Endocytic Ark/Prk Kinases Play a Critical Role in Adriamycin Resistance in Both Yeast and Mammalian Cells

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Abstract
To elucidate the mechanism of acquired resistance to Adriamycin, we searched for genes that, when overexpressed, render Saccharomyces cerevisiae resistant to Adriamycin. We identified AKL1, a gene of which the function is unknown but is considered, nonetheless, to be a member of the Ark/Prk kinase family, which is involved in the regulation of endocytosis, on the basis of its deduced amino acid sequence. Among tested members of the Ark/Prk kinase family (Ark1, Prk1, and Akl1), overexpressed Prk1 also conferred Adriamycin resistance on yeast cells. Prk1 is known to dissociate the Sla1/Pan1/End3 complex, which is involved in endocytosis, by phosphorylating Sla1 and Pan1 in the complex. We showed that Akl1 promotes phosphorylation of Pan1 in this complex and reduces the endocytic ability of the cell, as does Prk1. Sla1- and End3-defective yeast cells were also resistant to Adriamycin and overexpression of Akl1 in these defective cells did not increase the degree of Adriamycin resistance, suggesting that Akl1 might reduce Adriamycin toxicity by reducing the endocytic ability of cells via a mechanism that involves the Sla1/Pan1/End3 complex and the phosphorylation of Pan1. We also found that HEK293 cells that overexpressed AAK1, a member of the human Ark/Prk family, were Adriamycin resistant. Our findings suggest that endocytosis might be involved in the mechanism of Adriamycin toxicity in yeast and human cells. (Cancer Res 2006; 66(24): 11932-7)

Introduction
Adriamycin is an anticancer drug that is widely used in a clinical setting (1). The mechanism of action of Adriamycin, via inhibition of nucleic acid synthesis by intercalation into DNA and promotion of the cleavage of DNA by inhibition of DNA topoisomerase II, is well known, as is the mechanism of cytotoxicity, which involves the production of free radicals, but many other effects of the drug have also been reported, with many issues remaining to be clarified (2, 3). In cancer chemotherapy with Adriamycin, both the natural and the acquired resistance of cancer cells pose serious problems, as do the adverse effects of the drug (4). The acquisition of Adriamycin resistance involves promotion of the excretion of the drug from cells by overexpressed ATP-binding cassette transporters, such as P-glycoprotein and multidrug-resistance protein (5–7), and, in addition to the transporters involved in drug excretion, many other factors seem to be related to the mechanism of Adriamycin resistance. However, the properties of some Adriamycin-resistant cells cannot be explained by these phenomena, suggesting the presence of as yet unknown mechanisms of acquired resistance (8, 9). To shed further light on mechanisms of Adriamycin toxicity and the acquisition of Adriamycin resistance, we have identified genes involved in Adriamycin resistance in Saccharomyces cerevisiae. S. cerevisiae is widely used as a model eukaryote and its entire genome has been sequenced. Because many products of yeast genes have functions similar to those of human genes, information obtained from yeast cells might help to clarify the mechanism of acquisition by human cells of resistance to Adriamycin. We previously searched for genes that conferred Adriamycin resistance on yeast cells when such genes were overexpressed, using a yeast DNA genomic library, and we identified the SSL2 (10, 11) and BSD2 (12) genes. In the present study, we identified AKL1 as a gene of which the overexpression rendered yeast cells resistant to Adriamycin. Akl1 is considered to be a member of the Ark/Prk kinase family and to be involved in the regulation of endocytosis on the basis of certain characteristics of its deduced amino acid sequence (13), but, to our knowledge, its function has not been fully analyzed and its relationship to drug resistance has not been investigated.

This study revealed the involvement of Akl1 in the regulation of endocytosis via phosphorylation of Pan1 in the Sla1/Pan1/End3 complex, as is Prk1 (14, 15). In addition, our results suggest that overexpressed Akl1 inhibits the internalization step of endocytosis, leading to Adriamycin resistance in yeast cells. Because human cells that overexpressed AAK1 (16), which is a member of the human Ark/Prk kinase family that is known to be involved in the regulation of endocytosis, were also Adriamycin resistant, it is possible that the Ark/Prk kinase family might be involved in Adriamycin toxicity via the regulation of endocytosis not only in yeast cells but also in human cells.

Materials and Methods

Yeast strains and media. The wild-type yeast strains used in this study were W303B (MATa his3 can1-100 ade2 leu2 trp1 ura3; ref. 17) and BY4742 (MATa his3A1 leu2A0 lys2A0 ura3A0). The deletion strains (aak1A, arka1A, prk1A, end3A, sla1A, sla2A, vpr1A, rvs161A, yps23A, and vps27A), derived from the parent BY4742 strain, were obtained from Euroscarf (Frankfurt, Germany). Yeast cells were grown in synthetic dextrose medium with or without leucine. For the characterization of cells that harbored the expression vector pYES2 (Invitrogen, Carlsbad, CA) or genes expressed under the control of the GAL1 promoter, cells were grown in synthetic galactose medium that contained 2% galactose and 4% raffinose as the carbon source instead of dextrose.

Quantification of the toxicity of Adriamycin in yeast cells. Yeast cells were cultured (1 × 105/200 μL) in 96-well plates in synthetic dextrose medium that contained Adriamycin at various concentrations. After incubation for 48 hours, absorbance at 620 nm was determined spectrophotometrically. For the colony-formation assay, a suspension of yeast cells was incubated for 3 hours in liquid medium that contained...
Adriamycin. After washing with synthetic dextrose liquid medium, the cells were resuspended in synthetic dextrose liquid medium and spotted onto a plate of agar-solidified medium.

Construction of a point mutant of AKL1 (AKL1 D181Y). Construction of a point mutant of AKL1 (AKL1 D181Y) was done as described by Hwang et al. (18) with the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). For mutagenesis of AKL1, PCR was done using plasmid pBS315-AKL1 as the template and the following oligonucleotides as the mutagenic primers: 5′-CCCTTGATCACGTAGCTACAGAGTTG-3′ and 5′-CACTTGATATCTGGAGATGATGGC-3′. The mutation in AKL1 was verified with an automated DNA sequencer (LI-COR, Lincoln, NE).

Construction of an expression vector for various genes. The AKR and PRK1 genes were cloned separately by PCR with chromosomal DNA of S. cerevisiae as template. The following oligonucleotides were used as primers: 5′-GGCGGCTGCACTTTGGAAG-3′ and 5′-CAGTTGAAGGGGTTACGATGATG-3′ for AKR1, and 5′-AGTGGTCTCTACGGGATGATG-3′ and 5′-GCTTGTAGAAGACCACTGAT-3′ for PRK1. Each product of PCR was inserted into the pGEM-T easy vector (Promega, Madison, WI). Each insert was digested with the restriction endonuclease NotI and fragments were ligated into the single-copy plasmid pRS315. For the expression of each gene, we used the respective homologous promoter.

Construction of a PAV1-hemagglutinin (HA) vector. For construction of the PAV1-hemagglutinin (HA) vector, the PAV1 gene was amplified by PCR with chromosomal DNA of S. cerevisiae as template and primers 5′-GCTAATCTGTACAACTGAATATG-3′ and 5′-CACTCTAGGCATACTCTGG-3′ inserted into the pGEM-T easy vector to produce plasmid pGEM-PAV1-HA. The insert was digested with the restriction endonuclease NotI and fragments were ligated into the pYES2 expression vector.

Phosphorylation of Pani1-HA. Yeast cells (3 × 10⁶) expressing HA-tagged Pan1 (Pani1-HA) were cultured in 10 mL of synthetic galactose (−Ura, −Leu) medium for 4 hours and harvested. After washing with ice-cold water, cells were lysed by grinding with glass beads at 4°C. An aliquot of cell lysates (100 μg of protein) was immunoprecipitated with anti-HA agarose beads (Sigma, St. Louis, MO). Immunoprecipitates were fractionated by SDS-PAGE (7.5% polyacrylamide) and transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA). Immunoblotting was carried out using rabbit antibodies against phosphotheourine (Zymed, South San Francisco, CA) or rat antibodies against HA (Roche, Basel, Switzerland) and peroxidase-conjugated second antibodies (Dako A/S, Glostrup, Denmark). Immunoreactive proteins were detected with the enhanced chemiluminescence system from Amersham Pharmacia (Uppsala, Sweden).

Endocytic uptake of lucifer yellow. Fluid-phase endocytosis was assayed using lucifer yellow carbohydrazide (Molecular Probes, Eugene, OR). Yeast cells were incubated in synthetic dextrose medium in the presence of 4 mg/mL lucifer yellow carbohydrazide. After a 2-h incubation, cells were collected and washed thrice with PBS. Cells were resuspended in PBS and observed under a light microscope.

Construction of the AAK1-expression vector. The cDNA for human full-length AAK1 (clone KIAA1048) in pBluescript was provided by the Kazusa Research Institute (Kisarazu, Chiba, Japan). For construction of the AAK1-expression vector, the full-length AAK1 cDNA insert was amplified by PCR with primers 5′-CTCGAGAGAACATGACTATAATGAGATG-3′ and 5′-GAATTCTTAAA-CTCGGAGACTGGTTGATGGGCC-3′ and then the product of PCR was subcloned into the pCDNA3.1/Hygro expression vector (Invitrogen).

Measurement of the viability of AAK1-expressing HEK293 cells. Human embryonic kidney cells (HEK293 cells) were cultured at 37°C in DMEM supplemented with 10% fetal bovine serum and 2 mmol/L L-glutamine. Transfection of HEK293 cells with the plasmid that expressed AAK1 was done as described elsewhere (19) with TransIT-LT1 (Mirus, La Jolla, CA). For mutagenesis of AKL1, the respective homologous promoter. The mutation in AKL1 was done as described elsewhere (19) with TransIT-LT1 (Mirus, La Jolla, CA). For mutagenesis of AKL1, the respective homologous promoter.

Immunoblotting of HA-AAK1. Whole-cell extracts were prepared from individual transfectants by freezing and thawing. Immunoblotting with HA-specific antibody was done as described above.

Results

In a previous study, we searched for genes related to Adriamycin resistance using S. cerevisiae transfected with a chromosomal DNA library, and we obtained plasmids AR13 and AR17 that included genes that conferred resistance to Adriamycin on yeast cells when they were overexpressed (12). In the present study, we analyzed the DNA sequences of the genomic DNA fragments that had been verified with an automated DNA sequencer (LI-COR, Lincoln, NE). Results indicated that the SD fall within the symbol.

Figure 1. Identification of AKL1 as an Adriamycin resistance gene. A, restriction map of the genomic DNA insert in plasmid AR13. Thick black line, vector pRS425; thin line, genomic DNA insert. Vertical lines above the genomic DNA indicate the restriction sites used to generate different subclones. The ability of three subclones (AR13a, AR13b, and AR13c) to confer resistance to Adriamycin is indicated (+, conferred resistance; −, did not confer resistance). ORFs are indicated by arrows that point in the direction of transcription, with the name of each ORF given below the respective arrow. B, sensitivity to Adriamycin of yeast cells that harbored plasmids with the indicated inserts. Yeast strains (W303B) carrying plasmids AR13a, AR13b, AR13c, or pRS425 were grown in synthetic dextrose (−Leu) medium that contained Adriamycin. After incubation for 48 hours at 30°C, absorbance was measured spectrophotometrically at 620 nm. Points, mean of results from three cultures; bars, SD. The absence of a bar indicates that the SD falls within the symbol. C, effects of overexpression of AKL1 on the sensitivity of yeast cells to Adriamycin. Yeast cells were incubated in the presence of Adriamycin for 3 hours and washed with synthetic dextrose (−Leu) medium. Then yeast cells were grown on a plate of agar-solidified synthetic dextrose (−Leu) for 24 hours.

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inserted into these plasmids, referring to the *Saccharomyces* Genome Database. A region of ~5 kbp derived from yeast chromosome no. 2 was present in both AR13 and AR17, and the region contained three open reading frames (ORF), designated TSC3, AKL1, and ORC2 (Fig. 1A). To identify the gene involved in Adriamycin resistance, we cleaved AR13 with restriction enzymes and subcloned the resultant fragments (AR13a, AR13b, and AR13c) in pRS425. We introduced the three new plasmids into yeast W303B cells and examined the Adriamycin sensitivity. Only the yeast cells transfected with AR13b, which contained the AKL1 ORF, exhibited Adriamycin resistance, showing that overexpression of AKL1 conferred Adriamycin resistance on the yeast cells (Fig. 1B and C). Overexpression of the AKL1 gene from a single-copy plasmid, pRS315, also led to Adriamycin resistance, suggesting that a severalfold increase in the intracellular level of AKL1 is sufficient to allow wild-type yeast cells to become Adriamycin resistant (Fig. 2).

AKL1 is classified as a member of Ark/Prk kinase family, which consists of serine/threonine kinases involved in the regulation of endocytosis, on the basis of its deduced amino acid sequence (13). However, to our knowledge, its function has not yet been analyzed. To investigate the involvement of the activity of AKL1 kinase in the AKL1 overexpression–induced reduction of Adriamycin toxicity, we prepared plasmids that expressed mutants of AKL1 with point mutations in the AKL1 kinase domain. All members of the Ark/Prk kinase family have a kinase domain in the NH2-terminal half of the amino acid sequence, and the abolition of the kinase activity of Prk1 by substituting tyrosine (Y) for aspartic acid (D) at position 158 in this domain has been reported (14). Because the corresponding amino acid in Ak1 is aspartic acid at position 181 (Asp181), we generated a mutant (Ak1D181Y) with tyrosine at this position. When Ak1D181Y was overexpressed, the sensitivity to Adriamycin of the yeast cells was similar to that of the control strain (Fig. 3). As described below, yeast cells had Ak1 kinase activity, and Asp181 was essential for this kinase activity (Fig. 4A). In view of these findings, we postulated that Ak1 kinase activity might be necessary for the Ak1 overexpression–induced acquisition of resistance to Adriamycin.

Ak1 is considered to function similarly to Ark1 and Prk1 because the kinase domain of Ak1 is strongly homologous to those of Ark1 and Prk1, two members of the Ark/Prk kinase family (13). Therefore, we investigated the sensitivity to Adriamycin of yeast cells that overexpressed Ark1 and Prk1, respectively. The sensitivity of Ark1-overexpressing yeast cells was similar to that of the control yeast cells, but Prk1-overexpressing yeast cells were apparently resistant to Adriamycin, although the level of resistance was lower than that of Ak1-overexpressing cells (Fig. 2A). When we prepared yeast cells with defective members of the Ark/Prk kinase family and investigated their Adriamycin sensitivity, we found that Ark1-defective yeast cells were slightly sensitive and Ak1- and Prk1-defective yeast cells were markedly sensitive to Adriamycin (Fig. 2B). These observations suggested that Ak1 and Prk1 might be intimately involved in the mechanism of Adriamycin toxicity, whereas the involvement of Ark1 might be limited.

Prk1 seems to promote the dissociation of the Slal/Pan1/End3 complex via phosphorylation of both Slal and Pan1 (14, 15, 20). The Slal/Pan1/End3 complex loses its function on dissociation, and Slal- and End3-defective cells have low endocytic capacity (21, 22). When we investigated the sensitivity to Adriamycin of SLA1- and END3-defective yeast cells, we found that both defective lines of yeast cells were markedly resistant to Adriamycin (Fig. 5). Moreover, overexpression of Ak1 in these SLA1- and END3-defective cells did not increase the level of resistance (Fig. 5), suggesting that the presence of Slal and End3 is essential for the Ak1 overexpression–induced reduction in Adriamycin toxicity. In this series of experiments, we did not examine the effects of deletion of the *PAN1* gene because Pan1 is a protein that is essential for the proliferation of yeast cells.
Because our results suggested the involvement of the dissociation of the Sla1/Pan1/End3 complex via phosphorylation of Pan1 and Sla1 in resistance to Adriamycin, we investigated the effects of overexpression of Akl1 on the phosphorylation of Pan1-HA using yeast cells that expressed Pan1 tagged with HA at its COOH terminus (Pan1-HA) were transformed with pRS315, pRS315-AKL1, pRS315-ARK1, pRS315-PRK1, or pRS315-AKL1 D181Y. Pan1-HA was immunoprecipitated (IP) with anti-HA agarose and immunoblotted (IB) with phosphothreonine-specific antibody (α-phosphothreonine; top) or HA-specific antibody (α-HA; bottom). See text for details. B, yeast cells at the logarithmic phase of growth were incubated with lucifer yellow for 2 hours. The localization of lucifer yellow was visualized with FITC fluorescence optics. The fluorescent images (LY-CH; top) and the transmission images (bottom) are shown.

The overexpression of Prk1 is known to reduce endocytic ability, but, to our knowledge, no studies of the effects of overexpression of Akl1 on endocytosis have been done. We investigated the fluid-phase endocytic activity of Akl1-overexpressing yeast cells using the uptake of a fluorescent dye, lucifer yellow, as an index. The overexpression of Akl1 decreased the uptake of lucifer yellow (Fig. 4B), showing that the capacity for endocytosis was reduced in Akl1-overexpressed yeast cells as it was in Sla1-defective yeast cells. By contrast, no endocytic abnormalities were noted in yeast cells that overexpressed the Akl1 point-mutant (Akl1D181Y), which might not have any kinase activity (Fig. 4B). These findings suggest that Akl1 acts negatively to control cellular endocytosis by promoting the dissociation of the Sla1/Pan1/End3 complex through phosphorylation of Pan1, as does Prk1.

In mammalian cells, adaptor-associated kinase 1 (AAK1; refs. 23, 24) and cyclin G–associated kinase (25, 26) are both members of the Ark/Prk kinase family, and overexpression of AAK1 has been reported to reduce the endocytosis of membrane proteins, such as transferrin receptors and low-density lipoprotein receptor–related protein (16). To investigate the possible involvement of the Ark/Prk kinase family in the acquisition of Adriamycin resistance by human cells, we fused AAK1 with a HA tag at its NH2 terminus (HA-AAK1) and introduced it into HEK293 cells (Fig. 6A). The resultant AAK1-overexpressing HEK293 cells were found, as anticipated, to be resistant to Adriamycin (Fig. 6B). We confirmed that overexpression of AAK1 induced resistance to Adriamycin not
only in HEK293 cells but also in HeLa cells (data not shown). Our findings suggest that the Ark/Prk kinase family might be involved in the acquisition of Adriamycin resistance not only in yeast cells but also in human cells.

Discussion

In the present study, we showed that overexpression of Akl1 renders S. cerevisiae resistant to Adriamycin. Akl1 has a kinase domain in its NH2-terminal half and is considered to be a serine/threonine kinase that belongs to the Ark/Prk kinase family (13). Prk1 and Ark1 also belong to the Ark/Prk kinase family and these proteins phosphorylate different specific proteins (27, 28). In our study, overexpression of Prk1 rendered yeast cells Adriamycin resistant, but overexpression of Ark1 did not affect the sensitivity to Adriamycin (Fig. 2A). These findings suggest that similar mechanisms that are mediated by Akl1 and Prk1 might be involved in the resistance to Adriamycin.

This study is the first, to our knowledge, to reveal that Akl1, similarly to Prk1, phosphorylates Pan1 in the Sla1/Pan1/End3 complex, which is involved in endocytosis, and confirms that Asp181 is essential for the kinase activity (Fig. 4A). Cellular endocytic ability decreases when Pan1 is phosphorylated because the Sla1/Pan1/End3 complex dissociates (15). This dissociation might be responsible for the resistance of the yeast cells to Adriamycin.

The Sla1/Pan1/End3 complex regulates the internalization step of the endocytic pathway (15). Yeast cells with defects in genes for other factors that are involved in this internalization step of endocytosis, such as Sla2 (29, 30), Vrp1 (31), and Bys161 (32), also exhibited marked resistance to Adriamycin, whereas the Adriamycin sensitivity of yeast cells with defects in genes for Vps23 (33) and Vps27 (34), which are involved in the post-endocytosis step of endocytosis, was similar to that of control yeast cells (data not shown). The level of Adriamycin toxicity might be decreased when the process of endocytosis is inhibited.

Because endocytosis is involved in the cellular uptake of various substances, reduced endocytosis might decrease the cellular uptake of Adriamycin. Reduced endocytosis has also been reported to increase the stability of Pdr5 (35), a transporter involved in the excretion of Adriamycin (36, 37). However, the overexpression of Akl1 did not decrease the intracellular accumulation of Adriamycin and, indeed, the level of accumulation increased slightly (data not shown). Therefore, the resistance to Adriamycin of Akl1-overexpressing yeast cells might not be due to a decrease in the intracellular accumulation of Adriamycin. Chen et al. identified a carcioembryonic antigen–related membrane protein, p95, as an Adriamycin-resistance factor (38, 39). The mechanism of Adriamycin resistance that involves p95 has not yet been analyzed, but p95-overexpressing cells exhibit resistance to Adriamycin without any reduction in intracellular levels of Adriamycin (38). It is also possible that the Akl1 overexpression–induced reduction in the endocytic ability of the cell might have rendered yeast cells Adriamycin resistant by increasing the stability of membrane proteins, such as p95, that are not involved in drug excretion. Adriamycin is known to impair the functions of cell membranes, and some types of Adriamycin-resistant cell are resistant to such impairment, suggesting that some changes in cell membranes (such as changes in the lipid composition) might be involved in resistance to Adriamycin (40–42). Because the involvement of endocytosis in lipid homeostasis has been reported (43), endocytosis might be involved in the mechanism of Adriamycin toxicity via the regulation of the lipid composition of membranes.

Adriamycin also impairs formation of the cytoskeleton in human cells (44). The involvement of formation of the actin skeleton in the mechanism of cardiotoxicity of Adriamycin has also been reported (45). Ark1 and Prk1 are involved not only in the regulation of endocytosis but also in the formation of cytoskeleton (20). Therefore, we cannot rule out the possibility that some members of the Ark/Prk kinase family might be involved in Adriamycin toxicity.

Overexpression of AAK1, a member of the human Ark/Prk kinase family, rendered both HEK293 and HeLa cells resistant to Adriamycin. AAK1 is considered to regulate the internalization step of endocytosis via the phosphorylation of an adaptor protein, AP2μ (24). Thus, the kinase-dependent regulation of endocytosis...
might be involved in the mechanism of Adriamycin toxicity not only in yeast cells but also in human cells. To our knowledge, no studies of the relationship between endocytosis and resistance to Adriamycin have been reported. Our results suggest novel mechanism for Adriamycin toxicity and the acquisition of resistance to Adriamycin in human cells.

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