Fission yeast genes that confer resistance to staurosporine encode an AP-1-like transcription factor and a protein kinase related to the mammalian ERK1/MAP2 and budding yeast FUS3 and KSS1 kinases

Takashi Toda, Mizuki Shimanuki, and Mitsuhiro Yanagida
Department of Biophysics, Faculty of Science, Kyoto University, Kitashirakawa-Oiwake, Sakyo-ku, Kyoto 606, Japan

Staurosporine, a potent inhibitor of protein kinase C, arrests fission yeast cell elongation specifically at a stage immediately after cell division. We isolated two genes, which, when carried on multicopy plasmids, confer drug resistance in fission yeast. One, \( \text{spkl}^* \), encodes a protein kinase highly similar (54% identity) to those encoded by the mammalian ERK1/MAP2 kinase and the budding yeast KSS1 and FUS3 genes. It is not essential for vegetative growth of \( \text{Schizosaccharomyces pombe} \) cells but is required for conjugation. The \( \text{spkl}^* \) gene product is a 45-kD protein enriched in the nucleus, and its level increases 10-fold after addition of staurosporine. The other gene \( \text{papl}^* \) encodes an AP-1-like transcription factor that contains a region rich in basic amino acids followed by a ‘leucine zipper’ motif. The \( \text{papl}^* \) gene is required for \( \text{spkl}^* \)-conferred staurosporine resistance. These two genes appear to function as a part of the fission yeast growth control pathway.

[Key Words: Fission yeast; staurosporine; inhibitor of protein kinase C; AP-1-like factor; protein kinase; growth control]

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In the rapidly dividing cells of the early embryo, the cell cycle consists of basically only S and M phases, G1 and G2 being absent. These cells have little time to grow so that their size is reduced by the cycles of division. On the other hand, somatic cells grow during interphase, and after reaching certain critical cell volume, enter mitosis and divide. Therefore, in eukaryotic growth control, two classes of switches for cell division may exist, namely, entry into the growing stage and exit from that stage.

Mitogens trigger resting, quiescent cells to enter the cell division cycle, through complex networks involving protein phosphorylation/dephosphorylation [for review, see Edelman et al. 1987; Cohen 1989]. Several oncogenes are known to encode tyrosine- or serine/threonine-specific protein kinases [for review, see Bishop 1985]. Tumor promoters such as TPA, an activator of PKC [Nishizuka 1986], and some mitogens exert their biological effect by activating transcription of a series of genes, known as TPA- or serum-inducible genes. The nuclear oncogenes \( \text{c-jun} \) and \( \text{c-fos} \) are two such genes and form a heterodimer complex with each other, referred to as AP-1 [Angel et al. 1987, 1988; Bohmann et al. 1987]. The link between the protein phosphorylation cascade and the activation of nuclear transcription factors, however, remains unclear.

There are two distinct stages for growth and division in the cell cycle of the fission yeast \( \text{Schizosaccharomyces pombe} \) [Mitchison 1970]. The rod-like cells extend continuously for the three-quarters of the cell division cycle [Fig. 1D]. In the following constant cell-length stage, mitosis, septation, DNA synthesis, and cytokinesis occur [the G1 phase is negligible in exponentially growing wild-type yeast in rich media]. Growth [i.e., cell elongation] apparently ceases at the constant cell-length stage, the growing stage of wild-type \( \text{S. pombe} \) cells is restricted to the G2 phase. Thus, after the completion of cell division the early G2 cells begin to grow immediately.

The \( \text{S. pombe} \) genes involved in the exit from G2 and the entry into M phase have been well documented in recent years and shown to be conserved in higher eukaryotes. They include the \( \text{cdc2}^+ \) protein kinase [a component of M phase-promoting factor [MPF]], \( \text{cdc13}^+ \) [a cyclin homolog], and \( \text{suc1}^+ \ p13 \). The products of \( \text{cdc25}^+ \) [a mitotic inducer], \( \text{wee1}^+ \) [a protein kinase, putative mitotic inhibitor], and \( \text{nim1}^+ \) [a protein kinase, putative mitotic inducer, for review, see Nurse 1990] genes may also control the timing of entry into mitosis. The entry

\(^1\)Corresponding author.
into the M phase is blocked in cdc2, cdc13, and cdc25 mutants but cell elongation is not. In contrast, none of the genes involved in the initiation of cell elongation after cell division have been identified in this organism. No mutant has been isolated that exhibits an arrest phenotype specifically at the entry into the cell-elongation phase. Either such specific controlling genes do not exist or they may be functionally redundant, mutations in them not resulting in the arrest phenotype.

Staurosporine, an alkaloid compound produced in Streptomyces stauroporosus and originally found as an antifungal agent, has been shown to possess a very strong cytotoxicity when applied to some mammalian tumor cell lines [Omura et al. 1977; Tamaoki et al. 1986]. It has been shown that the drug is one of the most potent inhibitors for PKC in vitro [IC₅₀ = 2.7 nM, Tamaoki et al. 1986]. The drug also inhibits other protein kinases, including cyclic nucleotide-dependent protein kinases (protein kinases A and C) and src tyrosine-specific protein kinase, although the extent of inhibition for those kinases is severalfold lower than that for PKC [Nakano et al. 1987].

Recently the isolation of a PKC-like gene from the budding yeast Saccharomyces cerevisiae was reported [Levin et al. 1990], as was the purification of a PKC-like activity from that yeast [Ogita et al. 1990]. We have undertaken the isolation of mutants that show an altered sensitivity to staurosporine, expecting that some of them would be derived from PKC or other kinase gene homologs [Toda and Yanagida et al. 1988]. In this paper we will demonstrate that a low concentration of staurosporine arrests S. pombe cells at a specific cell-cycle stage immediately after cytokinesis. Subsequently, we have identified 11 genetic loci that display hypersensitivity to the drug. Using these mutants, we have obtained two genes that, when carried on a multicopy plasmid, confer drug resistance even to the wild-type cells. Nucleotide sequence determination and biochemical analysis indicate that one gene codes for a novel protein kinase and the other gene, surprisingly, encodes an AP-1-like transcription factor. The possible implications of these findings are discussed.

Results

Staurosporine arrests growth of S. pombe

To examine the effect of staurosporine on the growth of the fission yeast S. pombe, the drug (0.5 μg/ml) was added to an exponentially growing asynchronous culture. Most cells divided once and then arrested. Hence, the average cell length in the culture was strikingly reduced from 10.1 μm at 0 hr to 7.6 μm at 3 hr after addition of the drug [Fig. 1A]. Consistently, the percentage of septated cells decreased from 13% to 1%. Prolonged incubation caused cells to become fragile and lyse. Drug-treated cells lost viability [the percentage of viable cells was 14% and 4.7% after 3 and 6 hr, respectively]. In contrast, stationary-phase cells were insensitive to the drug (viability was 100% in a drug concentration of 1 μg/ml).

These results suggested that staurosporine inhibited cell elongation in actively growing cells.

To determine whether small cells immediately after cell division are specifically sensitive to the drug, synchronous culture analysis [Mitchison 1988] was done in the presence of the drug. Growing wild-type cells were fractionated by centrifugal elutriation, and the small, selected G₂ cells were regrown in a rich YPD medium in the presence of the drug (Fig. 1B). The cell number and size were monitored for the following 3 hr. Cell elongation and division did not take place; the average cell lengths were 7.1 and 7.6 μm after 0 hr and 3 hr, respectively [Fig. 1B]. In contrast, if the drug was added 60 min after the generation of synchrony when the average cell length had reached 10.4 μm, cells divided once within the following 80 min and then remained as small cells (average cell length, 7.4 μm) as shown in Figure 1C. As illustrated in Figure 1D, effect of staurosporine is dependent on the cell-cycle stage; cells immediately after division seem to become sensitive to the drug.

Genetic loci and genomic DNAs that affect staurosporine sensitivity

Wild-type S. pombe cells are capable of forming colonies on a solid medium containing 0.3 μg/ml of staurosporine. We used this criteria to isolate S. pombe mutants that are supersensitive to staurosporine, by replica-plating ~200,000 mutagenized colonies and selecting for mutants that were unable to grow on the plates containing 0.2 μg/ml staurosporine. Eighteen strains (ss1–ss18; see Materials and methods) were obtained and crossed with a mating strain, and the drug sensitivity of heterozygous diploids was examined. All of the mutants were recessive. Complementation tests by pairwise crosses showed that these mutants defined 11 loci [Table 1; designated sts1–sts11 for staurosporine supersensitive], indicating that staurosporine sensitivity is genetically complex.

During the course of isolating genes that suppress the sts mutations, we obtained two plasmids (pST1 and pST23) that suppress the drug supersensitivity of all the sts mutations except sts1 [Table 1; for details, see Materials and methods]. Furthermore, these two plasmids even conferred drug resistance to wild-type cells. Transformants containing these plasmids produce colonies in the presence of 1.0–1.5 μg/ml drug. pST23 confers resistance more effectively than pST1 [Table 1].

pST23 encodes a novel protein kinase

Determination of the nucleotide sequence of the 4.0-kb SalI fragment in pST23 [Fig. 2A] indicated one long open reading frame (ORF) that potentially encodes a polypeptide of 372 amino acids [Fig. 2B; see Materials and methods]. The predicted amino acid sequence contains consensus sequences of known protein kinases, as shown in Figure 2B for the ATP-binding site (●) and for the invariant kinase consensus (○) [Hanks et al. 1988]. A computer search for homologous proteins showed...
that this putative protein kinase is homologous to a number of protein kinases, with the highest homology (54% identity) to the products of the S. cerevisiae KSS1 and FUS3 genes that are proposed to be involved in the growth signal transduction pathway [Courchesne et al. 1989; Elion et al. 1990] and to a lesser extent (37%) to the human and yeast cdc2+/CDC28 protein kinases [Lee and Nurse 1987]. We designated the gene for this novel protein kinase spk1+ [staurosporine-related protein kinase 1].

Very recently, the mammalian ERK1 or MAP2 kinase was cloned and reported to be homologous to KSS1 and FUS3 (Boulton et al. 1990). spk1+ is also very homologous (54%) to ERK1. spk1+ seems to be equally homologous to ERK1, KSS1, or FUS3, the amino acid identity between any combination of the four proteins is 54%.

There are two regions where the amino acid sequences of ERK1, KSS1, and FUS3 are quite divergent; namely a short stretch located between subdomains VII and VIII, and the carboxy-terminal 50-amino acid residues (Fig. 2C; Boulton et al. 1990; Elion et al. 1990). The amino acid sequence of spk1+ in these regions is also very different from that of the other proteins, although the carboxy-terminal 20 amino acids of spk1+ have some weak homology with those of ERK1 and FUS3 (Fig. 2C). Note that these two divergent regions do not have any homology with those of cdc2+/CDC28, either. The remaining regions are very homologous among spk1+, ERK1, KSS1, and FUS3.

The spk1+ gene is nonessential but required for conjugation

To determine whether spk1+ is an essential gene, one-step gene disruption [Rothstein 1983] was done. A Ura− diploid was constructed and transformed with the disrupted spk1:: urad4+ sequence (Materials and methods). The transformant was sporulated in a poor medium, and tetrad analysis was carried out. Four viable spores were obtained (Ura+/Ura−, segregated 2:2), indicating that the spk1+ gene is nonessential. As confirmation, Southern hybridization of genomic DNAs of each of the four tetrad segregants was done, and Ura+ segregants showed the expected disruption pattern [data not shown]. The disrupted cells grew apparently normally and showed little difference in staurosporine sensitivity in comparison with wild-type cells (Table 1). However,
papl-staurpophine sensitivity was determined by streaking each transformant on YPD plates containing 0.2-2.0 μg/ml of the drug and cells for each transformation, respectively. The relative cells. (NT) Not tested.

Wild-type/ H123, TP106-3C, and TP108-3C were used as wild-type, spkl-, stsl, stslO, stsl9, stsl8, stsl7, stsl6, stsl5, stsl4, stsl3, stsl2, stsl1, and stsl0 genes can not confer staurosporine resistance in cells. We have never observed any diploids or spkl- those transformants which, after 3 days incubation at 33°C, formed normal-sized colonies were judged to be growing (represented by and those that did not form colonies were described as no growth (-)). Note that the plasmid pST23 that carries the spkl- gene product is a nuclear kinase

The spkl+ gene product is a nuclear kinase

We raised antiserum against fused spkl+ protein [Materials and methods] and identified the product of spkl+ gene in the S. pombe extracts by immunoprecipitation (Fig. 3A). A protein band of 45 kD was detected by immunoblotting only when using concentrated affinity-purified anti-spkl antibodies [lane 3] and not with diluted serum [lane 2] nor preimmune serum [lane 1]. When the ADH [alcohol dehydrogenase] promoter [Russell and Hall 1983] was connected with the coding sequence of the spkl+ gene to overproduce the protein [Materials and methods], we found >10-fold overproduction of a protein band of 43 kD [lanes 5 and 6]. An In vitro protein kinase assay was performed with anti-spkl immunoprecipitates using histone H1 as a substrate. After incubation with [γ-32P]ATP, reaction mixtures were run in SDS-PAGE. We found that histone H1 was specifically phosphorylated in immune complexes prepared from wild-type cell extracts [Fig. 3B, lanes 1 and 2] but not at all in those from spkl- [lanes 3 and 4]. No endogenous proteins were phosphorylated even after prolonged exposure [data not shown]. This result clearly demonstrates that the spkl+ gene product has a protein kinase activity for histone H1 as predicted from its nucleotide sequence. We tested casein as an alternate substrate. Although immunoprecipitates prepared from either wild-type or spkl- extracts seemed to contain a weak casein kinase activity, little difference, if any, was detected between the two extracts [lanes 5 and 6].

To determine the cellular localization of the spkl+ protein, indirect immunofluorescence microscopy was performed using affinity-purified anti-spkl antibodies. As shown in Figure 4, A and B, in cells carrying ADH promoter expression of the spkl+ gene, immunofluorescence was observed primarily within the nucleus and occasionally in the unidentified organelles other than nuclei. On the other hand, when cells carrying only vector plasmids were used, immunofluorescence was very faint, presumably due to the low level of the spkl+ protein [data not shown]. Fractionation of cellular components of S. pombe homogenates of cells containing a single-copy chromosomal spkl+ gene by Percoll gradient centrifugation [Hirano et al. 1988] indicated that

Table 1. Growth of transformants containing multicopy plasmid in the presence of staurosporine

| Loci number | Number of alleles | Drug concentration (μg/ml) | Plasmids |
|-------------|-------------------|---------------------------|----------|
|             |                   | pDB248 [vector] | pST1 [papl+] | pST3 [spkl+] |
| Wild-type/spkl+ | 0.2 | + | + | + |
|             | 0.3 | + | + | + |
|             | 0.5 | - | + | + |
|             | 1.0 | - | +/- | + |
|             | 1.5 | - | - | +/- |
|             | 2.0 | - | - | - |
| pap1-       | 0.2 | - | + | - |
|             | 0.3 | - | + | - |
|             | 0.5 | - | + | - |
|             | 1.0 | - | +/- | - |
|             | 1.5 | - | - | - |
|             | 2.0 | - | - | - |
| sts1        | 5 | 0.2 | - | - | - |
| sts2        | 4 | 0.2 | - | + | + |
| sts3        | 3 | 0.2 | - | + | + |
| sts4        | 1 | 0.2 | - | + | + |
| sts5        | 1 | 0.2 | - | + | + |
| sts6        | 1 | 0.2 | - | + | + |
| sts7        | 1 | 0.2 | - | NT | + |
| sts8        | 1 | 0.2 | - | + | + |
| sts9        | 1 | 0.2 | - | + | +/- |
| sts10       | 1 | 0.2 | - | + | NT |
| sts11       | 1 | 0.2 | - | + | + |

HM123, TP106-3C, and TP108-3C were used as wild-type, spkl+, and pap1- cells for each transformation, respectively. The relative staurosporine sensitivity was determined by streaking each transformant on YPD plates containing 0.2-2.0 μg/ml of the drug and those transformants which, after 3 days incubation at 33°C, formed normal-sized colonies were judged to be growing [represented by +] and small colonies [-/-] and those that did not form colonies were described as no growth [-]. Note that the plasmid pST23 that carries the spkl+ gene can not confer staurosporine resistance in pap1- cells. [NT] Not tested.
the spkl+ protein was broadly distributed throughout the fractions but preferentially accumulated in the nuclear fractions [data not shown].

Staurosporine induces synthesis of the spkl+ protein

We addressed the issue of whether the addition of staurosporine affected the levels of the spkl+ gene product in S. pombe cells. If the spkl+ protein kinase is directly or indirectly related to staurosporine inhibition, a feedback mechanism might exist to counteract the drug and thereby induce overproduction of the spkl+ kinase. Wild-type cells were grown exponentially and transferred to medium containing 0.5 μg/ml staurosporine. Aliquots of the culture were taken at intervals, and their extracts were analyzed by SDS-PAGE and immunoblotting using affinity-purified anti-spkl antibody.

As shown in Figure 3C, it was evident that the level of the spkl+ protein was increased by the drug; induction became clear 80 min after drug addition. Quantitative measurement of the amount of the protein showed that the spkl+ protein was induced ~10-fold 120 min after drug addition. Induction does not seem merely to be a consequence of growth arrest caused by the drug because the induction can be seen at levels as low as 0.3 μg/ml staurosporine [Fig. 3D], the level at which cells apparently grow and divide normally. Not all protein kinases are induced by the drug; for example, the level of the cdc2+ kinase does not change at all during drug treatment [data not shown].

Nucleotide and predicted amino acid sequences of papl+ gene

By subcloning pST1, a minimal complementing sequence was restricted to a 2.5-kb HindIII fragment [Fig. 5A], and its nucleotide sequence was determined by the dideoxy method [Sanger et al. 1977]. We found one ORF that encodes a protein of 544 amino acids beginning from a putative initiator methionine [Fig. 5B].

Analysis of the predicted amino acid sequence of pST1 indicated that it is likely to be a transcription factor similar to c-jun and c-fos. As shown in Figure 5B, the deduced sequence contains a “leucine zipper motif” in which leucine residues appear at every seventh position with the exception of threonine, which appears at the third position. A basic domain adjacent to the leucine repeat similar to that found in c-jun, c-fos, and GCN4 [Landschulz et al. 1988] is also present in the pST1 sequence. The homology between the basic regions of these proteins is ~40% if conservative replacements of amino acids are considered to be the same [Fig. 5D].

The basic region of pST1 particularly resembles that found in the recently identified S. cerevisiae YAP1 protein [a yeast AP-1-like gene; Harshman et al. 1988; Moye-Rowley et al. 1989], 19 of 29 amino acids in the basic regions are identical [Fig. 5C,D]. Note that the third position of the leucine repeat in YAP1 is also not leucine, but asparagine. Furthermore, comparison of the two sequences shows that there is one additional homologous region, the carboxy-terminal 50-residue-long stretch [Fig. 5C] in which 50% homology is observed [25 of 50 amino acids are identical; Fig. 5E]. It is worth noting, however, that the carboxy-terminal region is not essential to confer drug resistance; a truncated papl+ protein lacking the carboxyl domain produced drug resistance [Fig. 5A]. We designated the gene papl+ [pombe AP-1-like gene 1]. Jones and co-workers [1988] had previously identified an AP-1-like activity in fission yeast nuclear extract. We determined whether the isolated papl+ product behaved as an AP-1-like protein.

Specific binding of papl+ to AP-1 site

Approximately 80% of the papl+ coding sequence was expressed in Escherichia coli using a T7 RNA polymerase-dependent promoter [kindly provided by F.W. Studier [Studier and Moffatt 1986], see Materials and methods]. Note that when the same portion of papl+ was expressed in S. pombe using the strong ADH promoter [Russell and Hall 1983], it conferred drug resistance [Fig. 5A]. Using gel-purified fusion proteins, we examined the in vitro interaction between a DNA sequence containing an AP-1 binding site [Angel et al. 1987] and the papl+ gene product. We made oligonucleotides that contain an AP-1-binding site of human collagenase and SV40 early promoter [called Col-AP-1 and SV-AP-1, respectively; Jones et al. 1988]. We also made two other oligonucleotides [Col-mut1 and Col-mut2] that had point mutations in essential nucleotides of Col-AP-1 [Fig. 6A; Angel et al. 1987].

Gel retardation assays were done as described in Materials and methods. The papl+ protein was effectively bound to Col-AP-1 when it was used as a labeled probe [Fig. 6B, lane 2]. It binds similarly to SV-AP-1 [data not shown]. Binding of these labeled probes was entirely competed out by the addition of a 400-molar excess of either cold Col-AP-1 or SV-AP-1 [lanes 3 and 4]. In contrast, mutated AP-1 oligonucleotides [Col-mut1 or Col-mut2] were totally ineffective in competing with the intact AP-1 oligonucleotides [lanes 5 and 6]. A DNase I footprinting experiment using the SV40 promoter enhancer region as a probe confirmed that binding of the papl+ protein to these sequences was specific to the AP-1 site [Fig. 6C]. These biochemical characters are in exact parallel with those reported by Jones et al. [1988]. Therefore, we believe that the isolated papl+ gene encodes a fission yeast AP-1-like factor or at least one of those factors.

Disruption of the papl+ gene

The method for one-step gene replacement [Rothstein 1983] was used to disrupt the wild-type papl+ gene [Materials and methods]. Upon sporulation of Ura+ heterozygous diploids, we obtained four viable spores for all tetrads examined. The uracil marker segregated 2+ : 2−. Disruption of the papl+ gene in the Ura+ segregants was confirmed by Southern blotting [data not shown]. Although the deleted pap1− segregants were viable, the
Figure 2. (See following page for legend.)
growth rate of these cells was significantly lower than that of wild type, the average generation time of papl−
cells is 175 min at 33°C in a rich liquid YPD, whereas that of wild-type is 130 min. The nonessential standing of
papl+ may be caused by the functional redundancy of the AP-1-binding factors. Recently, we have detected a weak but reproducible AP-1-binding activity in extracts prepared from the papl− cells (T. Toda et al., unpubl.).

We found that papl− cells were hypersensitive to staurosporine. As shown in Figure 7A, papl− null mutants did not form colonies on plates containing 0.2 μg/ml of staurosporine, and drug supersensitivity and uracil prototrophy cosegregated in tetrads. We then examined whether multicopy spkl+ gene could confer staurosporine resistance in a papl− background. One set of tetrad segregants was transfected with multicopy plasmids containing either vector sequence alone or papl+ or spkl+ gene-containing plasmids (Fig. 7B, lanes 1, 2, or 3, respectively), and the drug sensitivity of each transformant was determined. It was evident that papl− cells carrying the spkl+ kinase gene on multicopy plasmids were still sensitive to the drug (right panel, lane 3; cf. a, d and b, c), although the same cells containing high-dose papl+ could grow on plates containing 0.3 μg/ml staurosporine (lane 2). These results indicate that the papl+ gene is required for spkl+ to confer staurosporine resistance (for a summary of drug resistance of each transformant, see Table 1). On the other hand, when carried on multicopy plasmids, the papl+ gene could confer drug resistance to spkl− cells [Table 1].

Discussion

In this study we report that staurosporine, an in vitro inhibitor of PKC, blocks growth of S. pombe cells specifically at a stage immediately after cell division, and we...
describe the isolation and characterization of the two fission yeast genes that confer resistance to staurosporine when carried on multicopy plasmids. One gene, $spkl^+$, encodes a novel 45-kD protein kinase enriched in the nucleus and resembling the mammalian ERK1/MAP2 kinase and budding yeast $KSS1$ and $FUS3$, and the other gene, $papl^+$ for an S. pombe AP-1-like factor.

The stage blocked by staurosporine is of particular interest in terms of growth control of fission yeast as cells enter the phase of cell elongation at this time. At first, tip growth occurs asymmetrically only at the old end of cells, which existed in the previous cycle (Streiblova and Wolf 1972). This early stage of cell elongation and not those at the stage referred to as NETO (new end take-off, Mitchison and Nurse 1985), when the new end of the cell starts to grow, appears to be staurosporine sensitive. It should be noted that in the rich medium used in this study, S. pombe apparently lacks a $G_1$ phase; cells immediately start to replicate DNA upon completion of nuclear division. Thus, our analysis does not rule out the possibility that staurosporine might affect $G_1$ cells. When higher drug concentrations (>10 $\mu$g/ml) were used, the block of cell growth was no longer cell-cycle-stage specific; cells arrested immediately after the addition of the drug. Hence, the target molecules most sensitive to staurosporine should be responsible for the early stage of cell growth. Although in vitro studies (Tamaoki et al. 1986; Nakano et al. 1987) indicated that PKC was the most staurosporine sensitive of the kinases examined, the situation might be quite different in vivo, and other kinases may well be targets of the drug.

Although much is now known about the regulation of late $G_2$, $G_2/M$ transition from the study of $cdc2^+\,$, $suc1^+\,$, and $cdc13^+\,$ as components of MPF (Booher et al. 1989; Moreno et al. 1989), regulation of the entry into the increase of cell mass in fission yeast is scarcely understood. Genes identified by staurosporine-hypersensitive mutations or high-dose resistance might provide the clue to understanding this process. So far, we have isolated 4 genes that confer resistance by high gene dose and identified 11 $sts$ genes that determine hypersensitivity. Two of the four former genes are reported in this paper, but the products of the remaining genes have not been identified except for $sts1^+$, which contains membrane-spanning domains (T. Toda and M. Shimomukai, unpubl.). The $sts1^+$ gene is required for the confering of high gene dose resistance by $spkl^+$ and $papl^+$; multicopy plasmids containing either $papl^+$ or $spkl^+$ cannot confer staurosporine resistance in the $sts1$ mutant background. The genetic and biochemical interrelationships between the resistance-conferring and hypersensitive genes have not been investigated. Mutants strongly resistant to staurosporine have not been isolated, probably because the number of the targets would increase under higher drug concentrations. The existence of PKC-like genes has been reported recently in budding yeast (Levin et al. 1990, Ogita et al. 1990). It is not known whether yeast PKC is highly sensitive to staurosporine. PKC might be an in vivo target for staurosporine if it exists in fission yeast, and it remains to be seen whether any of the $sts^+$ and high-dose resistance genes encodes a PKC-related protein.

An intriguing question is whether $spkl^+$ is a direct cellular target for the drug. We found that the $spkl^+$ protein is induced up to 10-fold by the addition of staurosporine. This may represent a close relationship between the drug and the expression of $spkl^+$ gene. In some cases, such as those between hydroxyurea and ribonucleotide reductase (Elledge and Davis 1987) and between pactamycin and HMG-CoA reductase (Brown and Goldstein 1980), inhibitors are known to induce synthesis of the target enzymes. It should be noted, however, that an $spkl$ null strain is viable and has a staurosporine sensitivity indistinguishable from that of wild type. Therefore, it is equally possible that $spkl^+$ is only indirectly related to the drug. The amount or susceptibility of other kinase(s) as the primary staurosporine target(s) may be regulated by the $spkl^+$ gene. This hypothesis is consistent with the nuclear localization of $spkl^+$ protein.

The amino acid sequence of $spkl^+$ is highly homologous to the recently reported S. cerevisiae $KSS1$ and $FUS3$ proteins and mammalian ERK1/MAP2 kinase. The growth-regulated properties of ERK1/MAP2 kinase have been well documented; mitogens and growth factors rapidly activate the MAP2 kinase (Boulton et al. 1990). High-dose $KSS1$ in budding yeast promotes recovery from pheromone-induced growth arrest in $G_1$ phase. The $KSS1$ protein acts positively on the mitotic cell cycle (Coursechesne et al. 1989). The $FUS3$ protein, however, works antagonistically to $KSS1$; $FUS3$ is required for both $G_1$ arrest and conjugation (Elion et al. 1990). Both $KSS1$ and $FUS3$ are involved in growth control of budding yeast. Although the disruption phenotypes of $spkl^+$ and $FUS3$ are essentially the same (viable and conjugation defective), it remains to be determined whether $spkl^+$ and $FUS3$ are interchangeable.

High-dose $papl^+$ confers staurosporine resistance, and we have shown that the $papl^+$ gene product behaves as an AP-1-like transcription factor. A comparison between $papl^+$ and budding yeast $YAP1$ indicates an extremely high degree of homology in the basic region adjacent to the leucine repeats; 11 continuous amino acid residues

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**Figure 4.** Cellular localization of the $spkl^+$ protein. Immunofluorescence microscopy of *S. pombe* cells that overproduce the $spkl^+$ protein is shown. Cells were stained with affinity-purified anti-$spkl$ antibody, followed by rhodamine-conjugated second antibody (A, denoted by Ab) and the DNA-binding fluorescent dye, DAPI (B). Bar, 10 $\mu$m.
Figure 5.  [See facing page for legend.]
in the basic region are identical. This region is believed to determine a sequence-specific DNA-binding activity of the c-jun/c-fos proteins (Kouzarides and Ziff 1988; Gentz et al. 1989; Turner and Tjian 1989; Vinson et al. 1989). A high degree of homology between pap1\(^+\) and YAP1 in this region may reflect similar target sequences.

One question raised is that of how overproduction of the transcription factor pap1\(^+\) confers staurosporine resistance. A simple explanation is that the pap1\(^+\) protein is one of the cellular targets for the drug. This is unlikely, because there is no indication for a direct interaction between bacterially produced pap1\(^+\) protein and the drug, pap1\(^+\) normally binds to the AP-1 site in the presence of an excess concentration of the drug (data not shown). The second possibility is that the pap1\(^+\) protein, in some way, activates target protein kinases, and as a result cells containing multicopy pap1\(^+\) plasmids become more resistant to the drug. Alternatively, there may be a feedback mechanism such that inactivation of a target protein kinase causes the activation of its transcription factor. These hypotheses are consistent with the drug hypersensitivity of a pap1\(^-\) null mutant that is perhaps unable to increase the level of target protein kinases.

The evidence suggests that the spkl\(^+\) protein kinase and pap1\(^+\) are functionally interrelated, although the link between them may not be direct. We showed that multicopy spkl\(^+\) genes could not confer drug resistance in the absence of the pap1\(^+\) gene. On the other hand, multicopy pap1\(^+\) plasmids could increase drug resistance in spkl\(^-\) cells. High-dose pap1\(^+\), however, could not complement the other spkl\(^-\) phenotypes; spkl\(^-\) cells containing multicopy pap1\(^+\) plasmids are still conjugation defective. A plausible hypothesis to explain our result is that a major target protein kinase of staurosporine, which is directly linked with pap1\(^+\), is yet to be identified. Conferring drug resistance may require a certain level of that major target kinase; both spkl\(^+\) and pap1\(^+\) genes regulate the amount of activity of the target kinase in different ways.

Materials and methods

Strains, media, and chemicals

*S. pombe* strains used in this study are listed in Table 2. Rich YPD (1% yeast extract, 2% peptone, 2% dextrose), minimal SD (0.67% yeast nitrogen base without amino acids, 1% dextrose), and SPA media (Gutz et al. 1974) were used. Plates contained 1.6% of agar. Staurosporine (2 mg/ml was provided by Dr. H. Nakano, Kyowa Hakko Co.) was dissolved in dimethyl sulfoxide (DMSO), and stock solutions of 0.5 ml each were stored at -20°C. The stock solution was diluted 10-fold with ethanol just before use.

Genetic techniques and nomenclature

Standard procedures for *S. pombe* were followed according to Mitchison (1970, 1988) and Gutz et al. (1974). Gene disruptions are denoted by lowercase letters representing the disrupted gene followed by two colons and the wild-type gene marker used for disruption. In this paper gene disruptions are abbreviated by lowercase letters representing the gene, followed by a superscript minus sign, such as pap1\(^-\).

Isolation and characterization of the staurosporine-supersensitive mutants

Wild-type HM123 (Table 2) cells were mutagenized with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine as described previously (Uemura and Yanagida 1984). Supersensitive mutants (called ss) that could not grow on YPD plates containing 0.2 mg/ml of staurosporine were isolated by replica plating. Approximately 200,000 colonies were screened, and 18 independent mutant strains were obtained (ss1-ss18). Backcrossing with a mating strain (JY6, Table 2) indicated that ss phenotypes of all the strains but two (ss1 and ss11) were due to a single chromosomal mutation. Each of the two strains, ss1 and ss11, contained double mutations, either of which alone showed a ss phenotype. Complementation test was done by crisscrossing each of the mutant strains. Segregants were examined for drug sensitivity, and 11 genetic loci (called *sts1*--*sts11*) were identified. Dominant-recessive tests were done by constructing prototrophic diploids between individual mutants and a meiosis-deficient mating strain (JY183, Table 2).

Cloning of genes that can suppress drug sensitivity

h\(^-\) leu1 strains of *sts1*-5, *sts2*-6, and *sts3*-15 (Table 2) were transformed with an *S. pombe* genomic library constructed in pDB248 (Beach and Nurse 1981, Beach et al. 1982, Hirano et al. 1988) using the lithium method (Ito et al. 1983). These alleles were chosen because their transformation efficiencies were the best. Leu\(^+\) transformants were replica-plated on YPD containing 0.2 mg/ml of staurosporine. Those that could grow on drug-containing plates were picked up, and their segregation pattern was analyzed. Plasmid DNAs were recovered as described (Nasmith and Reed 1980). We obtained one *sts1*-containing plasmid from *sts1*-5, one *sts2*-containing plasmid from *sts2*-6, one *spkl*-containing plasmid (Fig. 2A), and three different pap1\(^+\)-containing plasmids (Fig. 5A) from *sts3*-15.
Nucleotide sequence determination (Hattori and Sakaki 1986). Nucleotide sequence of the spk1+ gene was determined as follows. Subcloning analysis of pST23 suggested that either site of the 5.0-kb BglII must be located inside the coding region (see Fig. 2A). Nucleotide sequence was determined from both sides of the BglII fragment. One of the two sides (a rightward end side in Fig. 2A) was shown to be in the middle of an ORF, and computer search using NBRF revealed that amino acid sequences of that ORF have a significant homology to those of various known protein kinases. The 4.0-kb SalI fragment of pST23 was subcloned into Bluescript (Stratagene, designated as pRS4.0S), and an entire sequence was determined. For sequencing the papl+ gene, 2.5- and 2.0-kb HindIII fragments of pST1 and pST12 (Fig. 2A) were subcloned into pUC18 (Yanisch-Perron et al. 1985) and Bluescript (designated pPA2.5H and pPA2.0H, respectively). More than 80% of the coding regions of both the genes were sequenced, and in regions where only one direction was read, nucleotide sequence was confirmed by sequencing at least two different template plasmid DNAs.

**Figure 6.** DNA-binding specificity of the papl+ protein. (A) The AP-1-binding core sequences of the synthetic oligonucleotides used here are shown (Jones et al. 1988). SV-AP-1 contains the AP-1-binding site of the SV40 early promoter/enhancer region and Col-AP-1 contains that of the human collagenase gene. Col-mut1 and Col-mut2 are the two mutated forms of the collagenase AP-1 site. Underlined nucleotides indicate the replaced nucleotides. The asterisks (*) indicate those nucleotides essential for AP-1 binding [Angel et al. 1987]. (B) Gel retardation analysis was performed according to Fried and Crothers (1981), using the end-labeled Col-AP-1 as the probe. Labeled probes (1 ng, 3 x 10^6 cpm) were incubated with 0.25 µg of purified T7-papl+ protein made in *E. coli* (lanes 2–6). No protein was added in lane 1. Each lane contained 2 µg of poly[d(C·T)] as the carrier. Specific competitor oligonucleotides used were as follows: SV-AP-1 (lane 3), Col-AP-1 (lane 4), Col-mut1 (lane 5), and Col-mut2 (lane 6). The amount of each competitor added was to a 400-molar excess. (C) DNase I footprint analysis of the SV40 early promoter/enhancer region using sequences end-labeled in the noncoding strand as the probe. The amounts of the T7-papl+ protein used were 0 ng (lane 1), 25 ng (lane 2), 50 ng (lane 3) 100 ng (lane 4), and 250 ng (lane 5) in a 50-µl reaction volume.

**Figure 7.** Phenotypes of the papl+ gene disruptant. (A) Two sets of tetrad segregants were streaked on a minimal plate lacking uracil (—Ura, left) and a YPD plate containing 0.2 µg/ml of staurosporine (+STS, right). Note that colony formation on the two plates exhibits a positive/negative correlation; Ura+ cells grown in the left plate cannot grow on the drug-containing plate (right), while Ura- cells that cannot grow on the left plate can grow on the right plate. (B) One set of tetrad segregants [a–d] was transformed with three kinds of multicopy plasmids carrying only the vector sequence (lane 1), papl+ (lane 2), or spk1+ (lane 3) genes, respectively. Transformants were streaked onto either minimal plates lacking leucine (—Leu, left) or uracil (—Ura, middle), or a YPD plate containing 0.3 µg/ml of staurosporine (+STS, right). Ura- segregants (a and d) can grow on the drug-containing plate if they contain multicopy plasmids carrying either papl+ or spk1+. On the other hand, Ura+ segregants (papl+ null mutants, b and c) are still staurosporine sensitive when transformed with spk1+ containing plasmids (lane 3, right), while they became drug resistant when transformed with plasmids carrying papl+ (lane 2).
Gene disruption

For disruption of the spkl* gene, the 1.2-kb EcoRI fragment containing most of the coding region (Fig. 2A) was subcloned into pUC18 [designated pRS1.2R]. pRS1.2R (isolated from dam* GM33) was digested with EcoRI and the 1.8-kb Bcll-BglII fragment from pPA2.0H was inserted in EcoRI fragment of spkl* gene, the 1.0-kb BamHI fragment containing the S. pombe ura4* gene (Grimm et al. 1988) was inserted into the same site in the polylinker region of pSK248, yielding pAS248. The 1.2-kb EcoRI fragment containing disrupted papl gene was used for transformation of S. pombe ura4* diploids. In each case, stable Ura+ transformants were selected and disruption of the spkl* or papl* gene was checked by genomic Southern hybridization.

Expression of the fused protein in E. coli and preparation of antiserum

For construction of an expression plasmid for the fused protein of the spkl* gene, the 1.0-kb BamHI-BglII fragment from pRS1.2R was inserted into pET-3c (Rosenberg et al. 1987). This plasmid (pT7-spkl*) was introduced into E. coli BL21(DE3) (Studier et al. 1986). Induced extract of the E. coli strain was fractionated according to Watt et al. (1985). SDS-PAGE was performed as described (Laemmli 1970). The fused protein (p43) that was insoluble was recovered from the gel (Hirano et al. 1988). For production of the fused protein of the papl* gene, 1.4 kb of the BglII-BamHI fragment from pPA2.0H was inserted in pET-3a (Rosenberg et al. 1987). This plasmid (pT7-papl*) was used for purification of the fused protein as described above. The soluble fused papl* protein (p59) was purified from the gel and used for DNA-binding assay. Rabbit antisera were prepared as described previously (Hirano et al. 1988).

Overexpression of the spkl* and papl* proteins in S. pombe cells

The 2μ DNA-based, ADH promoter-containing vector, pAS248, was constructed as follows: The 5.8-kb HindIII/XhoI fragment that contains a part of 2μ DNA and the S. cerevisiae LEU2 gene was isolated from pDB248 (Beach and Nurse 1981) and was ligated with HindIII-XhoI-digested Bluescript, yielding pSK248. The 0.7-kb HindIII–SalI fragment that contains ADH promoter (Russell and Hall 1983) was inserted into the same site in the polylinker region of pSK248, yielding pAS248. The 1.2-kb HindIII fragment containing the spkl* gene (Fig. 2A) was subcloned into the Smal site of pAS248, yielding pADH-spkl*. For overexpression of the truncated papl* gene, the 1.4-kb BglII–BamHI fragment from pPA2.0H was inserted into the BamHI site of pAS248, yielding pADH-papl*. These plasmids were used to transform S. pombe cells.

Immunochemical assays

Immune complexes were prepared essentially as described by Booher et al. (1989), except that cell extracts [1 × 10^9 cell equivalents] were mixed with 20 μl of a 1:1 slurry of protein A–Sepharose beads (Pharmacia) that had been bound with appropriate serum. Immunoblotting was carried out by electrophoretically transferring the proteins to nitrocellulose filter (Towbin et al. 1979). Protein A labeled with ^125I (Amersham) or peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) and an immunostaining kit (Konica) was used to detect the bound first antibodies.

For an in vitro protein kinase reaction, procedures by Booher et al. (1989) were also followed with slight modifications. Histone H1 (Boehringer Mannheim) and casein was used at 0.5 and 2.5 mg/ml, respectively, 100-fold more ATP (final 20 μM) was used in the reaction mixture.

Table 2. S. pombe strains used in this study

| Name       | Genotype                                      | Deviation                              |
|------------|-----------------------------------------------|----------------------------------------|
| HM123      | h·leu1                                        | our stock                              |
| JY6        | h·leu1 his2                                    | our stock                              |
| JY183      | mei1-B102 lys3 arg1                           | our stock                              |
| TP38-1A    | h·leu1 sta5-1                                 | segregant of tetrads between ss5 and JY6 |
| TP39-1C    | h·leu1 sta5-6                                 | segregant of tetrads between ss6 and JY6 |
| TP52-1B    | h·leu1 sta3-15                                | segregant of tetrads between ss15 and JY6 |
| TP4-1D     | h·leu1 his2 ura4 ade6-M216                    | Ohkura et al. (1989)                   |
| TP4-5A     | h·leu1 ura4 ade6-M210                         | Ohkura et al. (1989)                   |
| 5A/1D      | h·h leu1/leu1 ura4/ura4 + his2 ade6-M216       | Ohkura et al. (1989)                   |
| TP20       | h·h·leu1/leu1 ura4/ura4 his2/+ ade6-M210/ade6-M216 | a transformant of 5A/1D with 2.6-kb EcoRI fragment of spkl::ura4* |
| TP21       | h·h·leu1/leu1 ura4/ura4 his2/+ ade6-M210/ade6-M216 | a transformant of 5A/1D with 2.8-kb EcoRI fragment of papl::ura4* |
| TP98-1A    | h·leu1 ura4 sta5-1                           | segregant of tetrads between TP4-1D and TP98-1A |
| TP101-3A   | h·leu1 his2 ura4 ade6                         | segregant of tetrads from TP20         |
| TP101-3B   | h·leu1 his2 ura4 ade6 papl::ura4+             | segregant of tetrads from diploids between TP101-3A containing pST23 and TP98-1A |
| TP101-3C   | h·leu1 ura4 ade6 papl::ura4+                  | segregant of tetrads from diploids between TP101-3B and TP98-1A |
| TP106-3C   | h·leu1 ura4 spkl::ura4+                       | segregant of tetrads from diploids between TP101-3B and TP98-1A |

Table 2. S. pombe strains used in this study

Fission yeast AP-1-like factor and protein kinase
Immunofluorescence microscopy

The procedures described by Hagan and Hyams [1988] were basically followed. S. pombe cells were fixed in methanol at −20°C for 8 min.

Assay for specific pap1+ binding to the AP-1 site

A gel retardation assay was performed as described [Fried and Crothers 1981]. Oligonucleotides synthesized were the same as in Jones et al. [1988]. After the oligonucleotides were annealed [Hurst and Jones 1987], they were end-labeled with T4 polynucleotide kinase. Approximately 1 ng (3 x 10⁴ cpm) of the labeled probe was used. Competitor DNA was added at a level of 400-fold molar excess. A DNase I footprinting experiment was carried out according to Lee et al. [1987], except that binding reaction was done in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 10% glycerol, and 0.5 mM DTT. pW2/Bgl (provided by Dr. M. Imagawa; Zenke et al. 1986), which contains the SV40 enhancer region including two copies of 72-bp repeats, was used for preparation of the probe fragment. pW2/Bgl was linearized with BanHI [at position −106] and labeled with [a-32P]dCTP and RAV-2 reverse transcriptase. The 240-bp fragment that contains the enhancer region was purified after the second digestion with EcoRI [−106 to −346]. In either assay, reaction mixture usually contained 2 µg poly [dT-C] (Pharmacia), unless otherwise stated. Binding reactions were incubated for 15 min at room temperature.

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T Toda, M Shimanuki and M Yanagida

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