the multicopy suppressor phenotype of TFS1 on galactose. When LRB27 Yep352T cells were transformed with pYXima2TBD, they could no longer grow at 32°C on YPGal (Fig. 5). This phenotype is specific of Ira2 TBD production, as the overproduction of the Ira1p domain homologous to the Ira2 TBD did not impair cell growth at 32°C (Fig. 5). These results show that Ira2 TBD overproduction directly affects TFS1 suppressor function. Therefore, the Ira2 TBD fragments appear to compete with the natural Ira2 protein for...
Tfs1p, thereby strongly suggesting that the TFS1 dose-dependent suppressor effect involves the inhibition of Ira2 activity.

Tfs1p does not interact with Ira1p or interfere with its function. The functional similarity between Ira1p and Ira2p suggested that they were both regulated by Tfs1p. The overall sequences of the two Ira proteins are 45% identical, and their TBD regions are 48.5% identical.

The two-hybrid technique revealed no interaction between Tfs1p and the Ira1p domain that is homologous to the Ira2 TBD (Table 4). As described above, furthermore, TFS1 could only exert its suppressor effect on cdc25-1 mutants via Ira2 TBD and the presence of a wild-type IRA1 gene in these strains did not allow the Tfs1p dose-dependent suppressor function to take place. Finally, the Ira1p domain homologous to the Ira2 TBD was cloned into the pYX243 plasmid (pYXira1) in frame with the HA epitope in the C terminus. We checked (by Western blot analysis using anti-HA antibodies) that the Ira1 fragment was indeed overproduced in the presence of galactose (data not shown). The overproduction of Ira1 fragment in cdc25-1 cells did not interfere with the multicopy suppressor phenotype of these mutants.

FIG. 3. 6His-Tfs1 and GST-Ira2p704 interact in vitro. Purified 6His-Tfs1 or YNL281W-6His (an aliquot of which was analyzed [lane 1] with anti-Tfs1 and anti-six-His antibodies, respectively) was incubated with glutathione beads previously used for affinity purification of GST-Ira2p704 produced from E. coli (10 μl of extracts was loaded in lane 1 and revealed with anti-GST antibodies). After several washes, the material bound to the beads was eluted with glutathione and analyzed by Western blot analysis with antibodies directed against GST, Tfs1, or six-His (as indicated) (lane 2). The molecular mass markers are indicated (in kilodaltons) on the left side of the figure.

FIG. 4. The TFS1 multicopy suppressor phenotype of cdc25-1 mutants is dependent on the presence of an intact Ira2 TBD. LRB27 cells expressing a wild-type Ira2p (LRB27), Ira2p lacking its 1,435 C-terminal residues (LRB27Δira2GAP), or Ira2 lacking its 847 C-terminal residues (LRB27Δira2TBD) was transformed or not transformed with the following plasmids: Yep352, Yep352T, pYXira2TBD, and pYXira1. They were then streaked out for single colonies and grown at 25°C or at the restrictive temperature of 37°C on YPD medium.
The suppressor phenotype of \textit{TFS1} (Fig. 5). Therefore, Tfs1p inhibits only Ira2p function; this is one of many functional differences between the two Ira proteins (46).

\textit{YLR179C} encodes a Tfs1p homolog that does not display the same function. \textit{YLR179C}, which is adjacent to \textit{TFS1} (\textit{YLR178C}) on chromosome XII, encodes a protein that is 40% identical to Tfs1p. In contrast to the results seen with \textit{TFS1}, \textit{YLR179C} has not been reported to be overexpressed in different stress conditions. Indeed, the promoter region of \textit{TFS1} bears two STREs whereas the \textit{YLR179C} promoter is devoid of them.

To test whether the product of \textit{YLR179C} shares some of the functional properties of Tfs1p, we used the two-hybrid system to assess its interaction with the Ira2 TBD and checked whether the overexpression of \textit{YLR179C} could mimic the dose-dependent suppressor phenotype of \textit{TFS1} in \textit{cdc25-1} strains. Whatever the vector chosen for cloning the corresponding DNA fragments (pEG202 or pJG4-5), the Ira2 TBD and the product of \textit{YLR179C} did not display any interaction in the two-hybrid system (Table 4). Similarly, \textit{cdc25-1} cells overproducing Tfs1p (LRB27 Yep352T), but not \textit{cdc25-1} cells overexpressing \textit{YLR179C} (LRB27 Yep352Y) or transformed with the corresponding empty vector (LRB27 Yep352), could grow on glucose at 37°C (Fig. 6). Indeed, the overproduction of \textit{YLR179C} gene product was verified by Western blot analysis using anti-\textit{YLR179C} antibodies (data not shown). Therefore, although they are homologous, the \textit{TFS1} and \textit{YLR179C} gene products interact with different proteins and fulfill different functions (at least with regard to the suppression of \textit{cdc25-1} mutants).

The deletion of \textit{TFS1} enhances the stress resistance of the cells and the overexpression of \textit{TFS1} causes the opposite phenotype. As the overexpression of \textit{TFS1} appeared to suppress \textit{cdc25}-deficient phenotypes by inhibiting Ira2 GAP activity, we predicted that cells overproducing Tfs1p would contain more Ras-activated proteins than cells lacking \textit{TFS1}. Robinson and Tatchell (33) showed that the overexpression of \textit{TFS1} had a small effect on the cAMP intracellular concentration, as cells that overexpressed \textit{TFS1} accumulated less glycogen than did wild-type cells and appeared to express the \textit{\beta}-galactosidase gene more from a cAMP-regulated promoter.

We investigated the resistance of cells to heat shock and hydrogen peroxide stress. The tests were performed in conditions that naturally allowed the high-level expression of \textit{TFS1} in wild-type cells. According to Gasch et al. (17), when cells that had been grown at 25°C were shifted to 37°C for 1 h STRE-responsive genes are strongly induced. Therefore, the heat shock and H$_2$O$_2$ resistance tests were performed after preincubating the cells at 37°C for 1 h. Cells overproducing Tfs1p were more sensitive (between 5- and 10-fold) to heat shock than wild-type cells, whereas \textit{TFS1} deletants were more resistant (between 5- and 10-fold) than wild-type cells (Fig. 7). As expected, the \textit{tfs1 ylr179c} double mutant was not more resistant to the stresses than the \textit{tfs1} single mutant (data not shown). The same results were obtained in the H$_2$O$_2$ stress tests (data not shown).

Although the effects were moderate, these results suggest that the overexpression of \textit{TFS1} is associated with a higher level of Ras activity and that the deletion of \textit{TFS1} is associated with the opposite phenotype. Furthermore, the phenotypes of \textit{TFS1} disruptants demonstrate that the inactivation of Ira2p by Tfs1p is physiological and is not a side effect of the artificial overproduction of one of the two proteins.

\textbf{Functional importance of conserved residues in Tfs1p.}

Tfs1p and the product of \textit{YLR179C} belong to a large family of proteins called PEBP. The three-dimensional structures of several members of this family have been resolved and appear to be remarkably similar (2, 3, 38–40). The conserved regions always delimit a unique and small binding pocket at the surface of the proteins and are thought to participate in the formation of the ligand-binding site, which can accommodate different anions. To determine whether the putative ligand-binding site
of Tfs1p is important for the interaction with the Ira2 TBD, we
created different point mutations in the conserved residues of
the PEBP family signature of Tfs1p (P99L, H111A, and R162A) (Fig. 8A). His111 corresponds to the His85 of the
bovine PEBP, which directly interacts with phosphorylethano-
mate PEBP (Self-Pruning gene product), resulting in drastic
protein and with three residues in the ligand-binding site (2).
The Pro99L mutation mimics the natural mutation of the toma-
to PEBP (Self-Pruning gene product), resulting in drastic
inflorescence anomalies (31). For each mutant, the interaction
with the Ira2 TBD was tested by the two-hybrid system (Table
4). Each mutant was also overproduced in cdc25-1 strains.
After the stability of each mutant protein had been checked by
Western blot analysis with anti-Tfs1p antibodies (data not
shown), we tested growth at 37°C on glucose (Fig. 8C). None
of the Ira1p mutants interacted with the Ira2 TBD (Table 4),
and none of them were able to restore the growth of cdc25-1
mutants at 37°C like wild-type Tfs1p. These results demon-
strate that different conserved residues of Tfs1p (thought to
participate in the formation of the ligand-binding site of the
protein) are involved in the interaction with Ira2 TBD.

**DISCUSSION**

This work shows that Tfs1p, previously characterized as a
multicopy suppressor of cdc25-1 mutation (Twenty-Five sup-
pressor) (33) and as a CPY inhibitor (26), specifically interacts
with Ira2p and inhibits its activity in vivo. The suppressor effect of
TFS1 was reported to be independent of its inhibitory ac-
tivity on CPY (26) and more efficient on cdc25-1 strains than
on other mutant alleles of CDC25, thereby suggesting an allele
specificity for the efficiency of the suppressor phenotype (22).
Our results (showing that Tfs1p directly interacts with Ira2p
and that such an interaction is absolutely required for the
suppressor effect of TFS1) allowed us to interpret this pheno-
type differently and to demonstrate by genetic means a new
physiological role for Tfs1p. The defect in Ras protein activa-
tion of the cdc25 mutants (more or less pronounced depending
on the cdc25 alleles) is compensated (more or less partially),
because Tfs1p inhibits a Ras inhibitor, Ira2p. *ira1* mutations
have been described as being more efficient at suppressing the
growth defect in cdc25 mutants than *ira2* mutations (6). This is
consistent with the fact that Tfs1p overproduction can partially
suppress cdc25 alleles but is unable to bypass the essential
requirement for CDC25 function.

Although Ira1p and Ira2p both inactivate Ras proteins, they
display functional differences (43). First, some preference
seems to exist between Ras and Ira proteins. Indeed, mutations
in ras1 or ras2 do not equally well suppress phenotypes asso-
ciated to mutations in *ira1* and *ira2*. Second, the normal pro-
duction or even the overproduction of Ira1p or Ira2p does not
completely suppress the effects of disrupting the corresponding
*RRA* gene. Third, *ira1* is more efficient at suppressing the
growth defect in cdc25 mutants than is *ira2* mutation, although
*ira2* phenotypes are more severe than *ira1* phenotypes. Our
results showing that Tfs1p inhibits Ira2p but not Ira1p repres-
ent a new functional difference between these two GAPs.
Together with the amino acid differences observed in the N-
terminal regions of the two proteins, Tfs1p-specific inhibition
of Ira2p might play a role in the phenotypic differences ob-
erved between the two *ira* mutants.

NF1, the closest human homolog to Ira1 and Ira2, is respon-
sible for the human autosomal dominant disease neurofibro-
matosis type I. Specific lipids modulate NF1 GAP activity in
vitro (5, 20). Furthermore, NF1 associates with microtubules
and the interaction with tubulin (thought to involve an 80-
residue segment upstream of the GAP domain) inhibits GAP
activity in vitro (6). Conversely, Parrini et al. (30) have shown
that the GAP activity of Ira2p is also inhibited by tubulin in
vitro and that the 95 N-terminal residues flanking its catalytic
domain might be involved in tubulin binding. In this work, we
describe a new physiological inhibitor of Ira2p in *S. cerevisiae*.
This also involves the 40 C-terminal residues of the Ira2p GAP
domain, which are important for the interaction with Tfs1p (H.
Chautard, personal communication). This Tfs1p-binding site
extends to a 324-residue C-terminal flanking region. By anal-
ogy with tubulin, one of the human homologs of Tfs1p might
inhibit the GAP activity of NF1 by interacting with a region located at the C terminus of its catalytic domain. The existence of an extended area of similarity (23.5% identity and 45.5% similarity) of around 800 residues immediately downstream of the GAP domain of NF1 and Ira suggests that this region has a conserved function (52). However, we cannot exclude the possibility that this type of inhibition is restricted to yeast. Indeed, the yeast Ira proteins display specific features such as
a strict specificity towards the yeast Ras proteins (42–44, 52, 30), whereas mammalian GAPs act on both mammalian and yeast Ras proteins (18, 52).

Biochemical and structural analyses have shown that the most important function of GAPs is to supply the active site of Ras with an arginine (the finger) localized at the tip of a loop (the finger loop) joining two α-helices, thus stabilizing the transition state of the GTPase reaction and playing a crucial role in catalysis (52, 1, 36). Te Biesebeke et al. (44a) subsequently showed that this 19-residue finger loop (centered on Arg1742 in Ira2p) is also a determinant for the specificity toward Ras proteins from yeast or mammals. The inhibitory action of Ts1p on Ira2p might be explained by sterical hindrance resulting from the formation of a complex close to the finger loop. Another possibility is that interaction with Ts1p induces a conformational change in the GAP domain, thereby impairing its ability to bind to Ras and/or inhibiting its stimulating role in catalysis. Another possibility is that Ts1p affects Ira2p localization or stability. We are currently testing these different hypotheses.

Ts1p belongs to the large PEBP family, members of which are structurally characterized by a small cavity at their surface that is able to interact with different anions. Two stretches of conserved residues are involved in the formation of this cavity. We mutated three of these residues in Ts1p, His111, which is directly involved in the interaction between the bovine PEBP and its ligands (phosphorylethanolamine and acetate) (39) was mutated into Ala. Pro99 was mutated into Leu to mimic the natural mutation of the tomato PEBP (Self-Pruning gene product) that results in drastic inflorescence anomalies (31). Arg162, which forms contacts with the N terminus and three residues of the cavity in the bovine PEBP, was mutated into Ala. Our results demonstrate that these mutations completely eliminate the interaction of Ts1p with Ira2p and its subsequent inhibition. Modeling experiments suggested that each of these mutations has a direct or indirect effect on the accessibility or the binding properties of the cavity (D. Sy, personal communication). Indeed, H111A might remove a binding site for negatively charged ligands; P99L (localized on a turn at the border of the cavity) might affect the accessibility of the cavity to ligands; finally, R162A might have an indirect effect on the ligand-binding site via its contacts with residues located in the cavity.

These results strongly suggest that the conserved cavity of Ts1p is important for its interaction with Ira2p. Given the possible contacts between R162 and the N terminus of the protein, however, we cannot exclude the possibility that the N terminus of Ts1p participates in the interaction with Ira2p. This idea is strengthened by the fact that Ira2p does not interact with YLR179c, a Ts1p homolog, even though its cavity seems to be quite similar (D. Sy, personal communication) despite the fact that its N-terminal extremity is quite different. Phosphorylated residues have already been identified as being possible ligands for members of the PEBP family (46, 47). Different putative PKA-dependent phosphorylation sites have been proposed for Ira2p; however, none of them are located in the Ira2 TBD fragment (43). Recently, the TAP (tandem affinity protein)-Tag technique showed that Ira2p interacts with the serine-threonine kinase Rim11p (23). However, RIM11 disruption in the cdc25-1 context does not prevent Ts1p from exerting its suppressor effect (H. Chautard, personal communication), thereby demonstrating that Ts1p could act independently of a putative Rim11p phosphorylation.

It is noteworthy that following the disruption or overexpression of TFS1, the phenotypes associated with carbohydrate storage or stress resistance are weak. This might be due to the fact that Ts1p regulates only one of the two GAPs. Another nonexclusive possibility is that Ts1p exerts its role only in certain physiological conditions (which differ from those used in the phenotypic analysis). These conditions might be related to stress conditions, because TFS1 is a STRE-regulated gene that is overproduced in many stress conditions. In conclusion, TFS1 constitutes a link between the general stress response pathway and the cAMP/PKA pathway. By helping to activate the cAMP/PKA pathway by downregulating Ira2p, Ts1p may participate in a feedback loop of the general stress response pathway.

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