

Fig. 3. Cell cycle progression and phosphorylation of Rad53p in the *orc5* degtron mutant. W303-1A (wild-type), YYM101 (*GAL-UBR1*), YYM104 (*orc3-td*) and YYM106 (*orc5-td*) cells were cultured in YP medium with 2% raffinose (YPRaff) to logarithmic phase at 24°C and then cultured in YP medium with 2% galactose (YPGal) for 1 h at 24°C and finally in the same medium at 37°C. A small portion of culture was taken when indicated. (A) Experimental outline and timing of sampling. (B) Cellular DNA contents analysed by FACS. (C) Whole-cell extracts were analysed by immunoblotting with monoclonal antibody for Rad53p. For control experiments, wild-type cells were cultured for 2 h at 24°C in YPRaff with hydroxyurea (HU) or without it (Cont.). Phosphorylated forms of Rad53p are shown by an asterisk.

non-permissive conditions, the upper-shifted band of Rad53p increased in the *orc5* mutant, but not in wild-type cells. Similar increases were observed in wild-type cells treated with HU. These results suggest that checkpoint systems are induced when Orc5p (ORC) is degraded, and the defect of cell cycle progression in the *orc5* mutant (Fig. 3B) may involve checkpoint systems.

Progression of Distinct Cell Cycle in *orc5* Mutants—We performed block and release experiments to examine the effect of the degradation of Orc5p (ORC) on cell

cycle progression. Asynchronously cultured *orc5* mutant and wild-type cells were synchronized at G1, S or G2/M phase by incubation with α -factor, HU or nocodazole, respectively. In the *orc5* mutants, Orc5p was supposed to be degraded by incubating cells in YP medium with galactose at 24°C, then at 37°C in the presence of each blocking agent, then cells were released into fresh YP medium with galactose (Fig. 4A). As shown in Fig. 4B, both S phase progression (from HU block) and G2/M phase progression (from nocodazole block) were the same in the *orc5* mutants and wild-type cells. G1/S transition (from α -factor block) was slightly slower in the *orc5* mutants (Fig. 4B). Furthermore, in nocodazole and HU block, although the entry into G1 phase was indistinguishable, the following G1/S transition was delayed in the mutant, suggesting that Orc5p (ORC) is necessary for this step. In *orc5* mutants, the unclear inhibition of G1/S transition (from α -factor block) is probably due to inhibition of the ORC degradation at late G1 phase (at the point of α -factor block); ORC degradation is less in cells blocked with α -factor than in cells blocked with nocodazole, or asynchronously growing cells (data not shown).

To test whether Orc5p (ORC) is necessary for G1/S transition, *orc5* mutant cells, and wild-type cells synchronized at G2/M phase, were incubated at 37°C in YP medium with galactose to degrade Orc5p (ORC), then released into fresh medium with α -factor, to be re-synchronized at late G1 phase, and finally released again into fresh medium to start S phase (Fig. 5A). As shown in Fig. 5B, the G1/S transition was clearly inhibited in *orc5* mutants. In wild type, cells with 2C DNA content peaked within 30 min, and cells with 1C DNA content re-appeared within 100 min of the final release (Fig. 5B). On the other hand, in *orc5* mutants, the peak of DNA content moved gradually from 1C to 2C and cells with 1C DNA content did not reappear (Fig. 5B). These results suggest that when Orc5p (ORC) is depleted from G2/M to late G1, cells can enter G1 normally, but subsequent entry into S phase is inhibited. Thus, ORC must be required for G1/S transition.

Formation of the Pre-RC in the *orc5* Mutant—*In vivo* foot-printing analysis revealed that a protein complex, called pre-RC, is formed in late M or G1 on each origin of chromosomal DNA replication (35–39). Pre-RC includes at least ORC, Cdc6p, Cdt1p and MCM (40). We here examined the formation of pre-RC in *orc5* degtron mutants. We used a chromatin-binding assay to examine the loading of Mcm2p onto chromatin. (Mcm2p is a subunit of MCM). Wild-type cells and *orc5* mutants were synchronized at G2/M, followed by ORC degradation, and finally cells were released into fresh medium (Fig. 6A). As shown in Fig. 6B, in the wild-type cells, Mcm2p bound to chromatin 40 min after the release, and the intensity of band decreased gradually (may be due to cell cycle progression into S and G2/M), consistent with our previous results (41). On the other hand, in *orc5* mutants, Mcm2p did not bind chromatin at least until 90 min after the release (Fig. 6B). Thus, when ORC is depleted from G2/M to late G1, pre-RC does not form efficiently. This inefficiency is perhaps what inhibits G1/S transition in the *orc5* mutants (Fig. 5B).

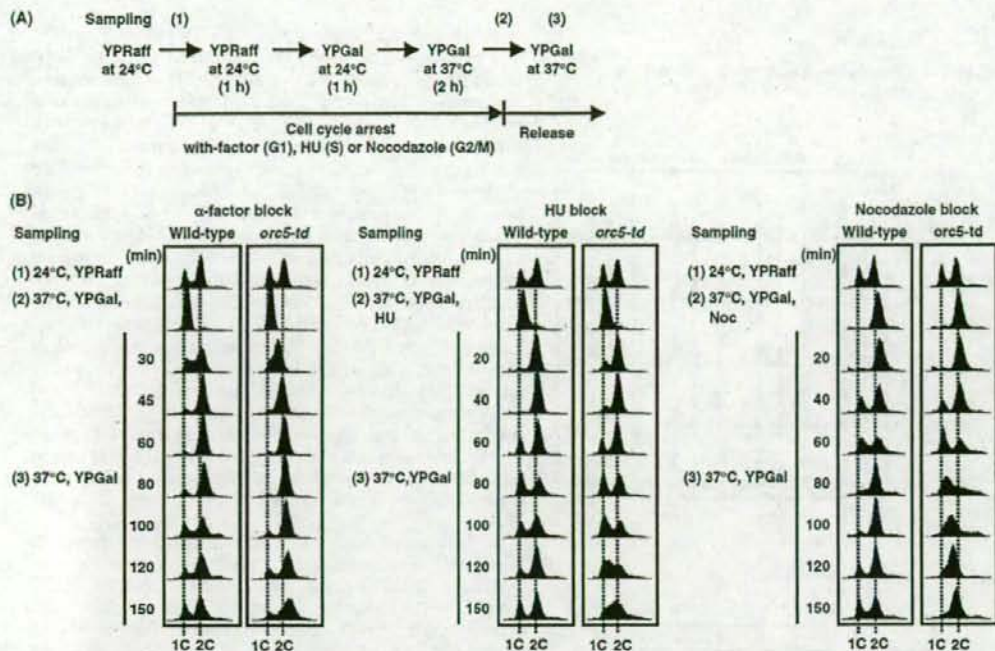


Fig. 4. Block and release analysis for cell cycle progression in the *orc5* degron mutant. W303-1A (wild-type) and YYM106 (*orc5-td*) cells were cultured in YP medium with 2% raffinose (YPRaff) to logarithmic phase at 24°C and synchronized at G1, S or G2/M phase by incubation with α -factor, HU or nocodazole, respectively. Cells were cultured first in YPRaff for

1 h at 24°C then in YP medium with 2% galactose (YPGal) for 1 h at 24°C, and finally in the same medium for 2 h at 37°C in the presence of each arrest agent. Cells were washed and released into the fresh YPGal and cultured at 37°C. (A) Experimental outline and timing of sampling. (B) FACS analysis was performed as described in Fig. 3.

ORC is Required at Late G1 Rather Than at Early G1 and G2/M, for Cell Cycle Progression and Pre-RC Formation—In experiments shown in Figs 5 and 6, ORC was absent in both G2/M and the following G1 phases. To define precisely when ORC is required for cell cycle progression (G1/S transition) and pre-RC formation, we designed experiments shown in Fig. 7A; wild-type cells and *orc5* mutants synchronized at G2/M phase, were first incubated in medium with galactose at 37°C to degrade Orc5p (ORC); then released into fresh medium with α -factor to be re-synchronized at G1 phase; then incubated at 24°C in medium with glucose plus α -factor (to restore ORC), or at 37°C in medium with galactose plus α -factor (to keep ORC absent); and finally released into fresh medium to examine cell cycle progression. We used glucose instead of raffinose after the final release, because under these conditions, even wild-type cells grow slowly with raffinose (data not shown). As shown in Fig. 7D, Orc5p was restored by the incubation at 24°C in YP medium with glucose (note the band of Orc5p-td at 60–120 min in the lower right panel in Fig. 7D). Under these conditions, cell cycle progression (G1/S transition) after the final release was identical in *orc5* mutants and wild type (right panel, Fig. 7B) and cells with nearly 2C DNA was not accumulated, even in *orc5* mutants

(right panel, Fig. 7B). On the other hand, with continued absence of Orc5p (left panel, Fig. 7B), inhibition of G1/S transition and accumulation of cells with nearly 2C DNA content were observed in the *orc5* mutants, as in Fig. 5B.

We also monitored pre-RC formation under these conditions (Fig. 7C). Following incubation in YP medium with galactose and α -factor at 37°C, both Orc5p and Mcm2p were loaded on chromatin in the wild-type strain, but not in the *orc5* mutant (Fig. 7D, sampling time 3). When *orc5* mutants were subsequently incubated in YP medium with glucose at 24°C to restore Orc5p, both Orc5p and Mcm2p were loaded on chromatin, (lower right panel, Fig. 7D). On the other hand, if the incubation conditions were not shifted (to keep Orc5p absent), neither Orc5p-td nor Mcm2p was loaded on chromatin (lower left panel, Fig. 7D). Thus, for cell cycle progression (G1/S transition) and pre-RC formation, Orc5p (ORC) is required at late G1 (the point of α -factor block), but not at early G1 and G2/M.

Finally, we examined whether pre-RC is degraded after ORC degradation in α -factor-blocked cells. After formation of pre-RC in α -factor-blocked cells, ORC was tried to be degraded by incubation of the *orc5* mutants at 37°C in YP medium with galactose and the level of MCM (Mcm2p) was monitored (Fig. 8A and B). As shown in

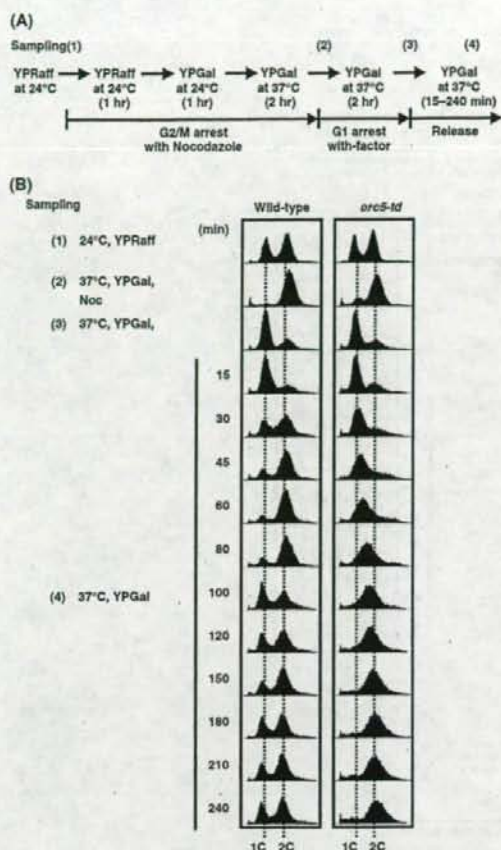


Fig. 5. Requirement of ORC for G/S transition. W303-1A (wild-type) and YYM106 (*orc5-td*) cells were cultured in YP medium with 2% raffinose (YPRaff) to logarithmic phase at 24°C and synchronized at G2/M phase by incubation with nocodazole. Cells were cultured first in YPRaff for 1 h at 24°C then in YP medium with 2% galactose (YPGal) for 1 h at 24°C and finally in the same medium for 2 h at 37°C in the presence of nocodazole. Then cells were washed and released into the fresh YPGal containing α -factor and cultured for 2 h at 37°C. Finally, cells were released into the fresh YPGal. (A) Experimental outline and timing of sampling. (B) FACS analysis was performed as described in the legend of Fig. 3.

Fig. 8C. Mcm2p released from chromatin more rapidly in *orc5* mutants than in wild-type cells, however, the difference was not so clear. This may be due to that the degradation of Orc5p in *orc5* mutants was not so clear in α -factor-blocked cells as in the nocodazole-blocked cells, as shown in Fig. 8D and E.

DISCUSSION

In yeast, the heat-inducible degron mutant system is very useful to reveal the exact roles of proteins essential

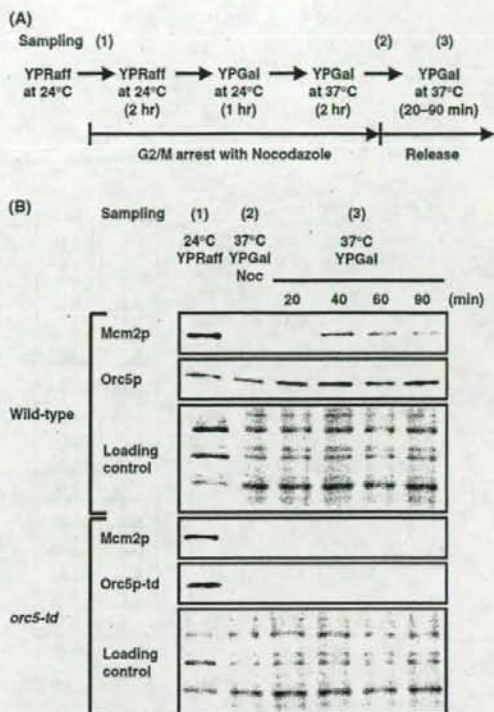


Fig. 6. The pre-RC formation in the *orc5* degron mutant. W303-1A (wild-type) and YYM106 (*orc5-td*) cells were cultured in YP medium with 2% raffinose (YPRaff) to logarithmic phase at 24°C and synchronized at G2/M phase by incubation with nocodazole. Cells were cultured first in YPRaff for 2 h at 24°C then in YP medium with 2% galactose (YPGal) for 1 h at 24°C and finally in the same medium for 2 h at 37°C. Cells were washed and released into the fresh YPGal and cultured at 37°C for indicated periods. (A) Experimental outline and timing of sampling. (B) The chromatin loading of Mcm2p and Orc5p monitored by immunoblotting, as described in the legend of Fig. 1. For loading control (bottom panel), gels were stained with silver.

for cell growth. This system has helped us to study the functions of various DNA replication-related proteins *in vivo* (21, 23, 24, 42), but its application to ORC has not yet been reported. Biochemical studies of ORC have revealed its functions *in vitro*, but, as described in the INTRODUCTION section, genetic studies using temperature-sensitive mutants have so far failed to reveal ORC's precise role *in vivo*. In this study, we constructed a heat-inducible degron mutant for every ORC subunit. All mutants showed temperature-sensitive growth phenotype, confirming that ORC is essential for budding-yeast cell growth. Using these mutants, we examined the role and function of ORC *in vivo*.

In *orc* degron mutants, under the non-permissive conditions, both the targeted ORC subunit, and other subunits, were degraded. Due to technical problems, we

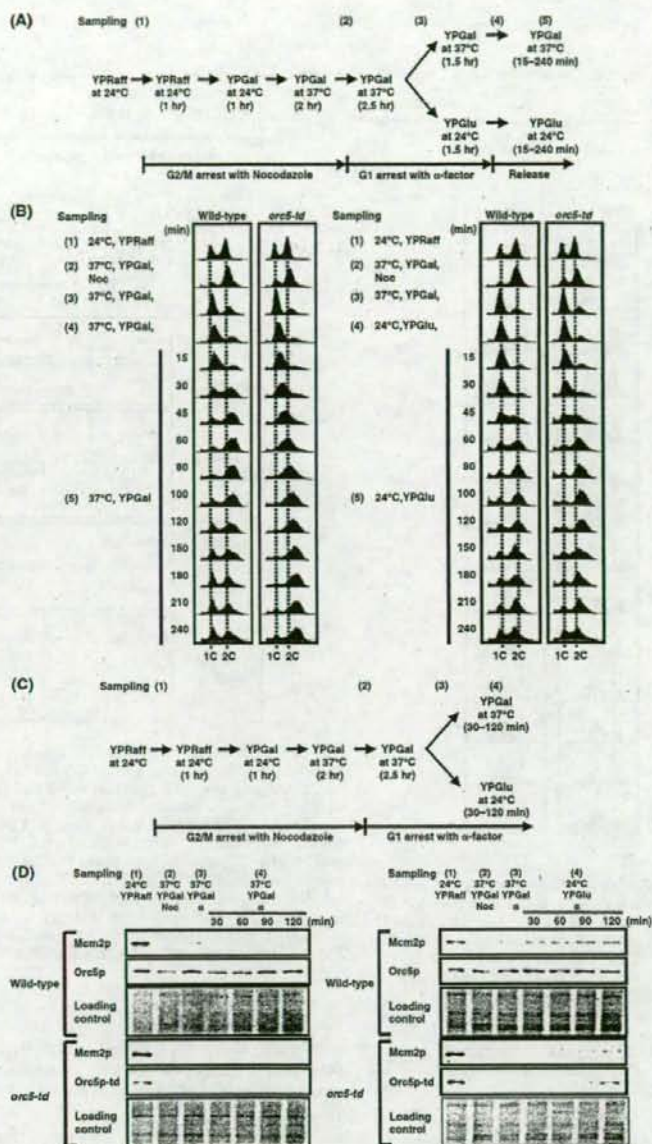


Fig. 7. The presence of ORC at late G1 rather than early G1 and G2/M phases is important for cell cycle progression and the pre-RC formation. YYM114 (wild-type) and YYM115 (*orc5-td*) cells were cultured in YP medium with 2% raffinose (YPRaff) to logarithmic phase at 24°C and synchronized at G2/M phase by incubation with nocodazole. Cells were cultured first in YPRaff for 1 h at 24°C then in YP medium with 2% galactose (YPGal) for 1 h at 24°C and finally in the same medium for 2 h at 37°C. Cells were washed and released into the fresh YPGal containing α -factor and cultured for 2.5 h

at 37°C. At this point, cell cultures were divided and incubated at 37°C in YPGal with α -factor, or at 24°C in YP medium with 2% glucose (YPGlu) plus α -factor, for 1.5 h (B) or indicated periods (D). Finally, cells were released into fresh YPGal or YPGlu and cultured for indicated periods (B). FACS analysis and chromatin-binding assay was performed as described in the legends of Figs 1 and 3 (B and D). For loading control, gels were stained with silver (D). Experimental outline and timing of sampling for Fig. 7B. (C) Outline and sampling for Fig. 7D.

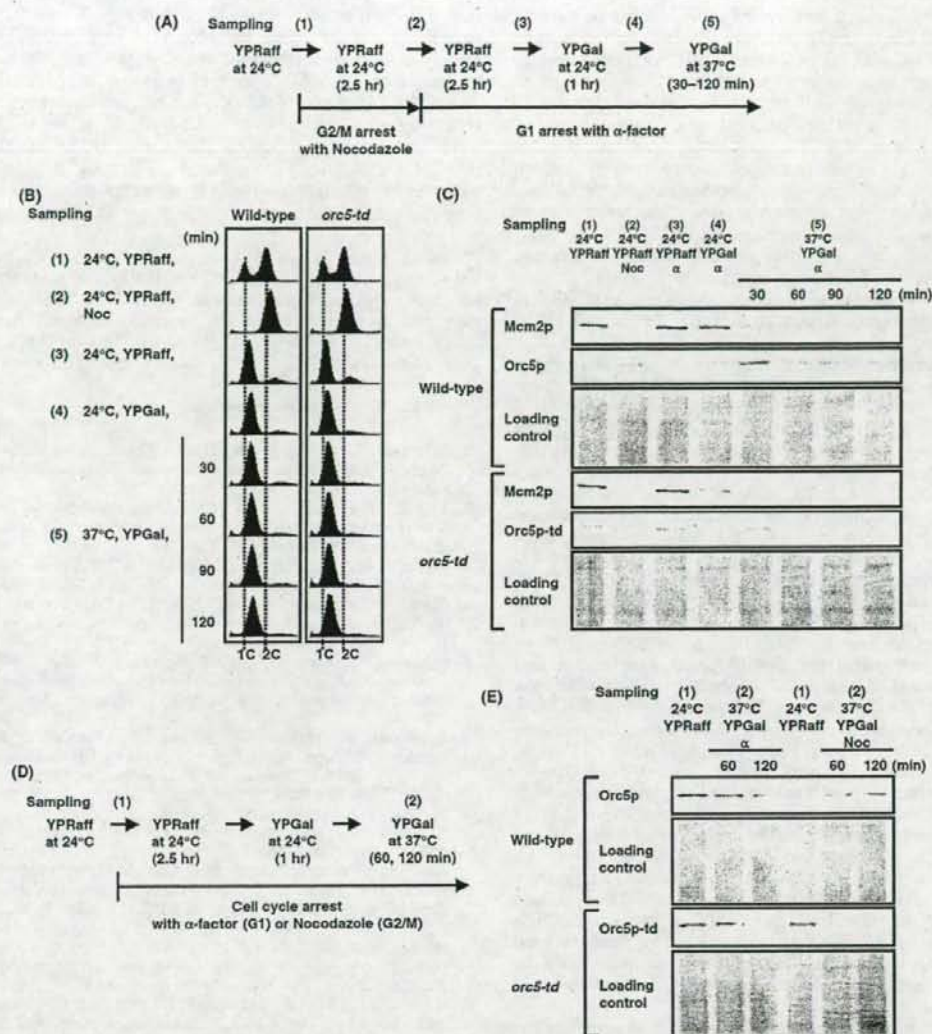


Fig. 8. Stability of pre-RC after degradation of ORC. YYM114 (wild-type) and YYM115 (*orc5-td*) cells were cultured in YP medium with 2% raffinose (YPRaff) to logarithmic phase at 24°C and synchronized at G2/M phase by incubation with nocodazole. Cells were washed and released into the fresh YPRaff containing α -factor and cultured for 2.5 h at 24°C, then YP medium with 2% galactose (YPGal) for 1 h at 24°C and finally in the same medium at 37°C for indicated periods. (A) Experimental outline and timing of sampling. (B) FACS analysis. (C) The chromatin loading of Mcm2p and Orc5p monitored by

immunoblotting, as described in the legend of Fig. 1. For loading control (bottom panel), gels were stained with silver. YYM114 (wild-type) and YYM115 (*orc5-td*) cells were cultured in YPRaff to logarithmic phase at 24°C and synchronized at G1 phase or G2/M phase by incubation with α -factor or nocodazole for 2.5 h at 24°C, then YPGal for 1 h at 24°C and finally in the same medium at 37°C for indicated periods. (D) Experimental outline and timing of sampling. (E) The Orc5p on chromatin was monitored by immunoblotting, as described in the legend of Fig. 1. For loading control (bottom panel), gels were stained with silver.

could not verify the degradation of all ORC subunits. However, it is reasonable to speculate that under the non-permissive conditions, all subunits of ORC would have been degraded in each of the *orc* degon mutants.

Thus, results suggest that ORC probably becomes unstable when any one of its subunits is degraded. We previously reported that in the temperature-sensitive *orc5-A* mutant (a strain expressing ORC with

Orc5pK43E, a mutation in the ATP binding domain of Orc5p, not only Orc5p but also all other subunits were rapidly degraded at non-permissive temperatures by ubiquitin-proteasome system (15, 43). Similar degradation was observed with other temperature-sensitive *orc* mutants (16, 44). Therefore, in yeast, when any one of the ORC subunits is degraded, other subunits may be also degraded by ubiquitin-proteasome system and we speculate that subunits of ORC other than targeted one, are degraded by this system in *orc* degenon mutants. But since the same system degrades the targeted subunit, we could not study the degradation of targeted and non-targeted subunits separately.

Block and release experiments suggested that ORC is required for G1/S transition but not for S and G2/M phase progression. Degradation of Orc5p (ORC) at S or G2/M phases did not inhibit the S or G2/M phase progressions, but elimination of Orc5p (ORC) from G2/M to late G1 phase, inhibited the subsequent G1/S transition. This conclusion is consistent with results in many previous studies using temperature-sensitive *orc* mutants, and genetic shut-off systems for Orc6p (13, 16-19, 34, 45). As well as various temperature-sensitive *orc* mutants, incubation of asynchronously growing *orc* degenon mutant cells under non-permissive conditions, caused accumulation of cells with nearly 2C DNA content. There are two possible explanations. First, as reported for other DNA replication-related mutants (41, 46), inefficient loading of MCM onto chromatin caused by the low level of ORC in *orc* degenon mutant cells is responsible for slow S phase progression and accumulation of cells with nearly 2C DNA. Second, the low level of ORC induces DNA damage and the DNA replication checkpoint systems that block cell cycle progression before mitosis. Supporting this idea, we showed that incubation of *orc5* degenon mutant cells under non-permissive conditions caused phosphorylation of Rad53p. Furthermore, in other temperature-sensitive *orc* mutants, non-permissive temperatures induce not only DNA damage and DNA replication checkpoint systems, but also spindle assembly checkpoint systems (14, 34). Both possibilities imply that ORC functions primarily as the initiator of DNA replication. The accumulation of cells with nearly 2C DNA content, seen in various temperature-sensitive *orc* mutants, may be due to inefficient MCM loading and/or induction of checkpoint systems.

For cell cycle progression, Orc5p (ORC) is required at late G1 phase rather than early G1 and G2/M phases. Even after elimination of Orc5p (ORC) from G2/M to late G1 (the point of α -factor block), α -factor-blocked *orc5* mutants could enter into S phase normally, after the release, as long as Orc5p (ORC) had been restored before the release. The heat-inducible degenon system did not work well in cells blocked with α -factor (i.e. blocked after pre-RC formation). Therefore, we could not test the effect of eliminating Orc5p (ORC) in late G1 on the following G1/S transition. Thus, we do not know whether ORC is required at late G1 for the subsequent G1/S transition. However, recent studies using genetic shut-off systems for Orc6p, showed that the elimination of Orc6p in cells blocked with α -factor did inhibit the subsequent G1/S transition (45), suggesting that ORC is required at

late G1. Furthermore, we showed that pre-RC can form in cells in late G1 phase provided that Orc5p (ORC) is restored. This is interesting because previous studies suggested that pre-RC is formed in late M or early G1 phase (29, 35). It seems that the pre-RC can be formed in relatively wide window in the cell cycle, from late G2/M (after the decrease in Cdc28p kinase activity) to late G1 (the point of α -factor block). This wide window may help yeast cells to maintain cell cycle progression under stress, which might otherwise inhibit pre-RC formation.

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Mini Review

Development of new type of NSAIDs with lower gastric side effects

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Non-steroidal anti-inflammatory drugs (NSAIDs) are a useful family of therapeutics. The anti-inflammatory actions of NSAIDs are mediated through their inhibitory effects on cyclooxygenase (COX) activity and resulting decrease in prostaglandins (PGs). On the other hand, NSAID use is associated with gastrointestinal complications. Although PGs have a strong protective effect on gastrointestinal mucosa, the inhibition of COX by NSAIDs is not the sole explanation for the gastrointestinal side effects of NSAIDs. In this study, we examined the COX-independent mechanism involved in NSAID-induced gastric lesions. Using DNA microarray analysis, we found that CHOP, a transcription factor with apoptosis-inducing ability is induced by NSAIDs and treatment of cells with NSAIDs caused apoptosis in wild-type cells but not in CHOP-knock out cells. We also found that NSAIDs have membrane permeabilization activity. Furthermore, intracellular Ca^{2+} chelator, BAPTA-AM inhibited the NSAID-induced apoptosis and induction of CHOP. *In vivo*, we proposed that both COX inhibition at gastric mucosa and direct gastric mucosal cell damage (such as induction of apoptosis) by NSAIDs are required for the production of gastric lesions. Results showed that NSAID-induced apoptosis is mediated by permeabilization of cytoplasmic membranes, increase in the intracellular Ca^{2+} levels and induction of CHOP. Furthermore, results suggest that NSAIDs without membrane permeabilizing activity have reduced gastrointestinal side effects.

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Key words NSAIDs, gastric ulcer, apoptosis, membrane permeabilization

INTRODUCTION

NSAIDs are one of the most frequently used classes of medicines in the world and account for nearly 5% of all prescribed medications¹⁾. However, NSAID administration is associated with gastrointestinal complications, such as gastric ulcers and bleeding, which sometimes become life-threatening diseases²⁾. About 15-30% of chronic users of NSAIDs have gastrointestinal ulcers and bleeding. In the United States, about 16,500 people per year die as a result of NSAID-associated gastrointestinal complications³⁾. Therefore, the molecular mechanism governing NSAID-

induced gastrointestinal damage needs to be elucidated in order to develop new NSAIDs that do not have these side effects.

Inhibition of COX by NSAIDs, which is responsible for their anti-inflammatory activity was previously thought to be fully responsible for their gastrointestinal side effects⁴⁾. There are at least two subtypes of COX, COX-1 and COX-2, which are responsible for the majority of COX activity at the gastric mucosa and tissues with inflammation, respectively. Therefore, it is reasonable to speculate that selective COX-2 inhibitors have anti-inflammatory activity without gastrointestinal side effects. In fact,

a greatly reduced incidence of gastroduodenal lesions was reported for selective COX-2 inhibitors (such as celecoxib and rofecoxib) both in animal and clinical data⁹. However, the increased incidence of gastrointestinal lesions and the decrease in PG levels induced by NSAIDs are not always linked with each other. For example, higher doses of NSAIDs were required for producing gastric lesions than were required for inhibiting COX at the gastric mucosa⁶. Understanding the additional mechanisms is necessary in order to establish an alternative method for development of gastrointestinally safe NSAIDs other than simply increasing their COX-2 selectivity. This new class of NSAIDs may be clinically beneficial because clinical disadvantages (ie. risk of cardiovascular thrombotic disease) of selective COX-2 inhibitors were recently suggested^{7,8} (see below).

In this study, we found that NSAIDs have the direct cytotoxic effect which is independent of the inhibition of COX and dependent on membrane permeabilization activity of NSAIDs and suggest that in addition to COX-inhibition by NSAIDs, direct cytotoxic effect of NSAIDs is involved in NSAID-induced gastric lesions *in vivo*.

RESULTS

1) Direct cytotoxic effect of NSAIDs

Using the primary culture of guinea pig gastric mucosal cells, we examined effect of indomethacin on cell death^{9,10}. As shown in Fig. 1A, treatment of cells with indomethacin for 1 h decreased cell viability in a dose-dependent manner, but did not affect the size of chromosomal DNA (Fig. 1B), even at high concentrations (2.5 mM), suggesting that short-term treatments of gastric mucosal cells with indomethacin induce necrosis. To confirm this finding, we carried out double-staining experiments with PI and Ho 342. Since necrotic cells lose their membrane integrity, PI staining causes pink nuclear staining in necrotic cells. Control cells did not stain with PI but cells treated with 2.5 mM indomethacin for 1 h showed pink nuclear staining (Fig. 1C). We also measured the caspase-3 activities by the use of specific fluorogenic peptide substrates. As shown in Fig. 1D, cells treated with 2.5 mM indomethacin for 1 h did not show higher activity of caspase-3. All these results show that short-term treatments of gastric mucosal cells with indomethacin induce necrosis.

On the other hand, treatment of cells with indomethacin for 16 h decreased cell viability in a dose-dependent manner and induced apoptotic DNA fragmentation, apoptotic chromatin condensation and caspase-3 activation (Fig. 1A-D), showing that long-term treatments of gastric mucosal cells with indomethacin induce apoptosis.

In order to test whether the cytotoxic effect of NSAIDs (necrosis and apoptosis) are dependent of their ability to inhibit COX, we examined the effect of exogenously added PGE₂ on necrosis and apoptosis induced by indomethacin. Exogenously added PGE₂ did not affect the extent of cell death by short-term or long-term treatment with indomethacin (necrosis or apoptosis, respectively) even at higher concentrations of PGE₂ than is present endogenously in medium¹¹. Results suggest that the cytotoxic effect of NSAIDs (necrosis and apoptosis) are independent of their ability to inhibit COX.

2) Membrane permeabilization activity of NSAIDs

We examined membrane permeabilization activity of more than 10 NSAIDs (Nimesulide, celecoxib, mefenamic acid, flufenamic acid, flurbiprofen, indomethacin, diclofenac, etodolac, ibuprofen and ketoprofen) using calcein-loaded liposomes^{12,13}. Calcein fluoresces very weakly at high concentrations due to self-quenching, so the addition of membrane permeabilizing drugs to a medium containing calcein-loaded liposomes should cause an increase in fluorescence by diluting out the calcein¹². All of NSAIDs tested increased the calcein fluorescence, suggesting that they have membrane permeabilization activity. We also examined the necrosis- and apoptosis-inducing ability of these NSAIDs as done for indomethacin in Fig. 1. To examine the relationship between NSAID-induced necrosis or apoptosis and membrane permeabilization, we determined ED₅₀ values of the 10 NSAIDs for necrosis or apoptosis (concentrations of NSAIDs required for 50% inhibition of cell viability by necrosis or apoptosis) and ED₂₀ values for membrane permeabilization (concentration of NSAIDs required for 20% release of calcein). We used ED₂₀ values instead of ED₅₀ values for estimating the activity of each NSAID for membrane permeabilization because ED₅₀ values of etodolac for calcein release could not be determined. Plotting ED₅₀ values for necrosis or apoptosis vs ED₂₀ values for membrane permeabilization (calcein release) yielded an r² value of 0.94 or 0.93, respectively (Fig. 2), which suggests that NSAID-induced necrosis and apoptosis is mediated by their ability to permeabilize membranes.

3) DNA microarray analysis

It is believed that necrosis is induced by drastic permeabilization of cytoplasmic membranes, however, mechanism how membrane permeabilization activity of NSAIDs induces apoptosis remained unclear. In order to understand molecular mechanism governing this apoptosis, we searched for genes whose expression is induced by indomethacin using DNA microarray analy-

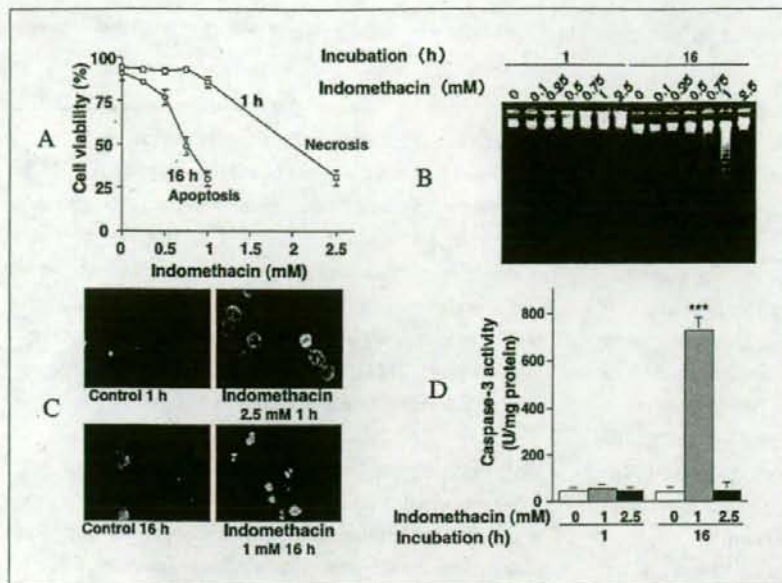


Fig.1 Induction of necrosis and apoptosis by NSAIDs *in vitro*

Cultured gastric mucosal cells were incubated with indicated concentrations of indomethacin for 1 hr or 16 hr. Cell viability was determined by the trypan blue exclusion test (A). Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (B). After staining with both Ho 342 and PI, cells were observed under fluorescence microscope (C). Cell lysates were prepared and their activity of caspase-3 was measured by fluorometric assay using Ac-DEVD-MCA. Values are mean \pm S.E.M. (n=3) (D).

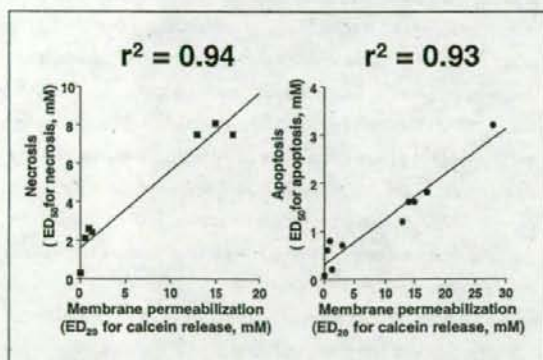


Fig.2 Relationship between necrosis- or apoptosis-inducing and membrane permeabilization activities of NSAIDs

ED_{20} values for membrane permeabilization (calcein release), ED_{50} values for apoptosis and necrosis are calculated and plotted.

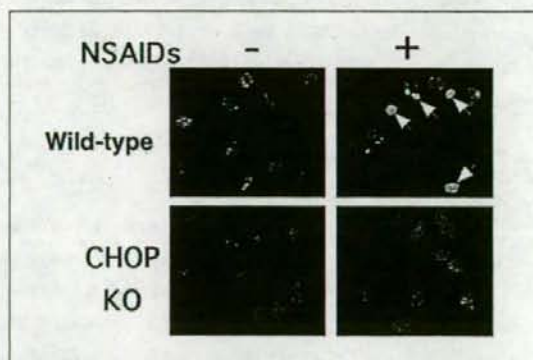


Fig.3 Involvement of CHOP-induction in NSAID-induced apoptosis

Peritoneal macrophages from wild-type or CHOP deficient mice were treated with 1 mM indomethacin for 24 h. Cells were stained with Hoechst dye 33258 and observed under a fluorescence microscope. Arrows indicate condensed chromatins.

sis and found that CHOP, a transcription factor with apoptosis-inducing ability is induced by various NSAIDs¹⁴⁻¹⁶. The analyses conditions were shown in other paper¹⁴. In order to test whether the induction of CHOP by indomethacin is involved in indomethacin-induced apoptosis, we used CHOP deficient mice. Peritoneal macrophages from wild-type mice or CHOP deficient mice were treated with indomethacin for 24 h. As shown in Fig.3, indomethacin-induced chromatin condensation was observed in

peritoneal macrophages from wild-type mice but not so apparently in those from CHOP deficient mice. This result strongly suggests that the induction of CHOP is involved in NSAID-induced apoptosis.

4)Contribution of the increase in the intracellular Ca^{2+} level
Permeabilization of cytoplasmic membranes causes an increase in intracellular Ca^{2+} levels by stimulating Ca^{2+} influx across the

cytoplasmic membrane and we showed that all of NSAIDs tested increases the intracellular Ca^{2+} level¹³. We used BAPTA-AM, an intracellular Ca^{2+} chelator that is permeable for cytoplasmic membranes to test the contribution of the increase in the intracellular Ca^{2+} level to NSAID-induced apoptosis. BAPTA-AM inhibited NSAID-induced cell death, apoptotic chromatin condensation and induction of CHOP in the presence of NSAIDs¹³, suggesting that the increase in intracellular Ca^{2+} levels caused by NSAIDs is involved in NSAID-induced CHOP induction and resulting apoptosis.

5) Development of gastric lesions by a combination of the oral administration of selective COX-2 inhibitors with the intravenous administration of non-selective NSAIDs

We considered that not only COX inhibition (inhibition of PG synthesis) but also the COX-independent direct cytotoxic effect of NSAIDs is involved in the development of gastrointestinal lesions *in vivo*. For testing this idea by pharmacological experiments, it is necessary to separate these two properties of NSAIDs (ie. COX inhibition and direct cytotoxicity) in the model of NSAID-induced gastric lesions *in vivo*. We tried to achieve this by employing intravenous administration of a non-selective NSAID (indomethacin) and oral administration of cytotoxic selective COX-2 inhibitors (such as celecoxib) in rats. Intravenous administration of non-selective NSAIDs may cause inhibition of both COX-1 and COX-2 (thus inhibition of PG synthesis) at the gastric mucosa without any direct cytotoxicity to the gastric mucosa, because the concentration of NSAIDs at the gastric mucosa following intravenous administration is much lower compared to when NSAIDs are orally administered. On the other hand, oral administration of celecoxib may cause direct cytotoxicity to the gastric mucosa without inhibition of COX-1 and thus PG synthesis may be maintained. We also used another selective COX-2 inhibitor, rofecoxib, which shows no cytotoxic effect on gastric mucosal cells¹¹.

Intravenous administration of indomethacin (3 mg/kg) in rats did not produce gastric lesions (Fig.4) even though the level of PGE₂ at the gastric mucosa was reduced by more than 90%. This data suggests that inhibition of COX is not sufficient to produce gastric lesions. On the other hand, oral administration of celecoxib did not by themselves (ie. without intravenous administration of indomethacin) produce gastric lesions (Fig.4). PGE₂ synthesis at the gastric mucosa was not inhibited by the oral administration of celecoxib. Therefore, the absence of gastric lesions only by oral administration of these selective COX-2 inhibitors can be explained by the fact that inhibition of PG syn-

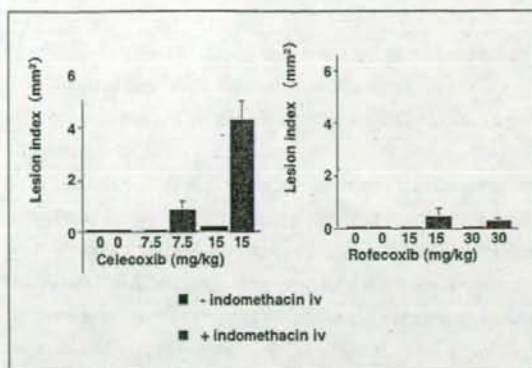


Fig.4 Production of gastric ulcers *in vivo*

Rats were intravenously administered with or without 3 mg/kg indomethacin. After 1 h, animals were orally administered with NSAIDs as indicated. After 6 h, the stomach was removed and scored for hemorrhagic damage. Values are mean \pm S.E.M. (n=6).

thesis is required for the development of gastric lesions by NSAIDs.

Interestingly, a combination of intravenous administration of indomethacin and oral administration of celecoxib clearly produced gastric lesions (Fig.4). On the other hand, a combination of intravenous administration of indomethacin and oral administration of rofecoxib did not significantly produce gastric lesions (Fig.4). Results in Fig.4 suggest that direct cytotoxicity of celecoxib but not rofecoxib is involved in production of gastric lesions. All these results support our idea that not only COX inhibition (inhibition of PG synthesis) but also the COX-independent direct cytotoxic effect of NSAIDs is involved in the development of gastric lesions *in vivo*.

CONCLUSION

Results showed that NSAID-induced apoptosis is mediated by permeabilization of cytoplasmic membranes, increase in the intracellular Ca^{2+} levels and induction of CHOP. Furthermore, results suggest that NSAIDs without membrane permeabilizing activity have reduced gastrointestinal side effects. We consider that the concentrations of NSAIDs required for necrosis and apoptosis *in vitro* are possible *in vivo* associating with gastric ulceration in animal models, as discussed in our previous paper⁹.

An issue that was recently raised concerning the use of COX-2-selective NSAIDs is their potential risk for promoting cardiovascular thrombotic events⁸. Prostacyclin, a potent anti-aggregator of platelets and a vasodilator, is mainly produced by COX-2 in vascular endothelial cells, while thromboxane A₂, a potent

aggregator of platelets and a vasoconstrictor, is mainly produced by COX-1 in platelets¹⁷. Until recently, rofecoxib and celecoxib were leading COX-2 selective NSAIDs in the market. Rofecoxib was withdrawn from the market due to the risk of it promoting cardiovascular thrombotic events and the U. S. Food and Drug Administration (FDA) advised physicians to consider alternatives to celecoxib due to the risk of it causing cardiovascular thrombotic events¹⁸. Based on our findings, NSAIDs that do not exhibit membrane permeabilization activity may be safe for the gastrointestinal tract even if they are not highly selective for COX-2. This type of NSAID may be of clinical benefit because they are predicted to be safe for both the gastrointestinal tract and cardiovascular system. We are now chemically synthesizing such NSAIDs.

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SmdAB, a Heterodimeric ABC-Type Multidrug Efflux Pump, in *Serratia marcescens*[†]

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We cloned genes, designated *smdAB*, that encode a multidrug efflux pump from the chromosomal DNA of clinically isolated *Serratia marcescens* NUSM8906. For cells of the drug-hypersensitive strain *Escherichia coli* KAM32 harboring a recombinant plasmid carrying *smdAB*, structurally unrelated antimicrobial agents such as norfloxacin, tetracycline, 4',6-diamidino-2-phenylindole (DAPI), and Hoechst 33342 showed elevated MICs. The deduced amino acid sequences of both SmdA and SmdB exhibited similarities to the sequences of ATP-binding cassette (ABC)-type multidrug efflux pumps. The efflux of DAPI and Hoechst 33342 from *E. coli* cells expressing SmdAB was observed, and the efflux activities were inhibited by sodium *o*-vanadate, which is a well-known ATPase inhibitor. The introduction of *smdA* or *smdB* alone into *E. coli* KAM32 did not elevate the MIC of DAPI; thus, both SmdA and SmdB were required for function. These results indicate that SmdAB is probably a heterodimeric multidrug efflux pump of the ABC family in *S. marcescens*.

Drug resistance in bacteria is a serious problem in the hospital setting. In particular, multidrug resistance causes difficulty in the treatment of infectious diseases. There are several mechanisms by which bacterial cells escape the toxicities of antimicrobial agents. Such mechanisms include the degradation or modification of the drugs, the alteration of targets, the emergence of alternative pathways, and the efflux of drugs out of the cells. Among the drug resistance mechanisms, drug efflux is a major cause of multidrug resistance and has been found to play a major role in the intrinsic resistance of many bacteria (28, 31).

Serratia marcescens is a cause of nosocomial and opportunistic infections. It has previously been reported to be associated with respiratory tract infections, urinary tract infections, septicemia, meningitis, and wound infections (9). This organism shows high-level intrinsic resistance to a variety of antimicrobial agents, which makes the treatment of infections with this bacterium very difficult. Previously, we compared the MICs of various antimicrobial agents for several strains of *S. marcescens* with those for *Escherichia coli* and *Pseudomonas aeruginosa* (6). Many antimicrobial agents, such as ampicillin, chloramphenicol, erythromycin, tetracycline, and ethidium bromide, showed higher MICs for *S. marcescens* than for *E. coli*. The levels of drug resistance in *S. marcescens* are roughly comparable to those in *P. aeruginosa*, which shows high-level intrinsic resistance to many antimicrobial agents. Since multidrug efflux pumps have been shown to contribute to the intrinsic resistance of *P. aeruginosa* (19, 20, 28), it may be possible

that these pumps are also important for the intrinsic drug resistance of *S. marcescens*.

We have previously reported that we succeeded in the cloning of nine distinct types of genes from the chromosome of *S. marcescens* and that such genes are responsible for drug resistance (6). These genes include *sdeXY* (5), which is a member of the resistance-nodulation-cell division family, and *smfY* (37), which is a member of the major-facilitator superfamily. In addition, Kumar and Worobec have reported the characterization of SdeAB (15). Given the genome sequence of *S. marcescens* Db11, which has been reported by the *S. marcescens* Db11-Sequencing Group at the Sanger Institute (<ftp://ftp.sanger.ac.uk/pub/pathogens/sm/>), many other multidrug efflux pumps that have not been physiologically characterized are expected to be present.

Another family of multidrug efflux pumps is the ATP-binding cassette (ABC) family. The ABC-type multidrug efflux pumps in eukaryotes have been well characterized and have previously been shown to be involved in tolerance to various cytotoxic agents (22). The human P-glycoprotein is a representative of the eukaryotic ABC-type multidrug efflux pumps (4, 41). The ABC-type multidrug efflux pumps utilize ATP as the energy source and are thus primary transporters. Meanwhile, most of the prokaryotic multidrug transporters are secondary transporters. Some ABC-type drug efflux pumps in prokaryotes, especially in gram-positive bacteria, have been characterized in detail previously (23, 25, 35, 38, 39, 42). These include LmrCD of *Lactococcus lactis*, which has been shown to be involved in intrinsic resistance to some antimicrobial agents (21). In addition, three ABC-type drug efflux pumps in gram-negative bacteria have been reported previously: MacAB, MsbA, and VcaM. MacAB is a macrolide-specific efflux pump and has been identified in several gram-negative bacteria, such as *E. coli*, *Salmonella enterica* serovar Typhimurium, and *Neisseria gonorrhoeae* (14, 29, 30, 34). MacA belongs to the membrane fusion protein family, and MacB is an integral mem-

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brane protein with a nucleotide-binding domain. MacAB seems to form a tripartite complex together with TolC, which is a multifunctional outer membrane protein in *E. coli* (13). MsbA is an essential ABC-type pump in *E. coli* and is involved in the transport of lipopolysaccharides and phospholipids (43). Furthermore, Reuter et al. demonstrated that MsbA confers multidrug resistance upon *E. coli* and mediates the transport of ethidium from cells (33). We have previously cloned and characterized a multidrug efflux pump, VcaM, from non-O1 *Vibrio cholerae* (10). MsbA and VcaM probably function as homodimers, similar to LmrA of *L. lactis*.

Here, we report the properties of an ABC-type multidrug efflux pump, SmdAB of *S. marcescens*. This pump rendered host *E. coli* cells resistant to various antimicrobial agents. Moreover, SmdAB probably functions as a heterodimer. To our knowledge, this is the first report of an ABC-type pump functioning as a heterodimer in gram-negative bacteria.

MATERIALS AND METHODS

Bacterial strains and growth. A clinically isolated *S. marcescens* strain, NUSM8906, was used as the source of chromosomal DNA (6). *E. coli* KAM32 (Δ acrB Δ yhjE Δ hsd Δ 5), which lacks the major multidrug efflux pumps AcrAB and YhjE, is hypersusceptible to many antimicrobial agents (7). *E. coli* KAM42 (Δ acrB Δ yhjE Δ hsd Δ 5 Δ tolC), a *tolC*-deficient strain derived from KAM32, was constructed as described previously (32). Cells were grown in Luria (L) broth (18) at 37°C under aerobic conditions.

An environmentally isolated strain, *S. marcescens* Db10, was a kind gift from Jonathan Ewbank of the Centre d'Immunologie de Marseille Luminy, France.

Cloning, sequencing, and gene manipulation. Genes responsible for resistance to antimicrobial agents were cloned from the chromosome of *S. marcescens* (6). Briefly, chromosomal DNA was prepared from *S. marcescens* NUSM8906 by the method of Berns and Thomas (2). The DNA was partially digested with *Sau*3AI, and the fragments from 4 to 10 kbp were separated by sucrose density gradient centrifugation. Plasmid pSTV28 (TaKaRa BIO Inc.) was used as a cloning vector. This vector carries *cat*, the chloramphenicol acetyltransferase gene. Plasmid pSTV28 was digested with *Bam*HI, dephosphorylated with bacterial alkaline phosphatase, and then ligated with the chromosomal DNA fragments by using a ligation kit (version 2; TaKaRa BIO Inc.). Competent cells of *E. coli* KAM32 were transformed with the recombinant plasmids and were spread onto 1.5% agar plates containing L broth, 20 μ g of chloramphenicol/ml, and 0.5 μ g of 4',6'-diamidino-2-phenylindole (DAPI)/ml. The plates were incubated at 37°C for 24 h. We obtained eight candidate hybrid plasmids and selected one of them, named pSDC6.

The DNA insert in plasmid pSDC6 was digested with several restriction endonucleases and subcloned into pSTV28. The resulting plasmids, which had shorter inserts than the original pSDC6 plasmid, were introduced into *E. coli* KAM32 cells, and all transformants were tested for their susceptibilities to DAPI. Of the plasmids that conferred resistance to DAPI upon *E. coli* KAM32, pSDC664 carried the shortest insert and was used for further analysis.

The nucleotide sequence was determined by the dideoxy chain termination method (36) using a DNA sequencer (ALF Express; Pharmacia Biotech). Sequence data were analyzed with GENETYX sequence analysis software (Software Development Co.).

Drug susceptibility tests. The MICs of various antimicrobial agents were determined by using the microdilution method according to the recommendations of the Japanese Society of Chemotherapy (12). Briefly, MICs were determined in Mueller-Hinton broth (Difco) containing each compound in a twofold serial dilution series. The cells were incubated in the test medium at 37°C for 24 h, and growth was examined visually. The MIC of each compound was defined as the lowest concentration that prevented visible growth.

Efflux assays. The DAPI efflux assay was carried out as described previously (16). Briefly, cells of *E. coli* KAM32 harboring control or recombinant plasmids were grown in 20 ml of L broth containing 20 μ g of chloramphenicol/ml and 0.5 mM isopropyl β -D-thiogalactopyranoside (IPTG) until the optical density at 650 nm reached 0.7 units. After the cells were harvested, they were washed with modified Tanaka buffer (27, 40) and were then resuspended in the same buffer containing 5 μ M DAPI and 1 mM 2,4-dinitrophenol (DNP) and incubated at 37°C for 10 h. DNP, which is a well-known conductor of protons across the

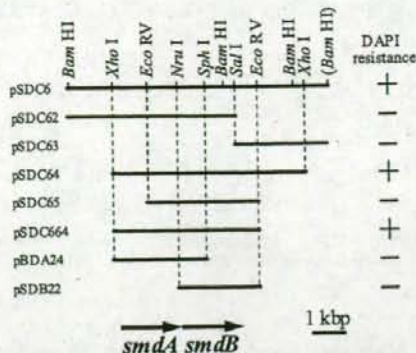


FIG. 1. Restriction map of pSDC6 and its derivatives. DNA regions derived from *S. marcescens* chromosomal DNA and carried by each plasmid are shown. The ability of *E. coli* KAM32 cells harboring each plasmid to grow in L broth containing 0.5 μ g of DAPI/ml is indicated on the right; plus signs indicate that cells grew, and minus signs indicate that cells did not grow. The positions and directions of the *smdAB* genes as revealed by sequencing are shown at the bottom.

cytoplasmic membrane (1), was used to de-energize the cells. The cells were washed with modified Tanaka buffer and then resuspended in the same buffer to obtain an optical density at 650 nm of 0.4 units. The fluorescence of DAPI was measured at excitation and emission wavelengths of 355 and 457 nm, respectively, with a fluorescence spectrophotometer, model F-2000 (Hitachi). The fluorescence intensity of DAPI is higher when DAPI binds to DNA molecules. Thus, the efflux of DAPI from the cell can be monitored by the detection of a decrease in the level of fluorescence over time. The cell suspension was incubated at 37°C for 5 min, and then glucose at 20 mM was added as an energy source to monitor the efflux of DAPI.

The assay for the efflux of Hoechst 33342 was carried out as described previously (10). Briefly, cells were cultured and washed as described above. Washed cells were resuspended in modified Tanaka buffer containing 1 μ M Hoechst 33342 and 1 mM DNP and incubated at 37°C for 10 h. The cells were washed with 100 mM 3-morpholinopropanesulfonic acid-tetramethylammonium hydroxide (MOPS-TMAH) containing 1 μ M Hoechst 33342 and then resuspended in the same buffer to obtain an optical density at 650 nm of 0.4 units. The fluorescence of Hoechst 33342 was measured at excitation and emission wavelengths of 355 and 457 nm, respectively.

To evaluate the effects of sodium *o*-vanadate on the efflux of DAPI or Hoechst 33342, cell suspensions were prepared in the same way as described above. The cell suspensions were preincubated for 5 min at 37°C with different concentrations of sodium *o*-vanadate (0 to 3 mM) prior to the addition of glucose.

RT-PCR analysis. Total RNA from cells of *S. marcescens* NUSM8906 and Db10 that were grown in L broth until the exponential growth phase was extracted by using the QIAGEN RNeasy mini kit. For efficient RNA extraction, the cells were well broken using a QIASHredder (QIAGEN Inc.) prior to the extraction of RNA. The extracted total RNA was used for reverse transcription-PCR (RT-PCR) with the QIAGEN one-step RT-PCR kit. PCR without the RT reaction was performed to confirm the lack of detectable DNA contamination. RT-PCR products were analyzed by 3% agarose 21 gel (Nippon Gene Co.) electrophoresis.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession no. AB360548.

RESULTS

Cloning of *smdAB* and sequence analysis. To understand the role of multidrug efflux pumps in the intrinsic multidrug resistance of *S. marcescens*, it is important to identify multidrug efflux pump genes. We previously cloned nine distinct types of genes from the chromosome of *S. marcescens* NUSM8906 and found that such genes are responsible for drug resistances (6).

		Walker A	
SmdA (<i>S. mar</i>)	334	-VADADIRAEHPEN- PHPAHDALTLKPGQML CPHCGKSTHLSLQRRQ DVGGQ	393
SmdB (<i>S. mar</i>)	340	-RDIITDLSAAL- RADKKVQHSLAVPSRQGV VCHCGKSTHLSLQRRQ DVGGQ	398
Md1A (<i>E. coli</i>)	334	-EDVNIHQETHPQT- DHPAENNFALKPGQML CPHCGKSTHLSLQRRQ DVSESG	393
Md1B (<i>E. coli</i>)	340	-TEVDNVSAAL- RDDNLVKGNLSVPSRNFV VCHCGKSTHLSLQRRQ DVSESG	398
LmrC (<i>L. lac</i>)	331	-SKFDFHVSSEPNPND- EETPKDSEFEVPGQMV VCAICGKSTHLSLQRRQ DVSESG	390
LmrD (<i>L. lac</i>)	424	-GQIENLDSEAL- LPKPKVKKINIDVKKQMV VCPHCGKSTHLSLQRRQ DVNGSA	482
AbcA (<i>B. bre</i>)	386	-DRFPAHVSAAAPDDPETPI- KDDFTVPAGSKL CPHCGKSTHLSLQRRQ DVSESG	446
AbcB (<i>B. bre</i>)	351	GKDFNDVVSRR- EDDGRNINLDFHVTGKTI VCPHCGKSTHLSLQRRQ DVSESG	411
EfrA (<i>S. fae</i>)	330	-YEFKNVTAAAPGHAESFV- RNSFKASPGETV VCHCGKSTHLSLQRRQ DVSESG	390
EfrB (<i>S. fae</i>)	344	-SEEFENVSSAAL- DPEKPLRNINFKVDAGQMV VCPHCGKSTHLSLQRRQ DVTEGA	402
LmrA (<i>L. lac</i>)	348	-TSAHVDAAAL- DDS-EQIHDSPFAQPNII VCPHCGKSTHLSLQRRQ DVSESG	406
BmrA (<i>B. sub</i>)	340	-PQLDRVSSGAL- KPD-QLIKGSAVIRAGKVT VCPHCGKSTHLSLQRRQ DVSESG	398
HorA (<i>L. bre</i>)	340	-TQMNHVSSEAL- DQ-HPIISQSPFAEPNSVI VCPHCGKSTHLSLQRRQ DVSESG	398
MabA (<i>E. coli</i>)	341	-DIEFRNVTITTPGR- DVPAERNLNKIPAKTV VCPHCGKSTHLSLQRRQ DVSESG	400
VcaM (<i>V. cho</i>)	360	-GTFDDVSSAAL- GENKG-VINHNLNLIKPGK VVRCPHCGKSTHLSLQRRQ DVSESG	418
Savi1866 (<i>S. aur</i>)	339	-RDIIDHVSAAAL- NDNEAPIKDNLSIEKGETV VCPHCGKSTHLSLQRRQ DVTSSE	398
		ABC signature	Walker B
SmdA (<i>S. mar</i>)	461	GYDTEVGRGVMSGQCR RSESRALLDSEELDGLL LAMEGRTHQLHNRQWGQ	520
SmdB (<i>S. mar</i>)	465	G1HTRVCGQNNISVGCCL LSESRVIVQAOQLDGLL LAMEGRTHQLHNRQWGQ	524
Md1A (<i>E. coli</i>)	461	GYDTEVGRGVMSGQCR RSESRALLDSEELDGLL LAMEGRTHQLHNRQWGQ	520
Md1B (<i>E. coli</i>)	465	G1YTPVCGQNNISVGCCL LSESRVIVQAOQLDGLL LAMEGRTHQLHNRQWGQ	524
LmrC (<i>L. lac</i>)	458	KYDSEVGRGNISGQCR RSESRALLDSEELDGLL LAMEGRTHQLHNRQWGQ	517
LmrD (<i>L. lac</i>)	550	KYETHSDESVESVGCCL LSESRVIVQAOQLDGLL LAMEGRTHQLHNRQWGQ	609
AbcA (<i>B. bre</i>)	514	GYDTEVGRGVMSGQCR RSESRALLDSEELDGLL LAMEGRTHQLHNRQWGQ	574
AbcB (<i>B. bre</i>)	479	GYDTEVGRGVMSGQCR RSESRALLDSEELDGLL LAMEGRTHQLHNRQWGQ	538
EfrA (<i>S. fae</i>)	458	GYDEPISGGTNSGQCR RSESRALLDSEELDGLL LAMEGRTHQLHNRQWGQ	517
EfrB (<i>S. fae</i>)	470	GYEMEDSEGDNISGQCR RSESRALLDSEELDGLL LAMEGRTHQLHNRQWGQ	529
LmrA (<i>L. lac</i>)	475	QLNTEVGRGVMSGQCR RSESRALLDSEELDGLL LAMEGRTHQLHNRQWGQ	534
BmrA (<i>B. sub</i>)	467	QFDTEVGRGVMSGQCR RSESRALLDSEELDGLL LAMEGRTHQLHNRQWGQ	526
HorA (<i>L. bre</i>)	467	GLDTEVGRGVMSGQCR RSESRALLDSEELDGLL LAMEGRTHQLHNRQWGQ	526
MabA (<i>E. coli</i>)	469	GLDTEVGRGVMSGQCR RSESRALLDSEELDGLL LAMEGRTHQLHNRQWGQ	528
VcaM (<i>V. cho</i>)	491	GYDAQVGRGVMSGQCR RSESRALLDSEELDGLL LAMEGRTHQLHNRQWGQ	550
Savi1866 (<i>S. aur</i>)	466	GYDTEVGRGVMSGQCR RSESRALLDSEELDGLL LAMEGRTHQLHNRQWGQ	525

FIG. 2. Multiple-sequence alignments of SmdA, SmdB, and similar or putative proteins. The amino acid sequence alignments of the Walker A motif, the Walker B motif, and the ABC signature sequences are shown. Identical and similar residues are indicated with black and gray backgrounds, respectively. Gaps in the alignment are indicated by hyphens. The numbers on the left and right of the sequences indicate the beginning and ending positions of each sequence, respectively. The sequences were aligned by using the EMBL ClustalW program (available at http://decypher.stanford.edu/decypher/algo-cw_ax.shtml). The names of the organisms from which the proteins are derived are shown in Table 1. *S. mar*, *S. marcescens*; *L. lac*, *L. lactis*; *B. bre*, *Bifidobacterium breve*; *E. fae*, *Enterococcus faecalis*; *B. sub*, *Bacillus subtilis*; *L. bre*, *Lactobacillus brevis*; *V. cho*, *V. cholerae*; *S. aur*, *Staphylococcus aureus*.

One of the recombinant plasmids, pSDC6, rendered *E. coli* KAM32 cells resistant to DAPI, norfloxacin, and tetracycline. Judging from the spectrum of drug resistance, it seemed that the plasmid pSDC6 carried other genes different from *sdeXY* and *smfY*, which we had already reported (5, 37). Thus, we analyzed pSDC6 further.

Plasmid pSDC6 carries a DNA insert about 8 kbp long. We constructed a series of deletion plasmids carrying various portions of the DNA insert in pSDC6 and tested whether those plasmids conferred DAPI resistance upon *E. coli* cells (Fig. 1). Plasmid pSDC664 carried the shortest DNA insert that conferred DAPI resistance. The sequencing of this insert revealed two open reading frames (ORFs). We designated the ORFs *smdA* and *smdB* (for *Serratia* multidrug resistance). The putative gene products were estimated to comprise 591 and 592

amino acid residues, respectively. Only the *smdA* gene has a promoter-like sequence in its upstream region, and both genes have ribosome-binding sequences (Shine-Dalgarno sequences), each of which is followed by a start codon. The *smdB* gene is followed by a transcription terminator-like (inverted repeat) sequence. The two ORFs overlap by 5 nucleotides. Hydropathy analysis by the method of Eisenberg et al. (8) suggested that both SmdA and SmdB possess six putative transmembrane segments followed by hydrophilic segments (data not shown). The hydrophilic segments of both SmdA and SmdB contain putative nucleotide-binding domains, Walker A and Walker B motifs, and ABC signature sequences (11) (Fig. 2).

The comparison of our sequence with the genome sequence of *S. marcescens* Db11 (a streptomycin-resistant mutant of Db10) (<ftp://ftp.sanger.ac.uk/pub/pathogens/sm/>) showed that

TABLE 1. Sequence similarities to SmdA and SmdB

Transporter	Organism	% Identity to SmdA	% Similarity to SmdA	% Identity to SmdB	% Similarity to SmdB	Reference or accession no.
SmdA	<i>S. marcescens</i>			27	70	This study
SmdB	<i>S. marcescens</i>	27	70			This study
MdIA	<i>E. coli</i>	79	96	26	68	P77265
MdIB	<i>E. coli</i>	26	70	79	96	P0AAG5
LmrC	<i>L. lactis</i>	26	68	22	67	AAK04408
LmrD	<i>L. lactis</i>	26	71	24	64	AAK04409
EfrA	<i>Enterococcus faecalis</i>	26	70	24	69	NP_816538
EfrB	<i>Enterococcus faecalis</i>	27	66	28	70	NP_816537
AbcA	<i>Bifidobacterium breve</i>	17	33	27	67	DQ486860
AbcB	<i>Bifidobacterium breve</i>	28	70	29	71	DQ486860
BmrA	<i>Bacillus subtilis</i>	28	68	27	69	D70031
HorA	<i>Lactobacillus brevis</i>	27	68	24	62	AB005752
LmrA	<i>L. lactis</i>	26	70	26	64	U63741
MsbA	<i>E. coli</i>	28	69	27	68	P27299
VcaM	<i>V. cholerae</i>	27	68	26	68	AB073220
MDR1 (N) ^a	<i>Homo sapiens</i>	23	56	23	58	P08183
MDR1 (C) ^a	<i>Homo sapiens</i>	26	67	25	66	P08183

^a N, N-terminal half; C, C-terminal half.

smdA corresponded to SMA0354 and *smdB* to SMA0355. Eighty differences in the nucleotide sequences of *smdA* and SMA0354 and 83 differences between those of *smdB* and SMA0355 were identified. Almost all of these differences were translationally silent, but nine and six amino acid residues were different, respectively. In addition, the ORF of *smdB* was 132 nucleotides shorter than that of SMA0355. SmdA and SmdB showed 27% identity and 70% similarity to each other. A BLAST search of the NCBI database for protein sequence similarities showed SmdA to be 79% identical to MdIA and SmdB to be 79% identical to MdIB of *E. coli*. The search also showed both SmdA and SmdB to have nearly 30% identity to other ABC-type multidrug efflux pumps (Table 1). The levels of similarity in the nucleotide-binding domains were much higher than those in the transmembrane domains.

We also investigated the expression of *smdAB* in *S. marcescens* NUSM8906 (a clinical isolate) and Db10 (an environmental isolate). RT-PCR analysis showed that *smdAB* was expressed to similar extents in the two strains (data not shown).

Drug susceptibility. To investigate the contribution of SmdAB to drug resistance, we measured the MICs of various antimicrobial agents for *E. coli* KAM32 cells to which the plasmid carrying *smdAB* had been introduced. The MICs for *E. coli* KAM32 cells harboring pSDC664 (which carries *smdAB*) or pSTV28 (control) are shown in Table 2. For *E. coli* KAM32 cells, the introduction of the plasmid pSDC664 elevated the MICs of several structurally unrelated drugs: norfloxacin, tetracycline, Hoechst 33342, and tetraphenylphosphonium chloride (TPPCI), in addition to DAPI. Therefore, we concluded that SmdAB conferred multidrug resistance upon *E. coli* KAM32.

MacAB is the sole pump in *E. coli* characterized as an ABC-type drug efflux pump (14). MacAB has previously been shown to require the outer membrane component TolC for function. We therefore investigated whether TolC was required for the function of SmdAB. *E. coli* KAM42 is a *tolC*-lacking strain derived from strain KAM32. The introduction of the plasmid pSDC664 into cells of *E. coli* KAM42 resulted in elevated MICs of various antimicrobial agents, similar to those

for *E. coli* KAM32 (data not shown). Thus, we conclude that TolC is not necessary for the function of SmdAB.

To test whether both SmdA and SmdB are necessary for the pump function, we constructed plasmids carrying each one of the corresponding genes. Plasmid pBDA24 carried *smdA*, while plasmid pSDB22 carried *smdB*. Both plasmids were introduced into *E. coli* KAM32 cells. The *smdA* gene was located under the control of the *tet* promoter in the pBR322 vector, and *smdB* was located under the control of the *lac* promoter in the pSTV28 vector (Fig. 1). Since vectors pBR322 and pSTV28 were compatible, both plasmids could be retained simultaneously. The drugs tested did not show elevated MICs for either the KAM32 transformant harboring pBDA24 (carrying *smdA*) or the KAM32 transformant harboring pSDB22 (carrying *smdB*) (Table 3). On the other hand, norfloxacin, tetracycline, TPPCI, and Hoechst 33342 showed elevated MICs for the KAM32 transformant harboring both pBDA24 and pSDB22, similar to those for KAM32 harboring pSDC664 (carrying *smdAB*). Thus, we conclude that both SmdA and SmdB are necessary for resistance.

TABLE 2. MICs of various antimicrobial agents for *E. coli* KAM32/pSTV28 and KAM32/pSDC664

Antimicrobial agent	MIC (μ g/ml) for:		Increase (<i>n</i> -fold) in MIC ^a
	KAM32/pSTV28	KAM32/pSDC664	
DAPI	0.25	8	32
Norfloxacin	0.016	0.125	8
Ciprofloxacin	0.002	0.004	2
Ofloxacin	0.016	0.016	1
Nalidixic acid	1	1	1
Tetracycline	0.25	2	8
Streptomycin	2	2	1
Erythromycin	4	4	1
Ampicillin	2	2	1
Hoechst 33342	0.25	4	16
TPPCI	4	16	4
Acridavine	2	2	1

^a Increase in MIC for *E. coli* KAM32/pSDC664 compared to that for *E. coli* KAM32/pSTV28.

TABLE 3. MICs of various antimicrobial agents for *E. coli* KAM32 cells carrying *smdA* and/or *smdB*

Antimicrobial agent	MIC ($\mu\text{g/ml}$) for:			
	KAM32/pSTV28 (negative control)	KAM32/pBDA24 (carrying <i>smdA</i>)	KAM32/pSDB22 (carrying <i>smdB</i>)	KAM32/pBDA24/pSDB22 (carrying <i>smdA</i> and <i>smdB</i>)
DAPI	0.25	0.25	0.25	8
Norfloracin	0.016	0.016	0.016	0.125
Tetracycline	0.25	0.25	0.25	2
Hoechst 33342	0.25	0.25	0.25	4
TPPCI	4	4	4	16

Efflux of DAPI and Hoechst 33342. In order to show that SmdAB is a multidrug efflux pump, we measured the efflux of DAPI and Hoechst 33342. Cells of *E. coli* KAM32 harboring either pSDC664 (carrying *smdAB*) or pSTV28 (control) were de-energized and preloaded with DAPI. The addition of glucose as an energy source caused the rapid extrusion of DAPI from KAM32 cells harboring pSDC664 compared with that from KAM32 cells harboring pSTV28 (Fig. 3A). When we measured the efflux of Hoechst 33342, we obtained a similar result (Fig. 3B). The addition of lactate instead of glucose as an energy source caused similar levels of extrusion (data not shown). These results indicate that SmdAB is an energy-dependent multidrug efflux pump.

Inhibition of SmdAB-mediated DAPI efflux by vanadate. From the primary amino acid sequence, SmdAB was categorized within the ABC family of multidrug efflux pumps. The activities of some ABC-type multidrug efflux pumps have previously been reported to be inhibited by sodium *o*-vanadate, an inhibitor of some ATPases (10, 17, 26, 35, 39, 42). We investigated the effect of sodium *o*-vanadate on the DAPI efflux activity of SmdAB. As shown in Fig. 4, sodium *o*-vanadate inhibited the activity in a concentration-dependent manner. The concentration causing 50% inhibition was approximately 1.1 mM. This 50% inhibitory concentration of sodium *o*-vanadate is similar to those for other ABC-type multidrug efflux pumps (10, 17). Meanwhile, Hoechst 33342 efflux activity was also inhibited by sodium *o*-vanadate (data not shown). Thus, it seems that SmdAB is an ATP-dependent multidrug efflux pump of *S. marcescens*.

DISCUSSION

We previously cloned genes that conferred multidrug resistance upon drug-hypersusceptible *E. coli* cells (6). We designated the genes *smdA* and *smdB* and characterized the properties of SmdAB. SmdAB was categorized into the ABC family of multidrug efflux pumps according to the primary structure. Both SmdA and SmdB were found to contain putative nucleotide-binding domains, Walker A and Walker B motifs, and ABC signature sequences (11) (Fig. 2). We observed the elevation of the MICs of several antimicrobial agents for cells into which *smdAB* was introduced and detected energy-dependent efflux of DAPI and Hoechst 33342 in these cells. The efflux of DAPI and Hoechst 33342 mediated by SmdAB was inhibited by sodium *o*-vanadate, which is a known ATPase inhibitor. Thus, we conclude that SmdAB is an ABC-type multidrug efflux pump. We found that both SmdA and SmdB were necessary for pump function. To our knowledge, SmdAB is the first example of a probably heterodimeric ABC-type multidrug efflux pump in gram-negative bacteria.

Several ABC-type multidrug efflux pumps have been cloned from gram-positive bacteria and characterized previously (10, 14, 17, 23, 25, 29, 33, 35, 38, 39, 42). Among them, LmrCD in *L. lactis* has been demonstrated to be a heterodimeric ABC-type multidrug efflux pump and to contain two structurally and functionally distinct nucleotide-binding domains (24). In LmrD, a canonical glutamate residue following the Walker B motif, which has been postulated to fulfill a critical catalytic role in the hydrolysis of ATP (3), is conserved, but in LmrC,

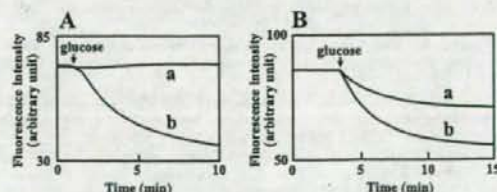


FIG. 3. Efflux of DAPI and Hoechst 33342 via SmdAB. Energy-starved *E. coli* KAM32 cells harboring pSDC664 (carrying *smdAB*) (curves b) or KAM32 cells harboring pSTV28 (control) (curves a) were loaded with 5 μM DAPI (panel A) or 1 μM Hoechst 33342 (panel B). At the time point indicated by the arrow, glucose (final concentration, 20 mM) was added to energize the cells. The fluorescence of dyes at 37°C over time was monitored with a fluorescence spectrophotometer. The downward deflection indicates the efflux of DAPI or Hoechst 33342 from the cells.

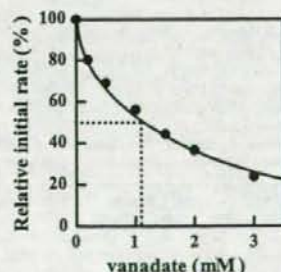


FIG. 4. Inhibition of DAPI efflux activity by sodium *o*-vanadate. Various concentrations of sodium *o*-vanadate were added to the assay mixture, and the mixture was preincubated with the cells for 5 min. Glucose (final concentration, 20 mM) was added to initiate the assay. The relative initial velocity of DAPI efflux was measured. The initial velocity observed in the absence of an inhibitor was set at 100%. Dotted lines indicate IC_{50} (approximately 1.1 mM).

this residue is replaced with a noncanonical aspartate residue. In each pair of heterodimeric transporters shown in Fig. 2, including SmdAB, one polypeptide contains a canonical glutamate residue and the other polypeptide contains a noncanonical aspartate residue instead of a glutamate residue. This pattern may be a feature of heterodimeric ABC-type transporters.

By searching with the BLAST system, we found homologues of SmdAB in other microorganisms, such as *E. coli*, *Yersinia pestis*, *Shigella flexneri*, *Salmonella enterica* serovar Typhimurium, and *Vibrio parahaemolyticus*, etc. In all cases, two ORFs were located in tandem, and many of the genes seemed to encode the multidrug resistance ABC-type proteins. Among them, only *mdlAB* in *E. coli* has been cloned (30). However, *mdlAB* did not confer any drug resistance upon *E. coli* host cells even if expressed from the high-copy-number plasmid pUC119 (30). We cloned *smdAB* into a middle-copy-number plasmid. Thus, it seems that the differences in copy number did not cause the phenotypic differences. It is possible that *mdlAB* by itself does not possess drug efflux activity; another possibility is that the expression of *mdlAB* is repressed by an unknown mechanism at the transcriptional or translational steps.

It has been reported previously that ABC-type efflux pumps have some roles aside from drug resistance (22). Since homologues of SmdAB are widely distributed in gram-negative bacteria, it may be possible that SmdAB-type ABC pumps have some important physiological roles. Further analyses of SmdAB and its homologues should be necessary to understand such unknown roles.

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Analysis of mutant origin recognition complex with reduced ATPase activity *in vivo* and *in vitro*

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In eukaryotes, ORC (origin recognition complex), a six-protein complex, is the most likely initiator of chromosomal DNA replication. ORC belongs to the AAA⁺ (ATPases associated with a variety of cellular activities) family of proteins and has intrinsic ATPase activity derived from Orc1p, one of its subunits. To reveal the role of this ATPase activity in *Saccharomyces cerevisiae* (baker's yeast) ORC, we mutated the Orc1p sensor 1 and sensor 2 regions, which are important for ATPase activity in AAA⁺ proteins. Plasmid-shuffling analysis revealed that Asn⁶⁰⁰, Arg⁶⁹⁴ and Arg⁷⁰⁴ are essential for the function of Orc1p. In yeast cells, overexpression of Orc1R694Ep inhibited growth, caused inefficient loading of MCM (mini-chromosome maintenance complex of proteins) and slowed the progression of S phase. *In vitro*, purified ORC-1R [ORC with Orc1R694Ep (Orc1p

Arg⁶⁹⁴→Glu mutant)] has decreased ATPase activity in the presence or absence of origin DNA. However, other activities (ATP binding and origin DNA binding) were indistinguishable from those of wild-type ORC. The present study showed that Arg⁶⁹⁴ of the Orc1p subunit is important for the ATPase activity of ORC and suggests that this ATPase activity is required for efficient MCM loading on to origin DNA and for progression of S phase.

Key words: ATPases associated with a variety of cellular activities family of proteins (AAA⁺ family of proteins), ATPase, DNA replication, origin recognition complex (ORC), origin recognition complex subunit 1p (Orc1p), sensor 1 and sensor 2 regions.

INTRODUCTION

The initiation of chromosomal DNA replication must be tightly regulated to maintain just one copy of each genome per cell; in prokaryotes, adenine nucleotides bound to the initiator protein control this process. In *Escherichia coli*, the DnaA protein, the initiator of chromosomal DNA replication, has a high affinity for both ATP and ADP [1]. The ATP–DnaA complex is active for DNA replication both *in vivo* and *in vitro*, but the ADP–DnaA complex is inactive [1–3]. DnaA has intrinsic ATPase activity and can hydrolyse bound ATP to ADP [1]. This hydrolysis inactivates DnaA, suppressing re-replication and thus regulating initiation of DNA replication [4–7]. Acidic phospholipids, such as cardiolipin [1,3-bis-(*sn*-3'-phosphatidyl)-*sn*-glycerol], interact with DnaA and decrease its affinity for adenine nucleotides and origin DNA [8–12].

In eukaryotes, ORC (origin recognition complex) is the most likely initiator of chromosomal DNA replication [13–16]. ORC was originally identified as a six-protein complex (subunits Orc1p–Orc6p) that specifically binds to *Saccharomyces cerevisiae* (baker's yeast) origins of chromosomal DNA replication [17]. ORC homologues have been found in various eukaryotes, including humans [18]. ORC in *S. cerevisiae* has been studied the most extensively, both biochemically and genetically. In this manuscript, 'ORC' refers to *S. cerevisiae* ORC, unless otherwise stated.

ORC has two subunits (Orc1p and Orc5p), which bind to ATP. Orc1p, but not Orc5p, has ATPase activity [19]. ATP binding to Orc1p is essential for specific ORC binding to origin DNA, whereas the ADP–ORC complex does not bind [19–21]. By

contrast, ATP binding to Orc5p is important for the stability of ORC in cells [22,23]. Furthermore, we found that acidic phospholipids, such as cardiolipin [1,3-bis-(*sn*-3'-phosphatidyl)-*sn*-glycerol], interact with ORC and decrease its affinity for adenine nucleotides and origin DNA [24,25]. These findings suggested that adenine nucleotides bound to ORC can regulate the initiation of DNA replication. The ATPase activity may regulate initiation by stimulating DNA replication rather than by suppressing re-replication (see below).

ORC binds to origins of chromosomal DNA replication throughout the cell cycle. It functions as a 'landing pad' and recruits other DNA-replication-related proteins [such as Cdc6p, Cdt1p and MCM (mini-chromosome maintenance complex of proteins)] to origin DNA to form a pre-RC (pre-replicative complex) [26]. Klemm and Bell [27] reported that overexpression of mutant ORC with reduced ATPase activity [ORC with Orc1D569Yp (Asp⁵⁶⁹→Tyr mutant) or with Orc1D569Fp; Asp⁵⁶⁹ is in Walker B motif (hhhhDEXX, where h is a hydrophobic residue)] inhibited the loading of MCM on to origin DNA. However, simultaneous overexpression of Cdc6p restores MCM loading, suggesting that the defect is due to titration of Cdc6p [27]. Bowers et al. [28] reported that another mutant ORC without ATPase activity (ORC with Orc4R267Ap) inhibits the reiterative loading of MCM on to origin DNA, both *in vivo* and *in vitro*. Furthermore, Speck et al. [29] showed that the ATPase activity of ORC changes how the ORC–Cdc6p complex binds to origin DNA *in vitro*; this may be required for pre-RC formation. Thus the ATPase activity of ORC apparently stimulates pre-RC formation, especially the loading of MCM on to origin DNA. However, the evidence supporting this mechanism is not

Abbreviations used: AAA⁺, ATPases associated with a variety of cellular activities; ARS1, autonomously replicating sequence 1; ChIP, chromatin immunoprecipitation; 5-FOA, 5-fluoro-orotic acid; HIS3, imidazoleglycerol-phosphate dehydratase gene; LEU2, β-isopropylmalate dehydrogenase gene; MCM, mini-chromosome maintenance complex of proteins; ORC, origin recognition complex; pre-RC, pre-replicative complex; Orc1p, ORC subunit 1p (etc.); ORC-1A, ORC containing mutant Orc1p with defective Walker A motif (GXXGXGK¹₁₅); ORC-1R, ORC with Orc1R694Ep (Orc1p Arg⁶⁹⁴→Glu mutant); SC, synthetic complete; TRP1, phosphoribosylanthranilate isomerase gene; URA3, uracil-biosynthesis gene.

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