Vol. 186, No. 1, 1992
July 15, 1992

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 467-474

IN VITRO INTERACTION BETWEEN *SACCHAROMYCES CEREVISIAE* CDC25 AND RAS2 PROTEINS

Maurizio D. Baroni, Giulia Marconi, M. Carla Parrini, Paolo Monti and Lilia Alberghina

Sezione di Biochimica Comparata, Dipartimento di Fisiologia e Biochimica Generali, Università degli Studi di Milano, via Celoria 26, 20133 MILANO, ITALY

Received May 30, 1992

In *Saccharomyces cerevisiae* the CDC25 protein is a positive regulator of RAS/cAMP pathway [1-4], enhancing the GDP-releasing rate of RAS2 protein [5]. In this work we have tried to detect a direct interaction between *CDC25* and *RAS2* gene products. The results indicate that both the whole RAS2 protein and a truncated version that lacks approximately 25 C-terminal residues interact specifically with the CDC25 protein. On the contrary, a derivative of RAS2 that lacks the 112 C-terminal residues as well as the $p21^{H-ras}$ is not able to bind the CDC25 protein in our assay conditions. The 310 C-terminal aminoacids of CDC25 bind RAS2 while a C-terminus deletion within this aminoacid stretch abolishes the binding. The possible physiological significance of these findings is discussed.

In all eukaryotic cells *ras* gene products are highly conserved regulatory proteins controlling fundamental processes like cellular growth and differentiation [6]. GTP binding induces the active *ras* conformation [7,8]: it requires the preliminary dissociation of *ras*-GDP complex, a critical regulatory step promoted by Guanine Nucleotide Release Proteins (GNRPs) [7-9].

In *Saccharomyces cerevisiae* there are two closely related *RAS* gene products, namely RAS1 and RAS2 of 309 and 322 aminoacids respectively [10,11]. These products are required for yeast adenylate cyclase activation [12,13]. The RAS proteins show about 80% homology in their N-terminal region in comparison with the highly conserved amino terminal domain of mammalian *ras* proteins (aminoacids 1-82 of $p21^{H-ras}$); this homology decreases to about 45% in a second less conserved domain (aminoacids 83-164 of p21) and completely disappears in their carboxy-terminal region (with the exception of the Cys-A-A-X-COOH sequence) [14].

The *CDC25* gene encodes for an essential product of 1589 aminoacids [2,4,15]. Cellular extracts from yeast strains overexpressing a fusion protein between β-galactosidase and about the last 700 CDC25 C-terminal aminoacids are able to stimulate *in vitro* the guanine nucleotide release on a purified RAS2 protein [5]. Nevertheless, the CDC25 catalytic core must be much shorter as different reports indicate that a CDC25 polypeptide of less than 480 C-terminal aminoacids [16] or even 286 CDC25 aminoacids, namely residues 1258-1543 [15,17], are still able to perform essential functions for the growth of yeast cells. Significantly, the above 286 aminoacids form the region that has been conserved during evolution among all the CDC25-like proteins [9] discovered so far in *S. cerevisiae* [16,18-20], in *S. pombe* [21], in *D. melanogaster* [22]

0006-291X/92 $4.00

and in mouse [23]. Moreover, the corresponding region of mouse CDC25 homologue (*CDC25^{Mm}*) is enough to allow growth of *cdc25*$^-$ yeast cells [23].

Although the above biochemical data and genetic analysis [24] are consistent with a direct interaction between *RAS2* and *CDC25* gene products, more straightforward evidence is still lacking. In this work we have set up an *in vitro* system to examine the interaction between RAS2 and C-terminal regions of CDC25 protein and identify CDC25 as well as RAS2 protein sequences that are required for their physical interaction. The same regions are also relevant for their *in vivo* functions.

Materials and Methods

In vitro transcription-translation. CDC25 sequences were subcloned into pGEM^{TM}-blue vector (Promega). p*CDC25-t8* plasmid was constructed by subcloning the *BglII-PvuII* 3'-terminal *CDC25* region. p*CDC25-t9* and p*CDC25-t11* plasmids are derived from p*CDC25-t8*. Within *CDC25* ORF a TAA stop codon is contained at the *BamHI* site in p*CDC25-t9* while a TAG stop codon is contained at the *HindIII* site in p*CDC25-t11* The plasmid p*CDC25-t10* was obtained from p*CDC25-t8* by deleting the *BglII-BamHI CDC25* region. Different *CDC25* sequences were transcribed *in vitro* from the above plasmids using SP6 RNA polymerase (Promega) at 37°C for 2h30' [25]. The corresponding CDC25 protein species (see fig.1) were translated using rabbit reticulocyte lysate (minus methionine) (Promega), as suggested by the manufacturer. 5-10 μg RNA, (purified on QUIAGEN-tip100 columns, Quiagen), 20 μM cold methionine and 60 μCi of [^{35}S]-methionine (specific activity 1000 Ci/mmol, Amersham) were added for each *in vitro* translation. The products were separated by free methionine on Sephadex G-50 columns (Pharmacia) at 4°C.

Western blots. ras blotted proteins were incubated sequentially with the anti-*ras* monoclonal antibody Y13-259 (kindly provided by D. Breviario, CNR, Milano-Italy) (2.8 μg/ml) and with the anti-rat peroxidase conjugate IgG (dilution 1:5000-Sigma). RAS-antibodies complexes were visualized with the ECL (Enhanced Chemiluminescent System) Western Blot detection kit (Amersham). The filters used for the interaction assay were washed once in TBS, Tween-20 0.1% (Merck) and saturated in TBS, Tween-20 0.1% and lyophilized milk 5% for 1 hr before being immunodecorated.

Interaction assay. RAS2 proteins and the N4830 *E. coli* strains containing pAV1 or pSK*cHras* plasmids [26,27] were kindly provided by O. Fasano (University of Palermo, Italy). The amount of purified RAS proteins absorbed on nitrocellulose filters (Schleicher and Schuell) was approximately determined by amido-black staining. *RAS2* and *H-ras* gene expression was obtained as described [27]; afterwards cells were harvested, directly resuspended in SDS-sample buffer and sonified to get crude extracts.

Filters with blotted proteins were pre-incubated in Interaction Buffer (IB) (TRIS-HCl 20mM, $MgCl_2$ 0.2mM, KH_2PO_4 12mM, DTT 0.3mM, pH=7.5,) in the presence of GDP 20μM, BSA 4%, methionine 30 μM at 30°C for 4-7 hrs. After this period, the pre-incubation buffer was removed and filters were incubated for 1h30' at 30°C with new IB buffer containing EDTA 1mM (while dispensable, the best signals are obtained with cation chelating compounds), BSA 1%, methionine 30 μM and one of the different CDC25 labelled species at a specific activity of 1.5-2x10^6 cpm/ml. Finally, nitrocellulose filters were washed 3 times for 15' at 30°C in IB buffer, GDP 20 μM, BSA 4% before exposition to autoradiographic films. The stability of the *in vitro* translated products was estimated before and after the experiments by 8% SDS-PAGE analysis.

Results

Figure 1A shows the different fragments of the *Saccharomyces cerevisiae* CDC25 protein, that have been translated *in vitro*. The apparent molecular weights of the products (Fig.1B) turned out to be identical (CDC25-t9 and CDC25-t10) or slightly lower (CDC25-t8 and CDC25-t11) than expected (see also Fig.1 legend).

The interaction between *in vitro* translated CDC25-t8 and the *S. cerevisiae* RAS2 protein was tested. Two different forms of RAS2 protein, namely p34 and p27, were used (Fig.2A). Both proteins, purified from the N4830/pAV1 *E. coli* strain [27], derive from partial proteolytic cleavage of the whole RAS2 (p37). The p34 species lacks approximately 25 C-terminal residues compared with the p37 while p27 is truncated at His210 (J.Jonak, as mentioned in [28]). In order

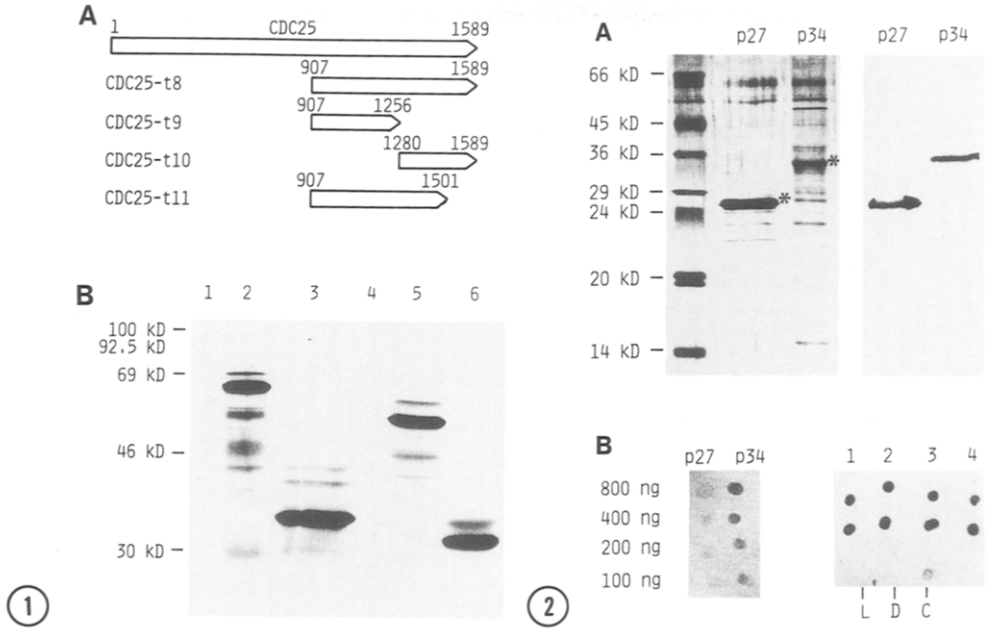

Fig.1. Different CDC25 protein species translated in vitro. A. The predicted *CDC25* translation products are shown in comparison with the whole CDC25. The first and the last aminoacid residues of each species are specified. The expected molecular weights are : 78.6 kDa for CDC25-t8, 41.1 kDa for CDC25-t9, 35.3 kDa for CDC25-t10 and 68.6 kDa for CDC25-t11. B. The *in vitro* translated CDC25 products labelled with [^{35}S]-methionine and analyzed on a 8% SDS-PAGE gel. Lane 1: control translation without mRNA addition. Lane 2: CDC25-t8 product. Lane 3: CDC25-t9. Lane 4: [^{14}C]-molecular weight markers. Lane 5: CDC25-t11. Lane 6: CDC25-t10. The apparent molecular weights of the products have been calculated as average values of at least 3 independent SDS-PAGE analyses. They resulted 70.9±1.6 kDa for CDC25-t8, 39.0±2.2 kDa for CDC25-t9, 34.0±1.5 kDa for CDC25-t10, 62.0±1.7 kDa for CDC25-t11.

Fig.2. Interaction between CDC25-t8 product and purified RAS2 protein. A. The p27 and p34 RAS2 proteins (about 1 μg) (indicated by an asterisk) were analyzed on a 15% Silver stained SDS-PAGE gel (left side) and by Western blot using anti-*ras* monoclonal antibody Y13-259 (right side). B. Left side: decreasing amounts of p27 and p34 RAS2 protein were spotted on a nitrocellulose membrane and probed with labelled CDC25-t8. Right side: 400 ng of p34-RAS2 protein (in duplicate) and control proteins, namely lysozime (L), DNAase (D), carbonic anydrase (C), were spotted on a nitrocellulose membrane to be probed with CDC25-t8. Before membrane absorption, the p34-RAS2 protein was subjected to different pre-treatments: EDTA-mediated GDP/GTP exchange (1), none (2), GDP/GTPγS exchange (3), nucleotide discharge (4)[29,37].

to perform the assay, the p27 and the p34 proteins were spotted on nitrocellulose filters which were incubated with labelled CDC25-t8 in appropriate conditions (see M & M). After 1.5 hours of incubation the filters were washed and exposed for autoradiography. Typical results are shown in Fig.2B: the CDC25-t8 protein was bound by the p34 containing spots in proportion to the amount of the spotted p34. On the contrary, the smaller p27-RAS2 form did not give a significant binding signal with CDC25. Different pre-treatment performed on p34 before the assay (described in Fig.2 legend) did not alter its ability to bind the CDC25-t8 protein (Fig.2B, right).

The p34-RAS2 protein is only partially purified (see Fig.2A), so in order to identify more accurately the species which might bind CDC25, the assay was modified as follows. First, the total proteins were extracted from the N4830/pAV1 *E. coli* strain, before and after the induction of RAS2 expression. In the latter case the whole RAS2 protein (p37) as well as the p34 and p27 species are present in comparable amounts (Fig.3A). Then, the proteins of the *E. coli* crude

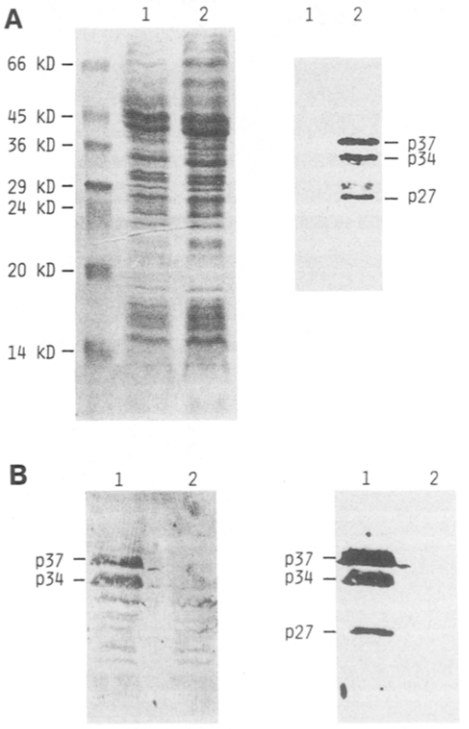

Fig.3. Interaction between CDC25-t8 and total protein extracts of a RAS2 expressing E. coli strain. A. Total proteins (about 100 μg) extracted from the *E. coli* N4830/pAV1 strain before (lane 1) and after (lane 2) induction of RAS2 expression, were analyzed on a 15% Comassie Blue stained SDS-PAGE gel (left side) or by Western blot (right side) with anti-*ras* monoclonal antibody Y13-259. Left and right parts represent two independent experiments. B. Total proteins (about 100 μg) extracted from the *E. coli* N4830/pAV1 strain, before (lane 2) and after (lane 1) induction of RAS2 expression, were separated on a 15% SDS-PAGE and electroblotted on a nitrocellulose membrane. The membrane was incubated with CDC25-t8 and exposed for autoradiography (left side). Then, the same membrane was washed and immunodecorated with the anti-*ras* monoclonal antibody Y13-259 (right side).

extracts were separated by SDS-PAGE, blotted on nitrocellulose membranes and finally incubated with the CDC25-t8 probe.

From this approach, it results that two protein migration bands were specifically bound by CDC25-t8. The immunodecoration of the same filter with the anti-*ras* Y13-259 monoclonal antibody demonstrated that these bands correspond to the p37 and p34 RAS2 proteins. On the contrary, the p27-RAS2 (even when blotted in much larger amounts than the experiment shown) was not able to bind a significant amount of CDC25 (Fig.3B - data not shown) consistently with the previously described results. Interestingly, in the same condition the p21$^{H\text{-}ras}$ wasn't able to bind CDC25 (showed in Fig.4, panels A and C).

Finally, to identify more precisely some of the regions of CDC25 involved in physical interaction with RAS2, the binding assay was repeated with labelled CDC25-t9, CDC25-t10 and CDC25-t11 proteins (Fig.1A,B). The results are shown in figure 4. A polypeptide corresponding to 310 C-terminal aminoacids of CDC25 (CDC25-t10) is still able to bind RAS2 efficiently. Significantly, it maintained the same specificity for the different *ras* species as occurred with CDC25-t8. The N-terminal part of CDC25-t8, namely CDC25-t9, is completely ineffective in

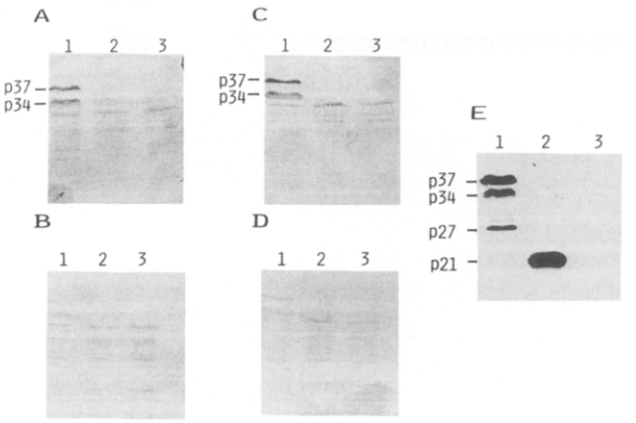

Fig.4. Interaction between CDC25-t8, CDC25-t9, CDC25-t10 and CDC25-t11 proteins and Ras proteins. Equal amounts of crude extracts (about 100 μg of proteins) obtained lysing the *E.coli* strain N4830/pAV1, before (lane 3) and after (lane 1) the induction of *RAS2* expression, or starting from the strain N4830/pSKc*Hras* after the induction of $p21^{H\text{-}ras}$ expression (lane 2), were processed as described for Fig.3B. Then identical filters were incubated with different probes: CDC25-t8 (A), CDC25-t9 (B), CDC25-t10 (C) or CDC25-t11 (D). Finally, all the membranes were washed and immunodecorated with the anti-*ras* monoclonal antibody Y13-259, giving identical results: filter A is shown as an example (E).

binding RAS2. Neither was any interaction signal between the proteins obtained, when the CDC25-t11 probe was used in order to test the effects of a C-terminal deletion impairing the *in vivo* CDC25 activity. In perfect keeping with this, the same results were obtained incubating the CDC25-t9, CDC25-t10 and CDC25-t11 probes with the purified p27 and p34 RAS2 proteins directly spotted on nitrocellulose filters (data not shown).

Discussion

The *in vitro* system presented in this work has allowed us to see direct evidence of the physical interaction between CDC25 and RAS2. The assay is essentially qualitative but it offers the important advantages that CDC25 and RAS2 protein purification can be avoided and that several different RAS2 forms can be tested for CDC25 binding at the same time. Moreover, in spite of the relatively non-physiological conditions in which the proteins interact during the assay, the obtained results can be well correlated with the *in vivo* functional activity of the two proteins, as is discussed below.

CDC25. All CDC25 polypeptides able to bind RAS2 can also perform essential growth functions in yeast cells, while the CDC25 forms that are inactive *in vivo* also fail to bind RAS2 in our assay conditions (Table I). Strikingly, the smallest sequence of CDC25 still able to bind RAS2, namely CDC25-t10, corresponds to the shortest known segment containing the CDC25 catalytic core (with the addition of 46 C-terminal aminoacids and without 22 N-terminal residues), as determined by functional analyses and by sequence comparison among CDC25-like proteins (see Introduction). On the other hand, the results obtained with CDC25-t11 indicate that some of the aminoacids in positions 1502-1543 are essential for the *in vitro* RAS2 binding as well as for CDC25 *in vivo* activity (with regard to this, we reasonably assumed that the last C-terminal 46 aminoacids of CDC25 are not required for RAS2 binding as they are dispensable *in vivo*). We

TABLE I. Complementing activity of different CDC25 truncated proteins compared to their *in vitro* RAS2 binding ability

CDC25 species	in vitro RAS2 binding	growth of $cdc25^-$ cells[a]
CDC25-t8	+	+
CDC25-t9	-	-
CDC25-t10	+	+
CDC25-t11	-	-

[a]see references [2,15,17].

should also mention that the CDC25-t11 C-terminal deletion might extend beyond the aminoacid 1501, as this species appears about 10% smaller than the predicted product. Even if this were the case, it would not alter the correlation between the CDC25 physiological activity and the *in vitro* RAS2 binding ability.

RAS2. *In vivo* complementation and biochemical analyses have shown that different CDC25-like proteins can functionally substitute in evolutionarily distant organisms and interact with *ras* proteins distinct from their physiological partners [16,19,23,29,30]. Conserved N-terminal *ras* sequences must therefore be involved in CDC25-like/RAS protein interactions, as it has also been indirectly suggested by genetic studies (ref. in [9]). On the other hand, substitutions in loops L2 and L4 of $p21^{H\text{-}ras}$ [8], strongly reduce the response to SDC25, a CDC25-like protein of *S.cerevisiae* [19] without decreasing the affinity of SDC25 for the mutated p21 [31].

Under our conditions, CDC25 cannot bind RAS2 if about 90 RAS2 C-terminal aminoacids, downstream to His210, are deleted. This result might explain why CDC25 also fails to bind $p21^{H\text{-}ras}$: in fact these aminoacids belong to the only evolutionarily variable region of *ras* proteins [6,14]. The inability to alter CDC25 binding by different RAS2 pre-treatments involving guanine nucleotide binding sequences (Fig.2B) also indicates that the latter play a minor role in our system. These results suggest that the variable *ras* protein domain could have a key role in the preferential interaction between a particular *ras* protein and its specific GNRP. Other experimental results are consistent with our data. A GNRP isolated from human placenta cells loses the ability to increase the nucleotides exchange rate on $p21^{H\text{-}ras}$ lacking the variable C-terminal region [32]. Another GNRP acting on smg-p21 (*rap1*) requires the presence of the C-terminal domain of this latter [33]. Finally, the guanine nucleotide releasing effect exerted on p21 by a non-physiological partner, namely SDC25, is not influenced by deletions of the last 23 C-terminal aminoacids of *ras* protein [31].

The hyper-variable RAS2 C-terminal region exerts a negative control on RAS2 activation *in vivo*. In fact a RAS2 version lacking residues 174-300 binds more GTP than is the case with the wild type protein [34] and allows the growth of $cdc25^-$ yeast cells [35]. These RAS2Δ174-300 properties do not depend on loss of sensitivity to GTPase Activating Proteins of budding yeast, namely IRA1 and IRA2 [36]. Consequently they might be explained by an altered GDP/GTP exchange rate in accordance with a possible recognition of RAS2 variable domain by CDC25.

The existance of an interaction between CDC25 and conserved *ras*-sequences in our system cannot be excluded. However, only more specific protein-protein interactions appear to be

effectively detected in these experimental conditions. In conclusion, the binding assay described offers an opportunity to analyze with a different approach the structure-function relationships in the interaction between CDC25 and RAS proteins, and provides extra information to complement the findings of previous studies.

References

1. Martegani, E., Baroni, M. and Vanoni, M. (1986) Exp. Cell. Res. 162, 544-548.
2. Camonis, J. H., Kalekine, M., Gondr, B., Garreau, H., Boy-Marcotte, E. and Jacquet, M. (1986) EMBO J. 5, 375-380.
3. Robinson, L. C., Gibbs, J. B., Marshall, M. S., Sigal, I. S. and Tatchell, K. (1987) Science 235, 1218-1221.
4. Broek, D., Toda, T., Michaeli, T., Levin, L., Birchmeier, C., Zoller, M., Powers, S. and Wigler, M. (1987) Cell 48, 789-799.
5. Jones, S., Vignais, M. L. and Broach, J. R. (1991) Mol. Cell. Biol. 11, 2641-2646.
6. Barbacid, M. (1987) Ann. Rev. Biochem. 56, 779-827.
7. Bourne, H. R., Sanders, D. A. and McCormick, F. (1990) Nature 348, 125-132.
8. Bourne, H. R., Sanders, D. A. and McCormick, F. (1991) Nature 349, 117-127.
9. Parmeggiani, A., Mistou, M. Y., Jacquet, E., Poullet, P. and Crechet, J. B. (1992, in press) In The ras superfamilies of GTPases (Lacal, J. C. and McCormick, F. Ed.), CRC Press.
10. DeFeo-Jones, D., Scolnick, E. M., Koller, R. and Dhar, R. (1983) Nature 306, 707-709.
11. Powers, S., Kataoka, T., Fasano, O., Goldfarb, M., Strathern, J., Broach, J. and Wigler, M. (1984) Cell 36, 607-612.
12. Toda, T., Uno, I., Ishikawa, T., Power, S., Kataoka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K. and Wigler, M. (1985) Cell 40, 27-36.
13. Broek, D., Samiy, N., Fasano, O., Fujiyama, A., Tamanoi, F., Northup, J. and Wigler, M. (1985) Cell 41, 763-769.
14. Gibbs, J. B. and Marshall, M. S. (1989) Microbiol. Rev. 53, 171-185.
15. Martegani, E., Baroni, M. D., Frascotti, G. and Alberghina, L. (1986) EMBO J. 5, 2363-2369.
16. Powers, S., Gonzales, E., Christensen, T., Cubert, J. and Broek, D. (1991) Cell 65, 1225-1231.
17. Petitjean, A. (1989) These, Universite Libre de Bruxelles.
18. Wickner, R. B., Koh, T. J., Crowley, J. C., O'Neil, J. and Kaback, D. B. (1987) Yeast 3, 51-57.
19. Boy-Marcotte, E., Damak, F., Camonis, J., Garreau, H. and Jacquet, M. (1989) Gene 77, 21-30.
20. Chant, J., Corrado, K., Pringle, J. R. and Herskowitz, I. (1991) Cell 65, 1213-1224.
21. Hughes, D. A., Fukui, Y. and Yamamoto, M. (1990) Nature 344, 355-357.
22. Simon, M. A., Bowtell, D. D. L., Dodson, G. S., Laverty, T. R. and Rubin, G. M. (1991) Cell 67, 701-716.
23. Martegani, E., Vanoni, M., Zippel, R., Coccetti, P., Brambilla, R., Ferrari, C., Sturani, E. and Alberghina, L. (1992) EMBO J., in press.
24. Powers, S., O'Neill, K. and Wigler, M. (1989) Mol. Cell. Biol. 9, 390-395.
25. Krieg, P. A. and Melton, D. A. (1987) Meth. Enzymol. 155, 397-415.

26. Gross, M., Sweet, R. W., Sathe, G., Yokoyama, S., Fasano, O., Goldfarb, M., Wigler, M. and Rosenberg, M. (1985) Mol Cell. Biol. 5, 1015-1024.
27. Kavounis, C., Verrotti, A. C., De Vendittis, E., Bozopoulos, A., Di Blasi, F., Zahn, R., Crechet, J. B., Parmeggiani, A., Tsernoglou, D. and Fasano, O. (1991) FEBS Lett. 281, 235-239.
28. Crechet, J. B., Poullet, P., Camonis, J., Jacquet, M. and Parmeggiani, A. (1990) J. Biol. Chem. 265, 1563-1568.
29. Crechet, J. B., Poullet, P., Mistou, M. Y., Parmeggiani, A., Camonis, J., Boy-Marcotte, E., Damak, F. and Jaquet, M. (1990) Science 248, 266-268.
30. Rey, I., Schweighoffer, F., Barlat, I., Camonis, J., Boy-Marcotte, F., Guilbaud, R., Jacquet, M. and Tocque, B. (1991) Oncogene 6, 347-349.
31. Mistou, M. Y., Jacquet, E., Poullet, P., Rensland, H., Gideon, P., Schlichting, I., Wittinghofer, A. and Parmeggiani, A. (1992) EMBO J., in press.
32. Downward, J., Riehl, R., Wu, L. and Weinberg, R. A. (1990) Proc. Natl. Acad. Sci. USA 87, 5998-6002.
33. Hiroyoshi, M., Kaibuchi, K., Kjawamura, S., Hata, Y. and Takai, Y. (1991) J. Biol. Chem. 266, 2962-2968.
34. Gibbs, J. B., Schaber, M. D., Marshall, M. S., Scolnick, E. M. and Sigal, I. S. (1987) J. Biol. Chem. 262, 10426-10429.
35. Marshall, M. S., Gibbs, J. B., Scolnick, E. M. and Sigal, I. S. (1987) Mol. Cell. Biol. 7, 2309-2315.
36. Tanaka, K., Nakafuku, M., Satoh, T., Marshall, M. S., Gibbs, J. B., Matsumoto, K., Kaziro, Y. and Toh-e, A. (1990) Cell 60, 803-807.
37. De Vendittis, E., Zahn, R. and Fasano, O. (1986) Eur. J. Biochem. 161, 473-478.