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Dis3, implicated in mitotic control, binds directly to Ran and enhances the GEF activity of RCC1

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Using the two-hybrid method, we isolated a *Saccharomyces cerevisiae* cDNA encoding a protein homologous to *Schizosaccharomyces pombe* protein Dis3sp, using as bait, human GTPase Ran. The *DIS3* gene is essential for viability and complements *S.pombe* mutant *dis3-54* which is defective in mitosis. Although Dis3sc has no homology to RanBP1, it bound directly to Ran and the *S.cerevisiae* Ran homologue Cnr1, but not to the *S.cerevisiae* RCC1 homologue Srm1. Upon binding to Ran with a 1:1 molar ratio, Dis3sc enhanced a nucleotide-releasing activity of RCC1 on Ran. In the presence of Dis3sc, the K_m of RCC1 on Ran decreased by half, while the k_{cat} was unchanged. *In vivo*, Dis3sp was present as oligomers of M_r 670–200 kDa as previously reported, and the 200 kDa oligomer of Dis3sp was found to include Spi1 and Pim1, the *S.pombe* homologues of Ran and RCC1, respectively. Although the biological function of the heterotrimeric oligomer consisting of Dis3, Spi1 and Pim1 is unknown, our results indicate that Dis3 is a component of the RCC1–Ran pathway.
Keywords: Dis3/Ran/RanGEF/RCC1

Introduction

Ran is an abundant nuclear G protein (Bischoff and Ponstingl, 1991b), which was originally isolated as a member of the Ras family designated as TC4 (Drivas *et al.*, 1990). Its guanine nucleotide is exchanged by RCC1 (Bischoff and Ponstingl, 1991a) which is also an abundant protein that localizes in the chromatin (Ohtsubo *et al.*, 1989; Dasso *et al.*, 1992). Similarly to Ras, the intrinsic GTP hydrolysis activity of Ran is low and is enhanced by a GTPase-activating protein (GAP) named RanGAP (Bischoff *et al.*, 1994) which is homologous to mouse fug1 (DeGregori *et al.*, 1994) and *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* Rna1 (Melchior *et al.*, 1993b; Becker *et al.*, 1995).

The RCC1 homologues, *SRM1/PRP20/MTR1*, *pim1*$^+$ and *BJ1* have been isolated from *S.cerevisiae*, *S.pombe* and *Drosophila*, respectively (reviewed by Dasso, 1993). The *rcc1*$^-$ phenotype is pleiotropic and includes the restoration of mating capacity to a receptorless mutant (*srm1*) (Clark and Sprague, 1989) and cell cycle arrest (tsBN2 and *pim1-d1*) (Nishimoto *et al.*, 1978; Matsumoto and Beach, 1991; Sazer and Nurse, 1994), in addition to defects in mRNA splicing (*prp20*) (Aebi *et al.*, 1990) and mRNA export (*mtr1*, *pim1-d1* and tsBN2) (Amberg *et al.*, 1993; Kadowaki *et al.*, 1993).

In order to clarify the RCC1–Ran pathway, proteins which directly bind to Ran have been identified either using an overlay assay (Coutavas *et al.*, 1993; Wu *et al.*, 1995) or using the two-hybrid method (Yokoyama *et al.*, 1995). These proteins possess a Ran binding domain homologous to RanBP1 (Dingwall *et al.*, 1995). In fact, RanBP2/Nup358 possesses four highly conserved Ran binding domains (Wu *et al.*, 1995; Yokoyama *et al.*, 1995). Consistent with the finding that Ran is required for nuclear import of proteins (Melchior *et al.*, 1993a; Moore and Blobel, 1993), RanBP2 locates on the top of cytoplasmic filaments of the nuclear pore complexes (NPC) (Melchior *et al.*, 1995), and the antibody to RanBP2 prevents nuclear protein import (Yokoyama *et al.*, 1995). Furthermore, a temperature-sensitive (ts) mutant of *YRB1*, the *S.cerevisiae* homologue of mammalian *RanBP1*, has a defect in the nuclear import of proteins (Schlenstedt *et al.*, 1995). Human RanBP1 has been purified as an inhibitor of RCC1-stimulated nucleotide exchange and functions as a co-activator of RanGAP (Bischoff *et al.*, 1995). In fact, overexpression of *YRB1* suppressed the *S.cerevisiae* RanGAP ts mutant, *rna1-1* (E.Noguchi, in preparation) which is synthetically lethal with a ts mutant of the NPC protein *nup1* (Bogerd *et al.*, 1994). *rna1-1* shows a similar phenotype to the *S.cerevisiae rcc1*$^-$ mutant *prp20* (Forrester *et al.*, 1992) and is defective in nuclear protein import (Corbett *et al.*, 1995). Taken together, these findings suggest that the RCC1–Ran pathway is involved directly or indirectly in the nucleocytoplasmic transport of proteins and mRNA. The pleiotropic phenotypes of *rcc1*$^-$ cells may thus be indirect consequences of the role of Ran in nucleocytoplasmic transport.

However, two groups independently have found that Ran locked in a GDP form inhibits the activation of maturation promoting factor (MPF) in *Xenopus* egg extract, even in the absence of the nuclei (Kornbluth *et al.*, 1994; Clarke *et al.*, 1995). We have also found that Cdc25C, which locates in the cytoplasm in tsBN2 cells and is essential for MPF activation, enters the nucleus upon loss of RCC1 (Seki *et al.*, 1992). These results cannot be explained from the point of view that Ran functions only in nucleocytoplasmic transport. In fact, Ohba *et al.* (1996) recently found that premature initiation of mitosis caused by loss of RCC1 can be inhibited not only by GTP-bound Ran which is essential for nuclear import of proteins (Moore and Blobel. 1993), but also by GTPγS-bound Ran

which blocks the nucleocytoplasmic transport of proteins and RNA (Melchior *et al.*, 1993a, 1995).

Ran does not have a lipid tail, which is conserved in most of the Ras superfamily (Drivas *et al.*, 1990), and so functions in solution, not in the membrane (Klebe *et al.*, 1995a). Ran locates predominantly in the nucleoplasm (Ren *et al.*, 1993) and also in the cytoplasm where it presumably functions in the nuclear import of proteins (Melchior *et al.*, 1993a; Moore and Blobel, 1993). Taken together, these results suggest that Ran may have multiple downstream pathways including nucleocytoplasmic transport and cell cycle regulation.

We have been isolating proteins which bind either RCC1 or Ran, in order to elucidate the RCC1–Ran pathway. By screening human and *S.cerevisiae* cDNA libraries by the two-hybrid method, we have already cloned human *RanBP1* and *RanBP2* (Hayashi *et al.*, 1995; Yokoyama *et al.*, 1995), and *YRB1* and *YRB2* (E.Noguchi, in preparation). In the present two-hybrid experiment using human Ran as bait to screen an *S.cerevisiae* cDNA library, we obtained a cDNA encoding a protein highly homologous to *S.pombe* Dis3sp (Kinoshita *et al.*, 1991), which complemented the *dis3-54* mutant isolated by Ohkura *et al.* (1988).

A cold-sensitive (cs) mutation of the *dis3*$^+$ gene, *dis3-54* has been reported to have a defect in mitosis similar to *dis2*$^-$, a cs mutant of the catalytic subunit of a type 1 protein phosphatase gene (Ohkura *et al.*, 1988; Kinoshita *et al.*, 1991). Although Dis3sp itself has no protein phosphatase activity, its overexpression reverses the *cdc25 wee1* double mutant to ts$^-$, and *dis3-54* shows synthetic lethality with *dis2* and *dis1*, both of which fail in chromosome segregation (Ohkura *et al.*, 1989; Kinoshita *et al.*, 1991; Nabeshima *et al.*, 1995). Taken together, these data implicate Dis3sp in mitotic control. However, the phenotype of the *dis3* deletion mutant is not mitotic specific, and therefore the cell cycle phenotype of *dis3-54* might be allele specific (Kinoshita *et al.*, 1991).

We found that Dis3sc directly bound to human Ran and the *S.cerevisiae* Ran homologue Cnr1, and enhanced the nucleotide-releasing activity of RCC1 on Ran. Dis3sp, of M_r 110 kDa, forms 670–160 kDa oligomers, as previously reported (Kinoshita *et al.*, 1991). The Dis3sp oligomer of lowest molecular mass was found to be a heterotrimeric complex consisting of Dis3sp, Spi1 and Pim1. Spi1 and Pim1 are *S.pombe* Ran and RCC1 homologues, respectively (Matsumoto and Beach, 1991; Sazer and Nurse, 1994).

Results

Isolation of S.cerevisiae DIS3, a structural homologue of S.pombe dis3$^+$

We screened ~5×10^5 colonies of the *S.cerevisiae* cDNA library with the two-hybrid method using human Ran as bait. Sixty-four colonies papillated on synthetic medium plates containing 25 mM 3-amino triazole (3-AT) and lacking histidine, tryptophan and leucine. We sequenced all 64 plasmids, and 37 of them contained the same cDNA encoding a peptide homologous to the C-terminal 212 amino acid residues of *S.pombe* Dis3sp (Kinoshita *et al.*, 1991) (Figure 1A, boxed region). Using the cloned cDNA fragment as a probe, a genomic library of *S.cerevisiae*, YCp50 (a gift from K.Matsumoto), was screened by colony hybridization, and a 4.5 kb genomic fragment was isolated as described in Materials and methods. The nucleotide sequence indicated that the 4.5 kb *Eco*RI fragment in YCp5072 contained an open reading frame (ORF) of 3003 nucleotides and a part of the ORF of *SCM2* (Chen *et al.*, 1994) (Figure 1B) which is located close to the *SUP3* gene on chromosome 15.

The identified ORF encodes a protein of M_r 110 kDa, which is 52.9% identical and 76.5% similar, including conserved amino acid residues, to *S.pombe* Dis3sp (Kinoshita *et al.*, 1991) (Figure 1A). Based on this high similarity, it was designated as *S.cerevisiae* Dis3sc.

DIS3 is essential for viability and complements the dis3-54 mutant of S.pombe

One-step gene disruption of the *DIS3* gene was carried out as shown in Figure 1B and C. Heterozygous diploid strains (*+/Δdis3*) in which genomic Southern hybridization produced hybridizing bands of the sizes expected from homologous recombination were selected and sporulated. Tetrad analysis indicated that fewer than two spores were viable and all the viable spores were His$^-$. Thus, *DIS3* is essential for viability, as reported for *S.pombe dis3*$^+$ (Kinoshita *et al.*, 1991). The observation that fewer than two spores survived suggested the possibility of a high frequency of chromosome non-disjunction in the *DIS3/Δdis3* heterozygous diploid, as has been observed previously in the *dis3-54* mutant (Ohkura *et al.*, 1988). In order to address this issue, we chose *pho80* as a genetic marker that is located near (~20 cM across the centromere) the *DIS3* gene on chromosome 15. *PHO80* is not essential, but, because it is a negative regulator of acid phosphatase, a previously described enzyme activity assay (Toh-e and Oshima, 1974) can be used to distinguish cells containing wild-type *PHO80* from those containing a disrupted copy of the gene. We prepared the heterozygous diploid, N83, in which *DIS3* was disrupted on one copy of chromosome 15 and *PHO80* was disrupted on the other (*DIS3/dis3::HIS3, PHO80/pho80::HIS3*) (Table I). When the N83 diploid strain was sporulated, 83 out of 232 spores survived. Because of the low expected frequency of recombination between the *DIS3* and *PHO80* loci, only three of these 83 surviving colonies were His3$^-$ whereas the remaining 80 were His3$^+$. Since *dis3::HIS3* is lethal, these surviving His$^+$ cells are expected to be carrying the *pho80::HIS3* chromosome 15 and to have high acid phosphatase activity. However, 25 of these 80 His$^+$ colonies were found to have low acid phosphatase activity, indicating that these *pho80::HIS3* spores also possess a wild-type copy of the *PHO80* gene. These haploid cells carrying both *pho80::HIS3* and *PHO80* are likely to contain two copies of chromosome 15 as the result of chromosome non-disjunction.

In order to investigate a functional relationship between Dis3sc and Dis3sp, we examined the ability of the *DIS3* gene to complement the cs *dis3*$^-$ mutant *dis3-54* of *S.pombe*. To do this, the full-length *DIS3* cDNA was inserted into the *S.pombe* expression vector pREP1 (Maundrell, 1990). The resulting plasmid pREP-DIS3 (*LEU2 nmt1-DIS3*) and, as controls, p(dis3$^+$) containing the wild type *S.pombe dis3*$^+$, and the vector alone, were introduced into *S.pombe dis3-54* (*leu1 ura4*). Transformants were selected on synthetic medium plates lacking

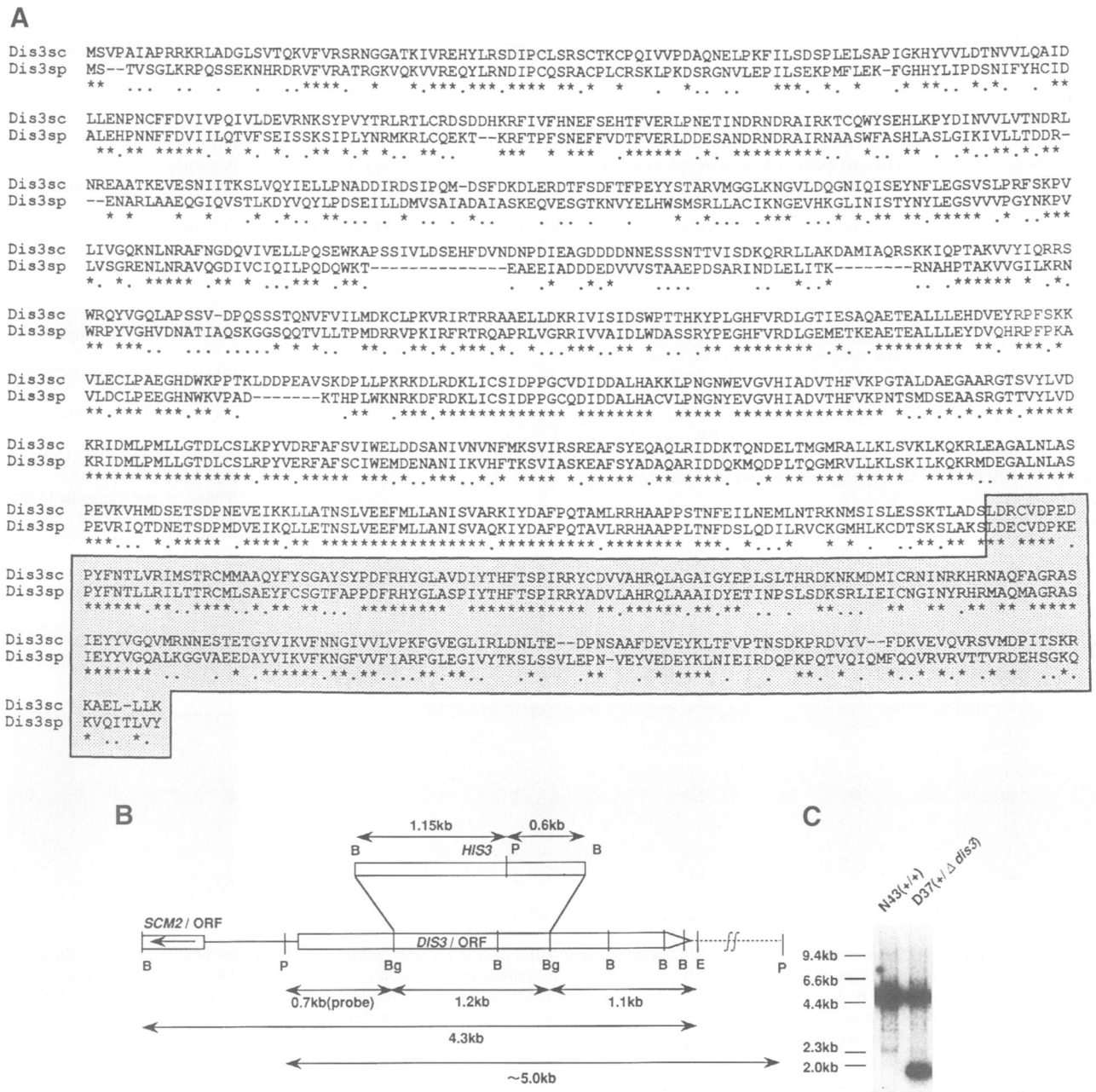


Fig. 1. *DIS3* is essential for viability of *S.cerevisiae*. (**A**) Amino acid sequence of *S.cerevisiae* Dis3 and its comparison with that of *S.pombe* Dis3. The star indicates the position of identical amino acid residues, and the region of *DIS3* cDNA which was isolated by the two-hybrid method is shown by the box. The nucleotide sequence data of *DIS3* is available in DDBJ, EMBL and GenBank with the accession number D76430. (**B**) Disruption of the *DIS3* gene. The 1.2 kb *Bgl*II fragment of the *DIS3* gene was replaced with the 1.75 kb *Bam*HI fragment of the *HIS3* gene to produce the plasmid pUC72BHIS containing the disrupted *DIS3* gene (*dis3::HIS3*). The 4.1 kb *Bam*HI fragment cut out from pUC72BHIS was introduced into a diploid strain, N43 (Table I) and the resulting heterozygous diploid strains D37 (Table I) were sporulated. The expected lengths of restriction fragments with or without the gene disruption are shown in kilobases. B, *Bam*HI; E, *Eco*RI; P, *Pst*I; Bg, *Bgl*II. (**C**) Genomic Southern hybridization of the wild-type (+/+) and His^+ integrant (+/Δ*dis3*) probed with the 0.7 kb *Pst*I–*Bgl*II fragment. Genomic DNAs were digested with *Pst*I and run on an agarose gel. The sizes of fragments are indicated in kilobases. The 5.0 and 1.85 kb fragments were derived from the intact and the disrupted *DIS3* gene, respectively.

leucine, and streaked at 20 or 33°C on EMM plates to induce expression by the *nmt1* promoter (Maundrell, 1990). The *DIS3* cDNA rescued the cs^- phenotype of the *S.pombe dis3*$^-$ mutant (Figure 2A). To confirm the ability of Dis3sc to complement the *dis3-54* mutation, Leu^+ transformants which were cultivated in synthetic liquid medium lacking leucine at 30°C were adjusted to the same cell number, and equal volumes of serially diluted cultures were dropped on EMM medium plates. After incubation at 20°C for 4 days, no *dis3-54* cells possessing the pREP1 vector alone grew after a 1:4 dilution, while several colonies papillated from transformants containing either p(dis3$^+$) or pREP-DIS3 even after a 1:16 dilution (Figure 2B). These results indicated that *DIS3* complemented the *dis3-54* mutation, although its ability seems to be weaker than that of *dis3*$^+$.

Table I. Plasmids and yeast strains used in this study

	Comment/Genotype	Source
Plasmids		
pTKS72E	pBluescript II TKS(+) with 4.5 kb *DIS3* fragment at *Eco*RI site	this study
pGEX-DIS3	GST-fused *DIS3* cDNA for *E.coli* expression	this study
pGEX-CNR1	GST-fused *CNR1* cDNA for *E.coli* expression	this study
pGEX-SRM1	GST-fused *SRM1* cDNA for *E.coli* expression	this study
pUC72BHIS	disruption of *DIS3* by replacement of 1.2 kb *Bgl*II fragment with 1.75 kb *HIS3* fragment	this study
pREP-DIS3	pREP1[a] with *DIS3* cDNA downstream of the *nmt1*$^+$ promoter	this study
p(dis3$^+$)	*LEU2* and *dis3*$^+$ plasmid	Kinoshita *et al.* (1991)
Yeasts		
S.cerevisiae N43	*MATα ade2 his3 leu2 + trp1 ura3* *MATa ade2 his3 leu2 lys2 trp1 ura3*	NBW5[b]×YPH499[c] diploid
S.cerevisiae D37	*MATα dis3::HIS3 ade2 his3 leu2 + trp1 ura3* *MATa + ade2 his3 leu2 lys2 trp1 ura3*	N43 disrupted with pUC72BHIS
S.cerevisiae N83	*MATα dis3::HIS3 + ade2 his3 leu2 lys2 trp1 ura3* *MATa + pho80::HIS3 ade2 his3 leu2 + trp1 ura3*	37C19×NBD80-1 diploid
S.cerevisiae 37C19	*MATα dis3::HIS3 ade2 his3 leu2 lys2 trp1 ura3* [YCp5072]	segregant from D37[YCp5072]
S.cerevisiae NBD80-1	*MATa pho80::HIS3 ade2 his3 leu2 trp1 ura3*	Bun-ya *et al.* (1991)
S.pombe HM123	*h*$^-$ *leu1-32*	Samejima and Yanagida (1994)
S.pombe dis3-54	*h*$^-$ *dis3-54 leu1 ura4*	Kinoshita *et al.* (1991)

[a]Maundrell (1990).
[b]Matsuzaki *et al.* (1990).
[c]Sikorski and Hieter (1989).

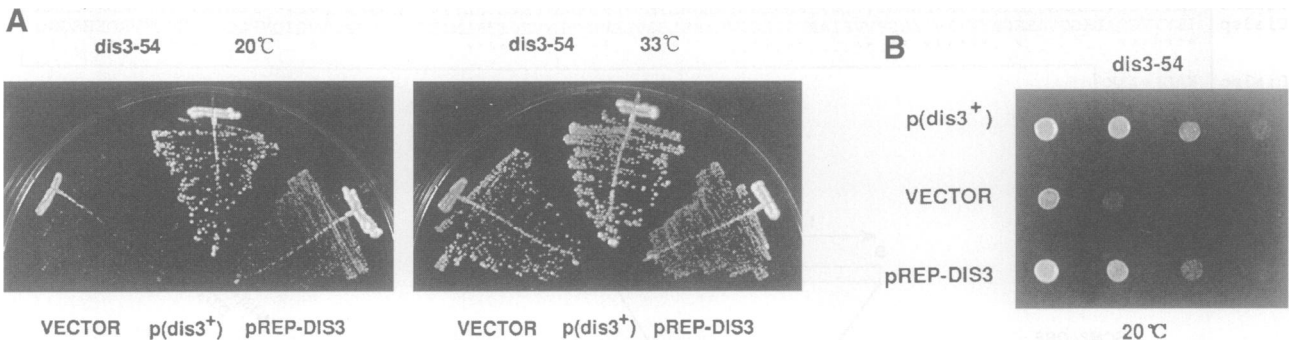


Fig. 2. *DIS3* complements *S.pombe dis3-54*. (**A**) The *DIS3* plasmid pREP-DIS3 and, as controls, p(dis3$^+$), and a vector pREP1, were introduced into *S.pombe dis3-54* (*h*$^-$ *dis3-54 leu1 ura4*). Transformants (Leu$^+$) selected on synthetic medium plates (*leu*$^-$) were streaked on EMM plates, and incubated at either 20 or 30°C. (**B**) Leu$^+$ transformants were cultivated in synthetic liquid medium lacking leucine at 30°C. After full growth, the cell numbers of all cultures were adjusted to $OD_{660\ nm}$ = 4.0 and then were serially diluted one to four as shown from the left to the right. One spot (5 μl) of each of diluted cultures was dropped on EMM medium plates and incubated at 20°C for 4 days.

Dis3sc directly bound to Cnr1 and Ran, but not Srm1

DIS3sc cDNA was isolated frequently by the two-hybrid method using human Ran as bait, although it has no domain homologous to the previously characterized Ran binding protein. In order to confirm a direct binding of Dis3sc to the *S.cerevisiae* Ran homologue Cnr1, GST-fused Dis3sc was engineered and expressed in *Escherichia coli*. GST–Dis3sc proteins purified on a glutathione column were mixed with bacterially produced Cnr1 and, as a control, also mixed with the bacterially produced *S.cerevisiae* RCC1 homologue Srm1. As shown in Figure 3, Dis3sc bound to Cnr1 but not to Srm1 (Figure 3, lanes 1 and 2). Interestingly, Dis3sc bound to Srm1 in the presence of Cnr1 (Figure 3, lane 3). The same results were obtained even in the presence of EDTA (data not shown). Since RCC1 forms a stable complex with Ran only in its nucleotide-free state (Bischoff and Ponstingl, 1991b), this finding suggests the possibibilty that Dis3sc binds to nucleotide-free Cnr1.

The next question is whether GTP- or GDP-bound Cnr1 can also bind to Dis3sc. In order to address this question, we used human Ran, since Cnr1 produced in *E.coli* had a very low nucleotide binding ability. While human Ran cannot replace Cnr1 in *S.cerevisiae*, overexpression of human Ran suppressed *prp20-1* (data not shown), indicating that human Ran is functionally similar to Cnr1. The ability of Dis3sc to bind nucleotide-bound Ran was examined both by filter binding and liquid binding assays. Our GTP-Ran preparation contained 70% GTP-Ran and 30% GDP-Ran, and the GDP-Ran preparation used contained no nucleotide-free Ran.

GST fusions of Dis3sc and, as controls, Yrb1 and Yrb2, *S.cerevisiae* RanBP1 homologues (Dingwall *et al.*, 1995; Schlenstedt *et al.*, 1995) that were isolated in the same two-hybrid screen as Dis3sc (E.Noguchi, in preparation), were produced in *E.coli* and purified using a glutathione column. These GST fusion proteins and GST alone were co-electrophoresed in SDS–PAGE and then transferred onto the filter. Following the renaturation of filter-bound

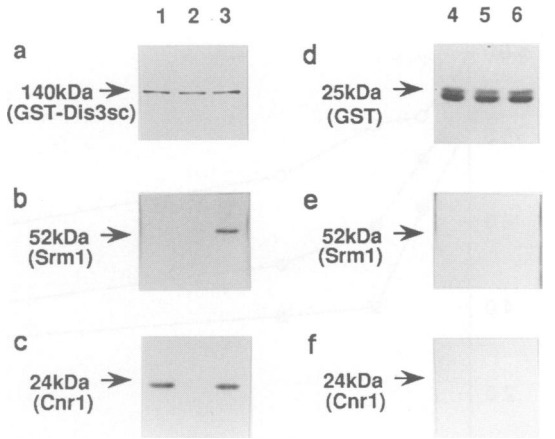


Fig. 3. Dis3sc directly binds to Cnr1, but not to Srm1. Purified GST–Dis3sc bound to beads was mixed with purified Cnr1 (lane 1), purified Srm1 (lane 2), Cnr1 plus Srm1 (lane 3) and, as controls, beads bound to GST alone were mixed with Cnr1 (lane 4), Srm1 (lane 5) and Cnr1 plus Srm1 (lane 6), incubated at 4°C for 60 min, and then the beads were spun down as described in Materials and methods. The beads were washed seven times with the binding buffer and were electrophoresed in 7.5% (for Dis3sc) and 12.5% (for Cnr1 and Srm1) SDS–PAGE, transferred onto a PVDF membrane and immunoblotted with the anti-GST, anti-Cnr1 and anti-Srm1 antibodies as indicated.

proteins, ^{35}S-labelled GTPγS- or GDPβS-Ran was overlaid as described in Materials and methods. Yrb1 bound to GTPγS-, but not GDPβS-Ran (Figure 4 insert) as previously reported for RanBP1 (Coutavas *et al.*, 1993). Dis3sc and Yrb2 bound neither GTPγS- nor GDPβS-Ran. In order to confirm these results, the binding to nucleotide-bound Ran was carried out in liquid. The GST fusion proteins were mixed with ^{35}S-labelled GTPγS- or GDPβS-Ran and precipitated with glutathione–Sepharose beads. The radioactivity that co-precipitated with the glutathione–Sepharose beads was quantified using a liquid scintillation counter, and the precipitates were assayed for the presence of Ran by immunoblotting using an antibody to human Ran as a probe. Again, high radioactivity was detected only in the case of GST–Yrb1 mixed with ^{35}S-labeled GTPγS-Ran (Figure 4A). Surprisingly, Ran was co-precipitated with Dis3sc, from mixtures of either [^{35}S]GTPγS- or [^{35}S]GDPβS-bound Ran (Figure 4B, lanes 4 and 8). Since there was no significant radioactivity in these precipitates (Figure 4A, columns 3 and 7), the nucleotides bound to Ran may have been removed from Ran upon binding to Dis3sc. Regarding Yrb1 and Yrb2, the result of the immunoblotting analysis is consistent with the radioactivity analysis: neither Ran nor any radioactivity were precipitated from the mixtures of [^{35}S]GDPβS-Ran and Yrb1, [^{35}S]GDPβS-Ran and Yrb2 and [^{35}S]GTPγS-Ran and Yrb2.

These results indicated that Dis3sc could bind to the nucleotide-free human Ran similar to the case of the *S.cerevisiae* Ran homologue Cnr1. In order to confirm the direct binding of Dis3sc to nucleotide-free Ran, GST-fused Dis3sc and, as controls, GST-fused Yrb1 and Yrb2 were mixed with GDP-Ran, in the presence of 2 mM EDTA to remove the nucleotide from GDP-Ran (Bischoff and Ponstingl, 1991b), and the GST fused proteins were precipitated by glutathione–Sepharose beads. Ran was co-precipitated with GST–Dis3sc and GST–Yrb1, but not with GST–Yrb2 (Figure 4C). Since GDP-Ran by itself

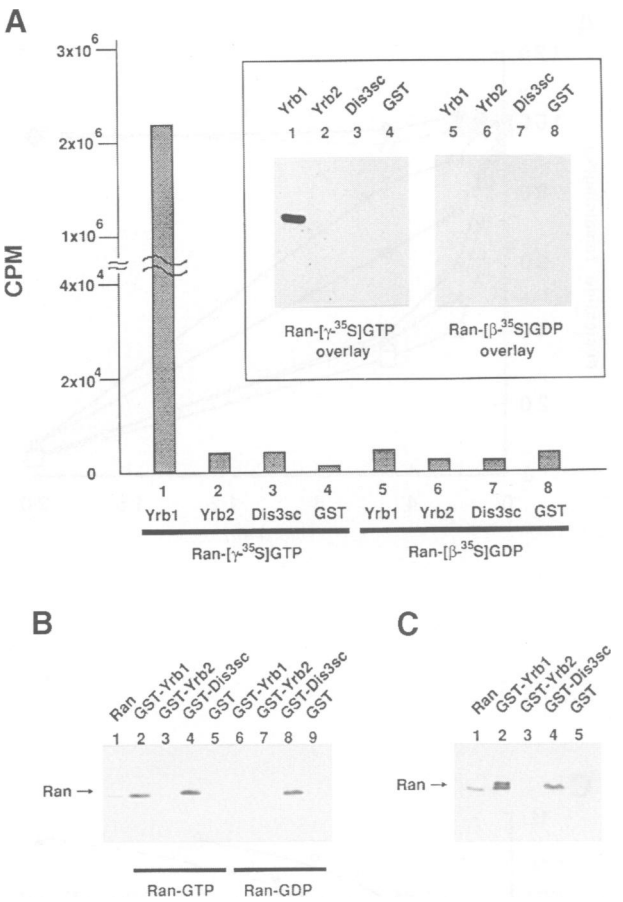
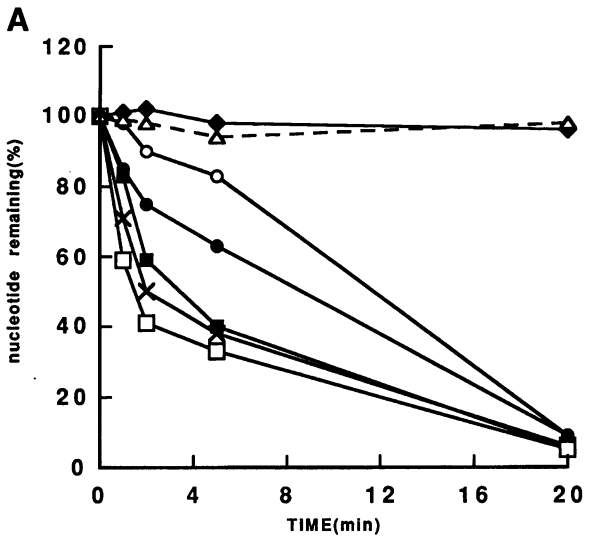
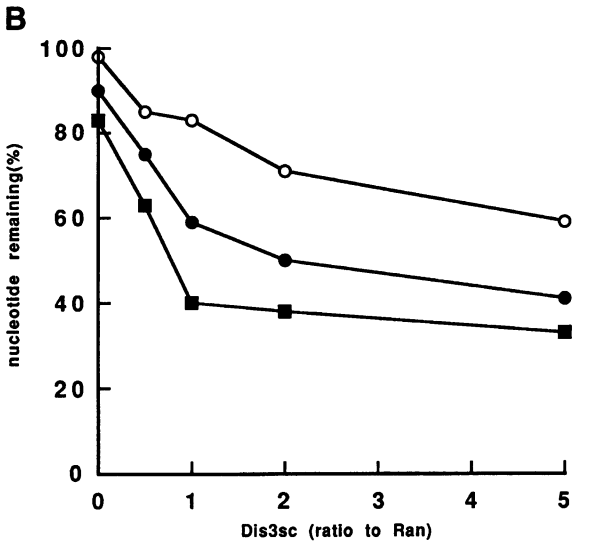


Fig. 4. Binding of Ran to Dis3sc. (**A**) The *E.coli*-produced GST fusion proteins bound to beads [Yrb1 (lanes 1 and 5), Yrb2 (lanes 2 and 6), Dis3sc (lanes 3 and 7)] and, as a control, GST alone (lanes 4 and 8) were mixed with ^{35}S-labeled GTPγS (lanes 1–4) or ^{35}S-labeled GDPβS (lanes 5–8), incubated at 4°C for 60 min, and then spun down as described in Materials and methods. The beads were washed seven times with the binding buffer and then the radioactivity of the beads was quantified by liquid scintillation counting. The c.p.m. of each sample is as follows: lane 1, 2 127 000; lane 2, 3390; lane 3, 3807; lane 4, 1307; lane 5, 3887; lane 6, 3186; lane 7, 2956; lane 8, 3566. Insert: 100 ng of *E.coli*-produced GST fusion proteins Yrb1 (lanes 1 and 5), Yrb2 (lanes 2 and 6), Dis3sc (lanes 3 and 7) and, as a control, GST alone (lanes 4 and 8) were electrophoresed in 10% SDS–PAGE and then overlaid with [^{35}S]GTPγS (lanes 1–4) or [^{35}S]GDPβS (lanes 5–8) as described in Materials and methods. (**B**) The precipitates of beads prepared as described in (A) and, as a control, human Ran were electrophoresed in 12.5% SDS–PAGE, transferred onto a PVDF membrane and immunoblotted with the anti-human Ran antibody. Lane 1, human Ran; lanes 2 and 6, GST–Yrb1; lanes 3 and 7, GST–Yrb2; lanes 4 and 8, GST–Dis3sc. Lanes 5 and 9, GST alone; lanes 2–5, mixed with [^{35}S]GTPγS; lanes 6–9, mixed with [^{35}S]GDPβS. (**C**) The *E.coli*-produced GST fusion proteins bound to beads (Yrb1, Yrb2 and Dis3sc) and, as a control, GST beads alone were mixed with GDP-Ran in the presence of EDTA, incubated at 4°C for 60 min, and then spun down as described in Materials and methods. The presence of Ran in the precipitates was analysed as described in (B). Lane 1, Ran; lane 2, GST–Yrb1; lane 3, GST–Yrb2; lane 4, GST–Dis3sc; and lane 5, GST alone.

did not bind to GST–Yrb1, as shown in Figure 4B lane 6, the co-precipitation of Ran with Yrb1 indicates that GDP was released from Ran in our experimental conditions so that Yrb1 bound to nucleotide-free Ran as previously reported for human RanBP1 (Bischoff *et al.*, 1995). These results are consistent, therefore, with the hypothesis that Dis3sc binds to nucleotide-free Ran.





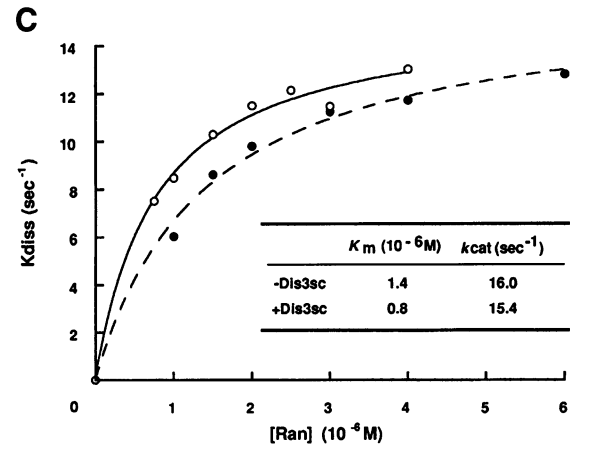


Fig. 5. Dis3sc co-activates the guanine nucleotide-releasing activity of RCC1. (**A**) Two pmol of [^{3}H]GDP-bound Ran were pre-incubated with 0 (○), 1 (●), 2 (■), 4 (×) and 10 (□) pmol of Dis3sc for 3 min at 30°C, and was then mixed with 2 fmol of human RCC1, as indicated. At the indicated time, the reaction was stopped and the remaining radioactivity on Ran was counted by liquid scintillation counting. As controls, 2 pmol of [^{3}H]GDP-bound Ran was mixed with 10 pmol of Dis3sc alone (△) and with buffer alone (◆), and incubated as indicated. (**B**) The ratio of nucleotide remaining after incubation for 1 min (○), 2 min (●) and for 5 min (■) is shown versus the concentration of Dis3sc which is indicated as the molar ratio to the concentration of [^{3}H]GDP-bound Ran. (**C**) Kinetic analysis of GDP (mantGDP) dissociation from Ran by RCC1 in the presence of Dis3sc and nucleotide-bound Ran with a 1:1 molar ratio. Various concentrations of mantGDP-Ran were either mixed with an equal molar concentration of Dis3sc (○) or not (●). After pre-incubation, 2.5 nM of RCC1 was added to start the reaction. The intensity of fluorescence emission at 450 nm, excited at 355 nm, was measured every second as described (Azuma *et al.*, 1996). The initial velocity of the reaction was estimated by linear fitting between zero-time and the time when 10% of the reaction had been completed for each reaction, and this was then divided by the concentration of RCC1 to estimate the dissociation coefficient (K_{diss}). The calculated K_{diss} values were plotted against the substrate concentration and fitted to the Michaelis–Menten equation to give the K_m and k_{cat}. The curvilinear fittings were achieved using the Kaleida Graph program on a Macintosh computer.

Dis3sc co-activates the nucleotide-releasing ability of RCC1

The above results suggested the possibilities that Dis3sc could bind to either GTP- or GDP-Ran and that the nucleotides bound to Ran were removed by binding to Dis3sc. The ability to release either GTP or GDP from Ran has been reported for RCC1 (Bischoff and Ponstingl, 1991b). We therefore addressed the question of whether Dis3sc can release the nucleotide from Ran.

As shown in Figure 5A, Dis3sc alone cannot release the nucleotide from Ran. However, the guanine nucleotide exchange (GEF) activity of RCC1 was enhanced by Dis3sc. In the presence of 2 fmol of RCC1, 2 pmol of ^{3}H-labelled GDP-Ran was mixed with 1, 2, 4 or 10 pmol of Dis3sc and incubated at 30°C. As shown in Figure 5, the GDP release from Ran was stimulated by Dis3sc in a dose-dependent manner. The ability of Dis3sc to enhance the GEF activity of RCC1, however, plateaued when Dis3sc and ^{3}H-labelled GDP-Ran were present in a 1:1 molar ratio (Figure 5B).

In order to examine the way in which Dis3sc stimulates the GEF activity of RCC1, we performed a steady-state reaction analysis of GEF activity of RCC1 on Ran in the presence of Dis3sc, and the kinetic parameters (K_m and k_{cat}) were calculated as described by Azuma *et al.* (1996). Based on the observation that the maximum effect of Dis3sc on Ran was obtained at a 1:1 molar ratio, we added to the reaction mixtures the same molar concentration of Dis3 as that of Ran. In the case of RCC1 alone, the K_m was 1.4 μM and the k_{cat} was 16/s, as previously reported (Klebe *et al.*, 1995b; Azuma *et al.*, 1996). Upon addition of Dis3sc, the K_m value was reduced to 0.8 μM, while the k_{cat} value was not changed (Figure 5C, insert).

Dis3sp was co-fractionated with Spi1

Schizosaccharomyces pombe Dis3sp, of M_r 110 kDa, has been found by gel filtration to be present in a complex of 250–350 kDa (Kinoshita *et al.*, 1991). In order to investigate whether Dis3sp forms a complex with Ran *in vivo*, a crude extract of *S.pombe* was fractionated by gel filtration

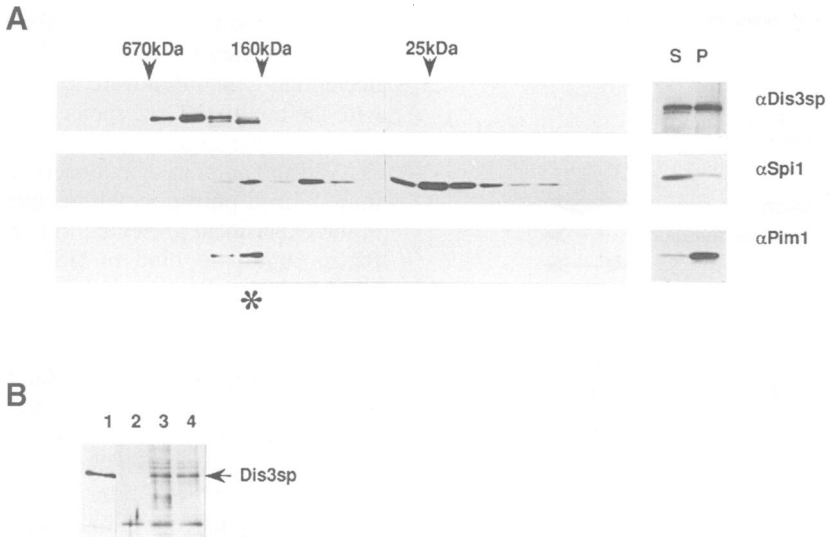


Fig. 6. *S.pombe* Dis3sp forms a heterotrimeric complex with Spi1 and Pim1. (**A**) Wild-type *S.pombe* cells were lysed by lysis buffer and centrifuged at 45 000 r.p.m. for 30 min. The supernatant (S) was fractionated with Pharmacia Hiload Sephacryl S-300HR and each fraction was electrophoresed with 7.5% (for Dis3sp) and 12.5% (for Spi1 and Pim1) SDS–PAGE, and assayed for the presence of Dis3, Spi1 and Pim1 by imunoblotting analysis using the antibodies to these proteins. As controls, the supernatant (S) and the precipitate (P) derived from the same number of cells were co-electrophoresed and assayed for the presence of Dis3sp, Spi1 and Pim1, respectively. (**B**) Co-immunoprecipitation of Dis3sp by antibody to either Spi1 or Pim1. Spi1 and Pim1 in the 200 kDa Dis3sp fraction were immunoprecipitated using antibody to Spi1 and Pim1, respectively. The precipitates and, as a control, the total cell extract were assayed for the presence of Dis3sp by immunoblotting analysis using an antibody to Dis3sp. Lane 1, total cell extract. Lanes 2, 3 and 4, total cell extract which was precipitated with the pre-immunue serum (2), anti-Spi1 (3) and anti-Pim1 (4) antibodies, respectively.

chromatography, and each fraction was assayed for the presence of Dis3sp and the *S.pombe* Ran homologue Spi1 by immunoblotting analysis using the antibodies to either Dis3sp or Spi1 (Figure 6). Dis3sp was recovered continuously in the 670–160 kDa fractions as previously reported (Kinoshita *et al.*, 1991). On the other hand, Spi1 was found in three distinct fractions, ~200, 100 and 25 kDa. The fraction of Spi1 with an M_r of 200 kDa overlapped with the Dis3sp fraction of lower molecular mass. In order to confirm the direct binding between Spi1 and Dis3sp, this fraction (marked by an asterisk in Figure 6A) was subjected to immunoprecipitation analysis using the antibody to Spi1. As expected, Dis3sc was co-precipitated with Spi1 (Figure 6B, lane 3).

The Spi1 fraction of M_r 25 kDa corresponded to the molecular mass of Spi1 (Matsumoto and Beach, 1991). Interestingly a considerable amount of Spi1 was fractionated into a oligomer of ~100 kDa, indicating that Spi1 also formed a complex with proteins other than Dis3sp.

The finding that Dis3sp can co-activate RCC1 suggested that Dis3sp may form a heterotrimeric complex with Spi1 and Pim1. In order to address this question, the same gel filtration fractions of *S.pombe* extracts were assayed for the presence of Pim1 by immunoblotting analysis, using an antibody to Pim1. As shown in Figure 6A, Pim1 was found to locate in the same fraction in which both Dis3sp and Spi1 were co-localized (shown by an asterisk). To confirm the presence of a heterotrimeric complex consisting of Dis3sp, Spi1 and Pim1, we immunoprecipitated this fraction using the antibody to Pim1 (Figure 6B, lane 4). Dis3sp was again co-precipitated with Pim1. Since the molecular mass of the light chain of immunoglobulin is similar to that of Spi1, we could not confirm the presence of Spi1 in the immunoprecipitated Pim1 fraction using the antibody to Spi1. As shown in Figure 3, however, Dis3sc, while not able to bind directly to Srm1, binds to Srm1 in the presence of Cnr1 (Figure 3, lane 3). Taken together with the finding that Dis3sc was co-precipitated with Spi1, the co-precipitation of Dis3sp with Pim1 indicates that Dis3sp formed a heterotrimeric complex with Spi1 and Pim1 *in vivo*.

The apparent molecular mass of the Dis3sp protein forming a heterotrimeric complex with Spi1 and Pim1 was lower compared with that of Dis3sp in other fractions (Figure 6A). This is not likely to be result of either dephosphorylation or degradation of Dis3sp, because extensive treatment of other Dis3sp fractions with both alkaline and acidic phosphatase did not produce a protein with the same molecular mass (data not shown), and this faster migrating version of Dis3sp is present only in Dis3sp fractions of the supernatant containing Spi1 and Pim1, but not in the precipitated fraction of the crude extract (Figure 5A, compare S and P on line Dis3).

Consistent with previous reports that Dis3sp is present in both the cytosol and the nucleus (Kinoshita *et al.*, 1991), and that RCC1, but not Ran, binds to chromatin (Ohtsubo *et al.*, 1989; Dasso *et al.*, 1992), the precipitated fraction of *S.pombe* cell lysates contained a majority of Pim1, around one-half of the total Dis3sp, but very little Spi1 (Figure 6A, P).

Discussion

By using human Ran as the bait in a two-hybrid screen, we have isolated *YRB1* and *YRB2* from a *S.cerevisiae* cDNA library (E.Noguchi, in preparation). In addition, we now report the isolation of a new cDNA encoding the C-terminal part of a protein which is highly similar to *S.pombe* Dis3sp. Since a full-length cDNA of this cloned cDNA complements a *S.pombe dis3*$^-$ mutant, the isolated

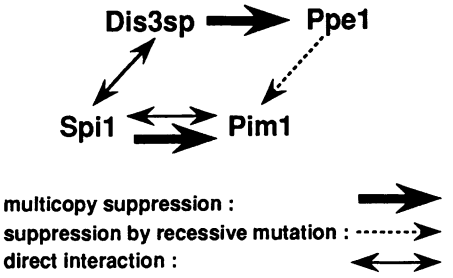


Fig. 7. Interaction of Dis3sp, Ppe1, Spi1 and Pim1.

cDNA was designated as *DIS3*. Although both Yrb1 and Yrb2 and several other Ran binding proteins share a domain homologous to RanBP1, Dis3sc does not have any homology to RanBP1.

RCC1–Ran pathway and protein phosphatase network

The C-terminal part of Dis3sc encoded by the cDNA isolated by the two-hybrid method corresponds to a region of Dis3sp essential to complement the *dis3*$^-$ mutant *dis3-54*, which has a defect in mitotic progression, and is synthetically lethal with both *dis2*$^-$, a cs mutant of a type 1 phosphatase, and *dis1*$^-$ (Kinoshita *et al.*, 1991; Nabeshima *et al.*, 1995). The amino acid sequence of this C-terminal region is highly similar to Srk1/Ssd1 which suppresses both the deletion of the essential phosphatase gene *SIT4* and a mutation in *BCY1* encoding a regulatory subunit of cAMP-dependent protein kinase (Sutton *et al.*, 1991; Wilson *et al.*, 1991). The C-terminal part of Dis3sp, therefore, has been suggested to play an important role in the protein phosphatase network. The isolation of the C-terminal part of Dis3sp using Ran as bait thus may reflect a functional relationship between Dis3 and Ran. In this context, it should be noted that a cs mutant *esp1-68* of the Ppe1 phosphatase, that has sequence similarity to Sit4 (Shimanuki *et al.*, 1993), suppressed the *S.pombe* *rcc1*$^-$ mutants *pim1-46* (Matsumoto and Beach, 1993) and *pim1-d1* (Sazer and Nurse, 1994). On the other hand, the disruptant of *ppe1*$^+$ can be suppressed by overexpression of either Dis3sp or Sit4 (Shimanuki *et al.*, 1993). The known genetic and biochemical relationships among components of the RCC1–Ran pathway and the protein phosphatase network are shown diagramatically in Figure 7. Reg1, which when mutated suppresses the ts mutation in *S.cerevisiae* RanGAP *rna1*, binds to a protein phosphatase type 1 but is not shown on this model (Tu and Carlson, 1995). Taken together, the Ran pathway seems to be involved in the phosphatase network which regulates cell cycle progression.

Dis3sp has been shown to form a complex with other proteins, since it was fractionated into multiple oligomers possessing a molecular mass higher than that estimated for a Dis3sp monomer based on its amino acid sequence (Kinoshita *et al.*, 1991). Both Spi1 and Pim1 were co-precipitated with the part of the Dis3sp fraction with the lowest molecular mass. It remains to be investigated why the Dis3sp bound to Spi1 shows an electrophoretic mobility faster than the Dis3sp proteins of other fractions.

Activation of nucleotide exchange on Ran by Dis3

While Dis3sc by itself does not show any GEF activity on nucleotide-bound Ran, it enhances the GEF activity of RCC1 by binding to Ran. The ability of Dis3sc to enhance nucleotide release from Ran plateaued when Dis3sc and nucleotide-bound Ran were in a 1:1 molar ratio, consistent with the binding of one molecule of Dis3 to one molecule of Ran. In the experiment shown in Figure 5A and B, we assayed the amount of radioactivity of labelled GDP-Ran immobilized on nitrocellulose filters. On the other hand, in the experiment presented in Figure 4, Ran was assayed for its ability to bind to GST–Dis3sc protein attached to glutathione–Sepharose beads. Prior to the assay for radioactivity, the beads were washed extensively several times with buffer to remove non-specifically bound proteins. Probably, the ability of Ran to bind guanine nucleotides was reduced upon binding to Dis3sc so that the nucleotides were released easily from Ran during extensive washing. However, the steady-state reaction analysis of GEF activity of RCC1 on Ran indicated that while the k_{cat} was not changed, the K_m was reduced to around half its original value by addition of Dis3sc, indicating that the affinity of RCC1 for the nucleotide-bound Ran was increased by addition of Dis3sc. Presently, we do not know why nucleotides were released from Ran by binding to GST–Dis3sc without RCC1.

The finding of a heterotrimeric complex consisting of Dis3sp, Spi1 and Pim1, even though its function is unknown, raises important and interesting questions regarding the RCC1–Ran pathway. One question regards the localization of Pim1/RCC1. Dis3sp is found to be present in both the cytosol and the nuclear fraction as previously shown (Kinoshita *et al.*, 1991). While it remains to be discovered where the heterotrimeric oligomer of Dis3, Spi1 and Pim1 exists in cells, our present finding indicates that it is not chromatin associated, suggesting that RCC1 locates to a place other than the chromatin, as has been demonstrated by immunolocalization of Pim1 in *S.pombe* (A.Matynia and S.Sazer, unpublished results). The other question regards the *in vivo* GEF function of RCC1. Since RCC1 has been shown to have a very strong GEF activity on Ran (Bischoff and Ponstingl, 1991a; Klebe *et al.*, 1995a) and since there was no evidence showing that RCC1 locates to a place other than the chromatin, we believed that chromosomal RCC1 has the GEF function *in vivo*. However, the finding that Dis3sc enhanced the nucleotide-releasing activity of RCC1 raises the question of whether RCC1 located on the chromatin functions as a GEF on Ran. It will be important to clarify whether RCC1 bound to the chromatin or to Dis3 functions in the cells as a real GEF on Ran, and how those activities are regulated in the cell cycle.

Additionally, our present results indicate that Spi1 forms another complex in cells which has an M_r of ~100 kDa. It is important for elucidation of the RCC1–Ran pathway to determine the identity of these other proteins that bind to Spi1 in this complex.

Materials and methods

Strains and media

Saccharomyces cerevisiae strains and plasmids used in this study are described in Table I. *Escherichia coli* strains XLI-blue and BL21(DE3) were used for plasmid engineering and for producing recombinant proteins as described previously (Hayashi *et al.*, 1995). The media used for yeast and bacteria have been described previously (Nishiwaki *et al.*, 1987; Samejima and Yanagida, 1994).

Two-hybrid screen

Yeast Y190 bearing pAS1-Ran (Yokoyama *et al.*, 1995) were cultivated in 5 ml of synthetic medium lacking tryptophan (*trp*$^-$) for 1 day, transferred into 100 ml of YPD medium and cultivated for another 6 h. Cells were harvested, washed with sterile water and treated with LiCl for transformation. An *S.cerevisiae* cDNA library fused with the GAL4 activation domain of the pACT vector (a gift from Dr Elledge) was then transfected into the cells. Transformants were cultured in synthetic medium lacking leucine and tryptophan (*leu*$^-$, *trp*$^-$) overnight, and then spread onto selection plates of synthetic medium lacking histidine, tryptophan and leucine (*his*$^-$, *trp*$^-$ *and leu*$^-$) containing 25 mM 3-AT.

Cloning of the full-length DIS3 gene

Using a cloned cDNA fragment as a probe, the *S.cerevisiae* genomic library, YCp50 (a gift from Dr K.Matsumoto), was screened and a 7.5 kb genomic fragment, YCp5072, was isolated. From the YCp5072 fragment, a 4.5 kb *Eco*RI fragment was cut out and inserted into the *Eco*RI site of pBluescript II TKS(+), resulting in pTKS72E (Table I).

Construction of GST-fused proteins

GST–Dis3. In order to engineer the *Nco*I site at the N-terminus of the *DIS3* ORF, 0.75 kb of the N-terminal fragment of *DIS3* carried on pTKS72E was amplified by PCR using a primer containing an *Nco*I site at the N-terminus. Amplified fragments were digested with *Nco*I and *Bgl*II and then ligated to the C-terminal 2.3 kb of the *Bgl*II–*Eco*RI fragment. The resulting 3.0 kb of the *Nco*I–*Eco*RI fragment was inserted to the *Nco*I–*Eco*RI site of pACT2 (a gift from Dr Elledge) and then, from the resulting recombinant plasmid, the *DIS3* ORF was cut out at the *Nco*I–*Xho*I site and inserted into the *Nco*I–*Xho*I site of pGEX-KG (Van Hoy *et al.*, 1993), resulting in pGEX-KG-DIS3.

GST–SRM1. The ORF of Srm1 of pSL785 (Clark and Sprague, 1989) was amplified and inserted into the *Sma*I site of pBluescript II SK (+). Then 1.5 kb of the *Nco*I–*Sal*I fragment of the resulting plasmid was inserted into the *Nco*I–*Sal*I site of pGEX-KG, resulting in pGEX-SRM1.

GST–CNR1. The ORF of Cnr1 of pBTCNR1-HB 4.1 (Kadowaki *et al.*, 1993) was amplified, digested with *Nco*I and *Hin*dIII, and then inserted into the *Nco*I–*Hin*dIII site of pGEX-KG, resulting in pGEX-CNR1.

The construction of GST-fused Yrb1 and Yrb2 is described elsewhere (E.Noguchi, in preparation).

Purification of Dis3sc, Cnr1 and Srm1

Escherichia coli BL21 (DE3)/pGEX-KG-DIS3 strains were cultured at 30°C up to an OD_{600} of 0.4, treated with IPTG (final concentration 0.2 mM) for 4 h and then collected by centrifugation. The cell pellet was washed with phosphate-buffered saline (PBS) and suspended in lysis buffer [40 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 2.5 mM $MgCl_2$, 10% glycerol, 0.25% Tween, 1 mM dithiothreitol (DTT), 1 mM *p*-amidinophenyl-methanesulfonyl fluoride (*p*APMSF)]. After sonication, the cell debris was spun down at 28 000 r.p.m. for 30 min. The collected supernatant was then added to glutathione–Sepharose 4B beads (Pharmacia) and rotated for 1 h. The beads were spun down, washed three times with lysis buffer, and then twice with thrombin cleavage buffer (25 mM Tris–HCl pH 7.5, 150 mM NaCl, 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.1% β-mercaptoethanol, 10% gylcerol). Washed beads were digested with thrombin (final 1% w/w GST–Dis3sc) for 2.0 h at 25°C, centrifuged and the supernatant was stored as Dis3sc solution. All procedures were carried out at 4°C except where indicated otherwise. The protein concentration was estimated on SDS–PAGE by staining with Coomassie brilliant blue.

Cnr1 and Srm1 were prepared using GST fusion proteins produced in *E.coli* similarly to Dis3sc.

Dis3sc, Cnr1 and Srm1 binding assay

Beads bound to GST-fused Dis3sc which were equilibrated with binding buffer A (20 mM HEPES-NaOH, pH 7.5, 120 mM NaCl, 20 mM $MgCl_2$, 0.05% Tween 20, 0.5% bovine serum albumin, 1 mM DTT, 1 mM *p*APMSF) or binding buffer B (the same as A except that 20 mM $MgCl_2$ was replaced with 2 mM EDTA) were mixed with Cnr1, Srm1 or Cnr1 plus Srm1, and incubated at 4°C for 1 h. After incubation, the beads were spun down and washed seven times with binding buffer A or B. Proteins bound to beads were separated by 12.5% SDS–PAGE, transferred to a PVDF membrane and analysed by immunoblotting using anti-GST, ant-Cnr1 and ant-Srm1 antibodies as a probe.

Ran binding assay

Ran overlay assay. GST-fused proteins were produced in *E.coli* strain BL21(DE3), bound to glutathione–Sepharose beads as described (Van Hoy *et al.*, 1993). Proteins bound to beads were electrophoresed in 10% SDS–PAGE, transferred to a PVDF membrane, and then renatured as described (Lounsbury *et al.*, 1994). Blots were rinsed with binding buffer alone and then overlaid with either [^{35}S]GTPγS- or [^{35}S]GDPβS-bound Ran for 30 min at room temperature as described (Yokoyama *et al.*, 1995). After washing with binding buffer, blots were dried and exposed to X-ray film.

Liquid binding assay. Beads bound to GST-fused proteins which were equilibrated with binding buffer A or binding buffer B, were mixed with either [^{35}S]GTPγS- or [^{35}S]GDPβS-bound Ran in the same buffer, and incubated at 4°C for 60 min. After incubation, beads were spun down, washed seven times with binding buffer and the radioactivity associated with beads was quantitated by scintillation counting. Proteins bound to beads were separated by 12.5% SDS–PAGE, transferred to a PVDF membrane and assayed for the presence of Ran by immunoblotting analysis using anti-Ran antibody as a probe.

Guanine nucleotide release assay

Human Ran and RCC1 were bacterially expressed and purified as described (Dasso *et al.*, 1994). Purified Ran (2 pmol) was mixed with 2 pmol of [^{3}H]GDP in buffer containing 25 mM Tris–HCl, pH 7.5, 50 mM NaCl, 5 mM EDTA, 1 mM DTT and 1 mM CHAPS. After incubation at 30°C for 30 min, 20 mM of $MgCl_2$ was added and the mixture was held on ice.

Two pmol of [^{3}H]GDP-Ran was mixed with Dis3sc in GEF buffer (25 mM Tris–HCl pH 7.5, 50 mM NaCl, 20 mM $MgCl_2$, 1 mM DTT, 1 mM CHAPS, 2 mM GDP). After incubation at 30°C for 3 min, 2 fmol of RCC1 was added to start the exchange reaction. The reaction was stopped by the addition of ice-cold stop buffer (20 mM Tris–HCl pH 7.5, 25 mM $MgCl_2$ and 100 mM NaCl), and the mixture was filtered through a nitrocellulose filter (0.45 μm, BA85, Schleicher & Schuell). The filters were dried and the radioactivity remaining with the proteins on the filter was counted in a liquid scintillation counter.

The steady-state kinetic analysis of the GEF activity of RCC1 in the presence of Ran and Dis3 with a 1:1 molar ratio was estimated as described by Azuma *et al.* (1996) using a fluorescent GDP analogue, 2′,3′-bis-*O*-(*N*-methylanthraniloyl)guanosine diphosphate (mantGDP).

Gel filtration chromatographic analysis of proteins

The wild-type *S.pombe* strain HM123 was cultivated in EMM medium (Samejima and Yanagida, 1994). Cells were spun down and broken in lysis buffer (50 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM DTT, 1 mM *p*APMSF). The crude extract was subjected to centrifugation at 45 000 r.p.m. for 30 min. The supernatant was collected and fractionated with Pharmacia Hiload Sephacryl S-300HR. Each fraction was assayed for the presence of Dis3, Spi1 and Pim1 by immunoblotting using the indicated antibodies as a probe. In the same buffer, the immunoprecipitation was carried out using the anti-Spi1 and the anti-Pim1 antibodies.

Immunoblotting

Proteins were loaded on SDS–PAGE, transferred onto PVDF membrane filters and then probed with the antibodies anti-Dis3sp (Kinoshita *et al.*, 1991), anti-Spi1 (X.He and S.Sazer, unpublished), anti-Pim1 (U.Mueller and S.Sazer, unpublished), anti-Cnr1 (prepared for us by Biologica Co.), anti-Srm1 (a gift from Dr K.Clark) and anti-Ran (Hayashi *et al.*, 1995) as described previously (Nishitani *et al.*, 1991).

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