A Family of Arf Effectors Defined as Suppressors of the Loss of Arf Function in the Yeast *Saccharomyces cerevisiae*

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Arf proteins are ubiquitous, eukaryotic regulators of virtually every step of vesicular membrane traffic. ADP-ribosylation factors are essential in yeast and the lethality resulting from either overexpression or underexpression (deletion) of Arf genes has previously been ascribed to dysregulation of the secretory process. We have identified a family of four genes (Suppressors of Arf (SAT) as high copy suppressors of a loss of function allele of ARF1 (arf1–2)). Those proteins with SAT activity were found to contain a minimal consensus motif, including a C2C2H2 cluster with a novel and specific spacing. Genetic interactions between members of this family and with ARF1 are consistent with each sharing a common cellular pathway. Included in this family is Gcs1, a protein previously described (Poon, P. P., Wang, X., Rotman, M., Huber, I., Cukierman, E., Cassel, D., Singer, R. A., and Johnston, G. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10074–10077) to possess Arf GTPase-activating protein (GAP) activity, demonstrating a direct interaction between Arf and at least one of these suppressors. The loss of the Arf function by overexpression of Gcs1 and demonstration of direct, preferential binding of Gcs1 to the activated form of Arf (Arf-GTP) lead us to conclude that the biological role of Gcs1 is as an effector of the essential function of Arf in mitotic growth, rather than a down-regulator as implied by the biochemical (Arf GAP) activity.

Suppression of the growth defect of arf1–3 cells was observed under conditions that did not alter the secretory defect associated with arf1– mutation, indicating that the essential role of Arf in eukaryotes can be distinguished from role(s) in the secretory pathway and appear to employ distinct pathways and effectors.

ADP-ribosylation factors (Arfs) are the family of monomeric, 21-kDa GTP-binding proteins originally identified as protein co-factors for cholera toxin-catalyzed ADP-ribosylation of Gs, the heterotrimeric G protein activator of adenyl cyclase (1, 2). Studies have implicated Arfs as regulators of a number of steps in the heterotrimeric G protein activator of adenylyl cyclase (1, 2). GTPase-activating protein; WT, wild type.

Gene expression (deletion) of Arf genes has previously been ascribed to dysregulation of the secretory process. We have identified a family of four genes (Suppressors of Arf (SAT) as high copy suppressors of a loss of function allele of ARF1 (arf1–2)). Those proteins with SAT activity were found to contain a minimal consensus motif, including a C2C2H2 cluster with a novel and specific spacing. Genetic interactions between members of this family and with ARF1 are consistent with each sharing a common cellular pathway. Included in this family is Gcs1, a protein previously described (Poon, P. P., Wang, X., Rotman, M., Huber, I., Cukierman, E., Cassel, D., Singer, R. A., and Johnston, G. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10074–10077) to possess Arf GTPase-activating protein (GAP) activity, demonstrating a direct interaction between Arf and at least one of these suppressors. The loss of the Arf function by overexpression of Gcs1 and demonstration of direct, preferential binding of Gcs1 to the activated form of Arf (Arf-GTP) lead us to conclude that the biological role of Gcs1 is as an effector of the essential function of Arf in mitotic growth, rather than a down-regulator as implied by the biochemical (Arf GAP) activity.

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1 The abbreviations used are: Arf(s), ADP-ribosylation factor(s); ER, endoplasmic reticulum; PLD, phospholipase D; PCR, polymerase chain reaction; bp, base pair(s); SGD, Saccharomyces genome data base; GAP, GTPase-activating protein; WT, wild type.

**MATERIALS AND METHODS**

**Yeast Culture**

Yeast were cultured using standard conditions, as described in Sherman et al. (12). Selective plates containing fluoride were prepared according to Stearns et al. (11). Yeast transformations were performed by thePEG/ LiCl method of Schiestl et al. (13).

**Mutagenesis**

**PCR Mutagenesis**—Random mutagenesis of the open reading frame of the ARF1 gene was achieved by PCR under conditions of reduced stringency, including 50 μM dATP and 0.1 mM M3Cl0, according to Leung et al. (14). Plasmids bearing the mutated ARF1 were produced in yeast by co-transfecting the mutant PCR products and gapped plasmid, pJCY1–31 (10), to allow gap repair by homologous recombination. The strain used, RT166, was deleted for both ARF1 and ARF2 and carried human Arf4 on another plasmid, under control by the GAL1 promoter. Conditional alleles of ARF1 were selected after replica plating transformants onto YEPD (1% yeast extract, 2% bacto-peatone, 2% glucose) plates (to turn off expression of human Arf4) and growing cells at 16, 28, and 37 °C. The arf1–3 mutation was introduced at the ARF1 locus by homologous recombination with replacement of the *arf1–3* allele.

**Site-directed Mutagenesis**—Specific mutations were introduced into plasmids using gene-specific primers encoding the desired changes with at least 18 bp of priming nucleotides in PCR reactions, as described in Kahn et al. (10). All PCR amplified fragments were completely se-
Gene Cloning and Deletion

A high copy, Arf-deleted, genomic library was constructed using genomic DNA (15) from RT166 (arf1 arf2 pGAL1-hArfl) that was partially digested with SmaI before size selection (3–20 kilobase pairs) and ligation into the BamHI site of YEp352, a high copy plasmid bearing the URA3-selectable marker.

The six yeast genes tested for SAT activity were amplified from genomic DNA by PCR using gene specific primers and cloned into the high copy (2 μ-containing, URA3-marked) plasmid, YEp352. The lengths of TERs and 3′-UTRs amplified varied with the distance to the adjacent genes. The length of 5′ upstream, open reading frame and 3′ downstream regions amplified were: SAT1 301/1190/880 bp; GCS1 243/1058/475 bp; GLO3 320/1481/125 bp; SPS18 162/902/201 bp; and GTS1 = 301/1190/880 bp.

The sat1::HIS3 and sat2::HIS3 deletions were generated by the method of Baudin et al. (16) after PCR amplification of the HIS3 gene using primers that encode 40 bp of gene-specific sequence from each end of the open reading frames. Auxotrophic markers were then “swapped” as described in Cross (27) to allow the ready isolation of double and triple deletants. Each deletion allele and swapped marker was confirmed by PCR.

Invertase Assay

The assay for processing of invertase was performed as described in Stearns et al. (9). Cells were grown in medium containing a high concentration (5%) glucose to log phase before being collected by centrifugation, washed, and resuspended into YEPD containing 0.1% dextrose. Half of the culture was then incubated at 30 °C and the other half at 37 °C. After 3 h, cells (2 A600 units) were collected, rinsed in 25 mM Tris-Cl, pH 7.4, 10 mM NaN3, 1 mM phenylmethylsulfonyl fluoride, and lysed by agitation with glass beads in Laemmli’s sample buffer before boiling for 5 min. Proteins were fractionated on 7.5% polyacrylamide-SDS gels before transfer to nitrocellulose membranes. The primary antibody used was a guinea pig anti-invertase polyclonal antiserum (the generous gift of D. Preuss), and the secondary antibody was goat anti-guinea pig IgG coupled to peroxidase. Detection of signal was accomplished with the enhanced chemiluminescence kit (ECL) from Amersham Pharmacia Biotech.

RESULTS AND DISCUSSION

The arf1–3 Allele Is ts—A temperature sensitive (ts) allele of ARF1, termed arf1–3, was generated by chemical mutagenesis of plasmids bearing the ARF1 gene and selection for sensitivity to growth at 37 °C. arf1–3 is a conditional loss of function mutation that is recessive to both ARF1 and ARF2. Sequencing of the open reading frame of arf1–3 revealed a single base pair mutation resulting in a mis-sense mutation, [T32I]Arf1. This residue is just downstream of the first consensus GTP binding domain, G24LDGAGK30, and immediately adjacent to Thr31, whose mutation results in a negative dominant phenotype (17). Thr32 lies in the nucleotide binding pocket of Arf1-GDP (18, 28 Å from an α-phosphate oxygen such that the introduction of the larger side chain of an isoleucine is predicted to disturb the nucleotide binding. Quantitative immunoblotting revealed only minor differences in the level of Arf1–3 versus Arf1, which were not affected by growth at the restrictive temperature. Thus, altered stability of the mutant protein does not explain the phenotype.

Cloning a Suppressor of arf1–3—A yeast genomic 2 μ library was screened for high-copy suppressors of arf1–3 at 37 °C. From 300,000 colonies screened, we obtained nine temperature-resistant (ts) colonies, all of which grew at near wild type levels but reverted to ts with loss of the library plasmid. Sequencing the inserts and comparison with the Saccharomyces Genome database (SGD) revealed the presence of a single, complete open reading frame present in all nine plasmids, labeled YDR524C in the SGD, and named suppressor of Arf1 ts or SAT1.2 Suppressor activity of SAT1 was confirmed with a high copy (2 μ) plasmid bearing the entire gene, obtained by PCR amplification of a 2.3-kilobase pairs genomic fragment, including the open reading frame of 1446 and 404 bp and 427 bp upstream and downstream, respectively (see Fig. 1). The same genomic fragment failed to complement arf1–3 when present on a low copy (CEN) plasmid.

Suppression by SAT1 was allele-specific as the growth rates at restrictive temperatures of strains carrying other Arf alleles, including five other ts alleles and one cold sensitive (cs) allele carrying a variety of other mutations, did not improve when bearing the 2-μ plasmid (not shown). Similarly, additional copies of SAT1 were unable to complement the arf1–3:HIS3 gene deletion, with regard to slower growth at several temperatures or supersensitivities to fluoride (not shown). arf1–2 is ts for growth on plates containing fluoride (40 μM), and growth under these conditions was not restored by SAT1. Interestingly, the decreased Arf activity in the arf1–3 cells resulted in fluoride sensitivity, which could be suppressed with overexpression of SAT1 (Fig. 1). Thus, growth defects of arf1–3 cells resulting from the stress of either elevated temperatures or fluoride were suppressed by SAT1 at high copy (Fig. 1). In addition to thermal and fluoride sensitivities, cells carrying ARF mutations, including arf1–3, often are defective in sporulation and/or growth on nonfermentable carbon sources. Overexpression of SAT1 was able to restore the diminished growth of arf1–3 cells on glycerol plates to near that of wild type cells, though it was unable to restore sporulation competence.

Sat1 Contains a Cysteine Repeat (Zinc Finger) Motif—SAT1 encodes a protein 482 residues in length, with a predicted molecular mass of 54 kDa. The DNA sequence of the open

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As this appears to be the first functional description for this gene we propose the name SAT1 for YDR524C and SAT2 for YIL044C.
reading frame of one of our genomic library inserts was identical to the SGD entry for YDR524C. BLAST analyses of Sat1 against the SGD revealed weak homology between Sat1 and a group of yeast proteins with a cysteine-rich zinc finger motif, one of which (GCS1) has been reported to possess Arf GTPase-activating protein (GAP) activity (19). The homologies between Sat1 and the members of this group, including Gcs1, Glo3, YIL044C, Gts1, and Sps18, are limited to the N-terminal, cysteine-rich, zinc finger domain, abbreviated as C2C2 (see Fig. 3). This is the region identified by Ireland et al. (20) in Gcs1, Glo3, and Sps18 and implicated in Arf GAP activity (19). The cysteine-rich domain of Sat1 is removed from the N terminus, by about 160 residues, with respect to the others. A similar degree of homology, again limited to the zinc finger domain, was observed in a number of proteins from other species, including a-centaurin and a mammalian Arf GAP, described by Cassel and colleagues (19, 21). We next explored the possibility that other members of the group of yeast proteins with the cysteine-rich motif possess effector activity for Arf1.

Each of the five other members of this group of proteins was tested for the ability to rescue growth of arf1–3 cells at 37 °C when present on high-copy plasmids. Three of these genes, GCS1, GLO3, and YIL044C, were found to have suppressor (SAT) activity (see Fig. 1). As this is the first description of a function for YIL044C, we propose to name it SAT2. Of this group, only SPS18 and GTS1 (Fig. 1) were inactive as suppressors of arf1–3. The fact that at least one protein (Gcs1) in this family interacts directly with Arf proteins (in the Arf GAP assay) is consistent with the notion that these are immediate downstream effectors of the essential Arf action in mitotic growth. Examination of the protein alignments, shown in Fig. 3, revealed that Gts1 lacks one and Sps18 lacks both of the histidine residues, downstream of the cysteine that are conserved in each of the proteins with SAT function. As histidine residues are often found to substitute for cysteines in various zinc finger motifs, we investigated the importance of these residues in defining a Sat motif.

The C2C2H2 Motif Is a Minimal Domain Required for Sat Activity—To test the importance of cysteine and histidine residues to Sat activity, mutants of the first and second cysteines and the two histidines were constructed in Sat1 and tested for suppressor activity. Mutation of the second cysteine in the motif in gcc1 (termed gcc1-1) to tyrosine results in a c− failure to re-enter the cell cycle phenotype, also observed with deletion of GCS1 (20, 22). As seen in Fig. 2, the [C186A]Sat1 or [C189Y]Sat1 mutants have lost SAT activity. Similarly, mutation of both of the histidines to residues present in Sps18 ([H214L,H220N]Sat1) also caused the loss of SAT activity. Thus, we propose a minimal consensus sequence for SAT activity to include the histidines, yielding the motif: C-X2-C-X16–17–C-X2–X3–X5–X7–H-X8–H. No other proteins containing this exact motif were found in either the yeast or Swiss-Prot data bases, although 22 others were found in SGD to contain the four cysteines with this spacing. Multiple human expressed sequence tags (ESTs) from distinct genes were found to contain the Sat consensus (C2C2H2) and include more extensive homology to Sat1 in the surrounding region than is present in the yeast family members. These human proteins are currently being tested for SAT activity.

Because of previous work documenting the importance of cysteine/histidine containing motifs in protein-DNA and protein-protein interactions, we focused initially on these residues and emphasize that we consider this a minimal SAT motif. As seen in the alignment shown in Fig. 3, other residues flanking or internal to the zinc finger motif are also conserved in this region and need to be evaluated for their importance to SAT activity. That the proposed SAT motif is not sufficient for SAT activity is evident from the finding that mutation of the two residues in Sps18 that align with the histidines in Sat1 to histidines ([L55H,N61H]Sat1) did not confer SAT activity to the mutant Sps18.

Genetic Interactions between the SAT Proteins and with arf1—A null allele of SAT1 was constructed in both haploid and diploid cells by replacement of the open reading frame of SAT1 with the HIS3 gene, as described under “Materials and Methods.” SAT1 is not an essential gene, as haploid strains of each mating type, carrying the disrupted gene, were viable. This was confirmed by dissection of spores, in which HIS3+ (sat1−) segregated 2:2 and all four spores were viable. Haploid strains deleted for SAT1 were found to grow at the same rates as wild type strains at 16, 28, or 37 °C and grew well on nonfermentable carbon sources, e.g. glycerol (not shown). Diploid cells homozygous for the deletion also appeared normal and sporulated as well as controls.

As Arfs are essential, and Sat1 overexpression compensates for the loss of Arf function associated with arf1–3, we originally predicted that SAT1 would also be an essential gene. The ability of three other genes to complement the arf1–3 allele may explain why sat1− strains are viable. Indeed, genetic redundancy in Arf effectors could have been predicted from the size of the Arf family in yeast (two genes) and mammals (six genes) and from the precedent of multiple effectors established for other small GTPases.

Tests for genetic interactions among the four genes with SAT activity were performed by combining null alleles of each, in all possible combinations. Initial tests for viability and thermal sensitivities revealed a number of interactions. Strains carrying either sat1 or sat2 deletions grew as well as parental controls at all temperatures (16, 28, and 37 °C), but deletion of either gcc1 or glo3 led to conditional thermal sensitivities (gecs1− = c−, glo3− = t+ and c+) and defects in cell cycling (20, 22,
Combinations of gcs1– with deletions of any of the other SAT genes revealed two types of genetic interactions. Two of the combinations of double deletions, gcs1 glo3 or gcs1 sat2, exhibited synthetic lethality. The double deletion gcs1 sat1 was weakly ts, in addition to the c– phenotype from the gcs1–.

Another phenotype that has been used in characterizing ARF1 mutations is fluoride supersensitivity (10, 11). As noted above, overexpression of Sat1 rescued arf1–3 cells from toxicity of 30 mM fluoride. The double delete strain, sat2– glo3–, was also found to be supersensitive to fluoride. The triple delete strain, sat1– sat2– glo3–, was viable, though it was c–, ts–, and fluoride-supersensitive. As first reported in Poon et al. (19), we also found that strains carrying the gcs1 null allele were supersensitive to fluoride. Thus, loss of Gcs1 function results in the same phenotype found for loss of Arf1 function; consistent with the two proteins acting in a common pathway but not in an antagonistic fashion. That is, loss of Gcs1 and its GAP activity should result in increased Arf activity in cells, not less, and thus the fluoride supersensitivity of gcs1– cells appears incompatible with its GAP activity being biologically relevant in this context.

Whereas no phenotype was ascribed to sat1– alone, these cells became much more ts when this deletion was combined with arf1–3. In contrast, the double deletion, sat1– arf1–, made cells neither more c– nor more fluoride-supersensitive than arf1–3 alone (not shown). We conclude that arf1–3 retains some, though clearly diminished, Arf activity, which is compromised further with loss of Sat1. The rank order potency for high-copy suppression of ts in arf1–3 cells or arf1–3 sat1– cells was Sat1 > Glo3 > Sat2 > Gcs1. Sps18 and Gts1 were inactive in each assay.

Separation of Secretory and Mitotic Growth Defects—The sat1– deletion alone had no obvious effect on the secretory pathway, as visualized by the processing of invertase during its transit of the secretory pathway, at either 30 or 37 °C (Fig. 4; WT versus sat1–1). In contrast, the deletion of ARF1 has previously been shown to cause a partial defect in invertase glycosylation, consistent with a defect in transit through the Golgi (Ref. 9; Fig. 4, arf1 versus WT). Cells carrying the arf1–3 allele processed invertase to the same (incomplete) level at permissive and restrictive temperatures, comparable with that of arf1– cells (see Fig. 4; compare arf1 to arf1–3). This defect was unaffected by the introduction of SAT1 on high copy plasmids at either temperature (arf1–3 SAT1). Thus, under restrictive conditions (37 °C) that result in little or no cell growth (arf1–3) or near WT growth rates (arf1–3 + SAT1), there is no discernible difference in the ability to process invertase through the Golgi. The sec18–1 strain is shown as a control that shows the ER form (core glycosylated) of invertase as SEC18 encodes the yeast homolog of the NSF protein, required for exit from the ER. Although we cannot completely exclude the possibility that it is Arf acting at other membrane traffic sites (e.g. endocytosis) that is required for cell growth, we consider this unlikely because the phenotypes observed previously with depletion of Arfs were most severe on the early secretory pathway and readily observed with this same invertase assay (9). Thus, we interpret these data as indicating an essential role for Arf proteins in cell growth that can be separated from its role in membrane transport and that Sat1 and related proteins are effectors of this essential Arf function.

The finding of effector functions for proteins with defined GAP activity has preceded in other systems. In at least two cases (Gna and phospholipase C-β and phosphodiesterase γ subunits and transducin) G protein effectors have strong GAP activity (24, 25). The situation with Ras and its GAPs is a bit confusing, at least in part because Ras GAP and Ras effectors have overlapping binding sites on the GTPase, but the expression of GAP suppresses cell transformation by Ras, and it thus can work as a negative regulator (e.g. Ref. 26). The identification of a GAP activity is a useful biochemical tool to identify GTPase-interacting proteins, but the name implies the down-regulation of the GTPase activity, which is clearly not the physiologically relevant activity of the Arf GAP in this case. The identification of SAT activity is a step toward making this biologically important distinction. Further genetic and biochemical tests of the genes/proteins in the SAT family will likely reveal higher orders of specificity and overlapping as well as distinct signaling roles in the control of the cell cycle and secretory pathways.

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A Family of Arf Effectors 19795

FIG. 4. Defects in the processing of invertase in its transit through the Golgi are associated with Arf mutations but are not corrected by overexpression of Sat1. The varying degrees of glycosylation of the induced form of invertase were monitored at permissive (30 °C) and restrictive (37 °C) temperatures by immunoblotting of total yeast cell extracts with a guinea pig anti-invertase polyclonal antiserum, as described under “Materials and Methods.” The strains used include the parental PSY315 (WT; ARF1 ARF2; Ref. 11), T704 (arf1; arf1::HIS3 ARF2; Ref. 11), YZC133 (sat1; sat1::HIS3), YZC214 (arf1 sat1; arf1::URA3 sat1::HIS3), RT364 (arf1–3; arf1–3 arf2– ), YZC190 (arf1–3 + SAT1; RT364 transformed with YEp352 carrying SAT1), and CKY58 (sec18–1; sec18–1; Ref. 9). Only the relevant genotypes are specified. No differences were observed when invertase processing was compared in ARF1ARF2 and ARF1arf2 cells (not shown), so only the true parental control is shown (WT). The two lower bands shown on the immunoblot represent the noninduced forms of invertase. This experiment was repeated several times with similar results, though differences in the intensity of signal and the broadness of the invertase signal were seen to vary between experiments.
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