[CANCER RESEARCH 61, 6982–6986, October 1, 2001]

Advances in Brief

SKY1 Is Involved in Cisplatin-induced Cell Kill in *Saccharomyces cerevisiae*, and Inactivation of Its Human Homologue, *SRPK1*, Induces Cisplatin Resistance in a Human Ovarian Carcinoma Cell Line[1]

Paul W. Schenk, Antonius W. M. Boersma, Jourica A. Brandsma, Hans den Dulk, Herman Burger, Gerrit Stoter, Jaap Brouwer, and Kees Nooter[2]

Department of Medical Oncology, University Hospital Rotterdam-Daniel den Hoed Cancer Center, Josephine Nefkens Institute, 3000 DR Rotterdam [P. W. S., A. W. M. B., H. B., G. S., K. N.], and Department of Molecular Genetics, Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, 2300 RA Leiden [J. A. B., H. d. D., J. B.], the Netherlands

Abstract

The therapeutic potential of cisplatin, one of the most active and widely used anticancer drugs, is severely limited by the occurrence of cellular resistance. In this study, using budding yeast *Saccharomyces cerevisiae* as a model organism to identify novel drug resistance genes, we found that disruption of the yeast gene *SKY1* (serine/arginine-rich protein-specific kinase from budding yeast) by either transposon insertion or one-step gene replacement conferred cellular resistance to cisplatin. Heterologous expression of the human *SKY1* homologue *SRPK1* (serine/arginine-rich protein-specific kinase) in *SKY1* deletion mutant yeast cells restored cisplatin sensitivity, suggesting that *SRPK1* is a cisplatin sensitivity gene, the inactivation of which could lead to cisplatin resistance. Subsequently, we investigated the role of SRPK1 in cisplatin sensitivity and resistance in human ovarian carcinoma A2780 cells using antisense oligodeoxynucleotides. Treatment of A2780 cells with antisense oligodeoxynucleotides directed against the translation initiation site of *SRPK1* led to down-regulation of SRPK1 protein and conferred a 4-fold resistance to cisplatin. The human *SRPK1* gene has not been associated with drug resistance before. Our new findings strongly suggest that SRPK1 is involved in cisplatin-induced cell kill and indicate that SRPK1 might potentially be of importance for studying clinical drug resistance.

Introduction

Cisplatin is one of the most widely used anticancer drugs. Platinum-based chemotherapy is active against cancers of the lung, ovary, bladder, head and neck, esophagus, cervix, and endometrium and is curative for the vast majority of patients with testicular cancer (1). Unfortunately, cellular resistance to cisplatin, either intrinsic or acquired, is encountered regularly and severely limits the therapeutic potential of the drug (2). A better understanding of the cellular mechanisms of cisplatin sensitivity and resistance could lead to the development of effective specific biological and pharmacological intervention and thus to better treatment results with regard to long-term survival. Molecular pathways leading to cisplatin resistance need to be defined in detail, which will be largely sustained by the identification and characterization of genes regulating cisplatin sensitivity. Multiple mechanisms by which cells may overcome the cytotoxic action of cisplatin have been identified *in vitro*. These include decreased intracellular drug accumulation, inactivation by glutathione or metallothioneins, aberrations in repair, enhanced tolerance, and defects in pathways modulating cell death (2–4). Despite the many potentially important resistance mechanisms that have been identified *in vitro*, hardly any data demonstrate correlations between presently known *in vitro* mechanisms of cisplatin resistance and clinical outcome. Apparently, these processes do not fully account for the observed *in vivo* unresponsiveness of particular tumors to platinum-based chemotherapy. Therefore, additional cisplatin resistance mechanisms for which the genes involved have yet to be identified may exist.

We used the budding yeast *Saccharomyces cerevisiae* as a model system to study drug sensitivity and resistance (5). In this study, we performed a genome-wide screen to identify and characterize novel cisplatin sensitivity genes, and we found that disruption of the yeast *YMR216C* locus corresponding to the *SKY1* (SR[3] protein-specific kinase from budding yeast) gene conferred cellular resistance to cisplatin. We demonstrate that down-regulation of the human Sky1p homologue SRPK1 (SR protein-specific kinase) by antisense ODNs in human ovarian carcinoma cells also confers cisplatin resistance. This is the first report in which *SKY1* and *SRPK1* are associated with alterations in cisplatin sensitivity, and our findings indicate that SRPK1 might potentially be of importance for studying clinical drug resistance.

Materials and Methods

Generation of Cisplatin-resistant Yeast Mutants. Yeast cells were cultured on complete YNB medium (Difco Laboratories, Detroit, MI) at 30°C as described previously (5). Cisplatin-sensitive *S. cerevisiae* strain MGSC131 (6) was transformed with a yeast genomic mini-Tn*3::lacZ::LEU2* transposon insertion library (7), referred to as Δ35 (kindly provided by Dr. P. B. Ross-Macdonald, Yale University, New Haven, CT), by means of a high-efficiency protocol. Transformants were selected on complete YNB medium plates lacking leucine. Leucine-proficient *S. cerevisiae* cells were replated at a density of 10^4 cells/94-mm dish on selective YNB medium plates containing 4 μg/ml cisplatin [Platosin; *cis*-diamminedichloroplatinum(II); Pharmachemie, Haarlem, the Netherlands]. Colonies surviving this one-step drug selection were picked and retested for cisplatin resistance in semiquantitative spot assays and quantitative clonogenic survival assays as described previously (5). Inverse PCR (8) was used to obtain sequences flanking the transposon elements. PCR products were purified and sequenced using transposon-specific primers (7). A gene-specific *SKY1* disruption was generated in strain MGSC283 (an isogenic *ura3-1* derivative of MGSC131) by one-step gene replacement performed as described by Rothstein (9), which yielded MGSC-*sky1*Δ cells. The construct used in the one-step gene replacement procedure was made using primers SKY1-5A (5′-GTA-AGA-AAG-CTG-GGA-TGG-GGC-CAC-TTC-TCA-TCT-TTG-ACA-GCT-TAT-CAT-C-3′) and SKY1-3 (5′-CGG-AAC-CAC-TCC-CGT-ACA-ACT-CTC-TAT-CAG-GTA-CCC-ACT-

Received 5/4/01; accepted 8/13/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported in part by Grant DDHK 97-1397 from the Dutch Cancer Society.

[2] To whom requests for reprints should addressed, at Department of Medical Oncology, University Hospital Rotterdam, Josephine Nefkens Building Room Be422, P. O. Box 1738, 3000 DR Rotterdam, the Netherlands. Phone: 31-10-4088357; Fax: 31-10-4088363; E-mail: nooter@oncd.azr.nl.

[3] The abbreviations used are: EPEI, ethoxylated polyethylenimine, SR, serine/arginine-rich; ODN, oligodeoxynucleotide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; YNB, yeast nitrogen base.

CGT-GCA-CCC-3′). A probe hybridizing to the 0.5-kb 5′ *Spe*I-*Hin*dIII *SKY1* fragment was used to confirm successful disruption by Northern blotting. Sequences were analyzed using the *Saccharomyces* Genome Database. Database entries used for amino acid comparisons between Sky1p and its human counterpart, SRPK1, were RefSeq accession number NP_013943 and Protein Information Resource accession number S45337, respectively.

Construction of Expression Plasmids. A 2.3-kb *SKY1* PCR product was ligated into the low-copy *S. cerevisiae* expression vector pYCTEF, which consists of the 4.5-kb *Pvu*II backbone fragment from centromeric plasmid YCplac22 (10) and the 1.0-kb *Pvu*II fragment from p424TEF (11), containing the constitutive translation elongation factor 1α promoter. The PCR product was generated using primers SKYex-5 (5′-ATA-GTG-GAT-CCT-GGT-ATA-AAT-AGA-CAC-CCC-C-3′) and SKYex-3 (5′-CTA-ACC-TCG-AGA-GGG-CAA-AAT-AAA-GGT-ATA-AAG-G-3′). The full-length human *SRPK1* coding region, homologous to *SKY1*, was kindly provided by Drs. X-D. Fu and H-Y. Wang (University of California at San Diego, La Jolla, CA), and dn*SRPK1* (12) was made available by Drs. X-D. Fu and J-H. Ding (University of California at San Diego). Both constructs were also cloned into the pYCTEF expression vector, enabling heterologous expression in yeast.

Treatment of Cells with Antisense ODNs. The human ovarian carcinoma A2780 cell line was obtained from the American Type Culture Collection (Manassas, VA), and maintained in HEPES-buffered RPMI 1640 supplemented with 10% fetal bovine serum (Life Technologies, Inc., Rockville, MD) in a humidified incubator at 37°C with 8.5% CO_2. Morpholino phosphorodiamidate antisense ODNs directed against the translation initiation site of *SRPK1* (referred to henceforth as AS-SRPK1; sequence, 5′-CAT-GGT-GAG-ACC-CAA-CAA-AAG-CAG-G-3′), control random antisense ODNs (referred to henceforth as NS; sequence, 5′-CCT-CTT-ACC-TCA-GTT-ACA-ATT-TAT-A-3′), and control antisense ODNs with four mispairs distributed along the sequence (referred to henceforth as MIS; sequence, 5′-CAT-**C**GT-G**T**G-ACC-CAA-CAA-**T**AG-C**T**G-G-3′; mismatches are shown in *bold*) were purchased from Gene Tools (Corvallis, OR). Incubation of A2780 cells with ODNs was performed using the manufacturer's Special Delivery protocol. Cisplatin sensitivity of ODN-treated A2780 cells was determined by MTT assay (13). Briefly, the cells were seeded in 96-well tissue culture plates. The next day, the culture medium was replaced by medium containing ODNs and 0.6 μM ethoxylated polyethylenimine delivery agent but lacking serum, and the cells were incubated at 37°C with 8.5% CO_2 for 3 h. Subsequently, the medium was exchanged with standard culture medium containing serum, as described above. After 16 h, cisplatin was added at a dose range of 0–24 μM, and the cells were allowed to grow for another 72 h. Growth inhibition was determined by the use of MTT (Sigma-Aldrich, Zwijndrecht, the Netherlands) as described by Perez *et al.* (13).

Statistical Analysis. Relationships between cisplatin concentration and cisplatin-induced cytotoxicity were evaluated using Siphar version 4.0 software (InnaPhase, Philadelphia, PA) and the Number Cruncher Statistical System version 5.X package (Dr. J. L. Hintze, University of Utah, East Kaysville, UT). Data were fitted to a sigmoidal maximum effect (E_{max}) model based on the modified Hill equation, as follows: $E = E_0 + E_{max} \times [(C^\gamma)/(C^\gamma + EC_{50}{}^\gamma)]$. In this equation, E_0 is the "no drug" effect, E_{max} is the maximum drug effect, C is the drug concentration to which cytotoxicity is related, EC_{50} is the drug concentration predicted to result in half-maximal cytotoxicity, and γ is the Hill constant describing the sigmoidicity of the curve. The difference in EC_{50} between two cytotoxicity tests was evaluated statistically using a two-tailed (unpaired) Student's t test.

Immunofluorescence Detection and Western Blot Analysis of SRPK1. After 3 h of incubation with 5.6 μM ODNs as described above, followed by recovery in standard culture medium, a monolayer of A2780 cells was deposited on slides in a Cytospin 2 Cytocentrifuge (Shandon Inc., Pittsburgh, PA) at 750 rpm for 10 min. Cells were fixed in methanol:acetone (1:1) at −20°C for 10 min, rehydrated in PBS at room temperature for 15 min, and then blocked in blocking buffer (10% rabbit serum, 1% goat serum, and 1% normal human serum in PBS) at room temperature for 15 min. Next, they were incubated at room temperature for 45 min with primary monoclonal anti-SRPK1 (mouse IgG1; 1:50 dilution of the 250 μg/ml stock purchased from BD Transduction Laboratories, Lexington, KY) or nonspecific mouse monoclonal IgG1 (ICN/Cappel, Costa Mesa, CA) at the same final concentration, washed three times with PBS, and developed at room temperature for 30 min with secondary FITC-conjugated goat antimouse IgG (1:50 dilution of the 1 mg/ml stock obtained from Nordic, Tilburg, the Netherlands). Finally, the slides were washed three times in PBS and mounted in VECTASHIELD medium containing 1 μg/ml 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) for examination under a Leica DMR fluorescence microscope with the CGH photoimaging system (Leica Microsystems, Wetzlar, Germany). For Western blotting, A2780 cells were incubated similarly, and total protein was isolated by adding SDS-loading buffer to cell pellets and boiling the samples for 3 min. Western blots were blocked in PBS containing 0.2% Tween 20 and 5% Protifar (Nutricia, Zoetermeer, the Netherlands) at 37°C for 1 h and then incubated with a 1:2000 dilution of mouse monoclonal anti-SRPK1 at 4°C for 16 h. SRPK1 was visualized using horseradish peroxidase-linked goat anti-mouse IgG (1:4000 dilution of the 400 μg/ml stock purchased from Santa Cruz Biotechnology, Santa Cruz, CA) and SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). Blots were then stripped in 63 mM Tris (pH 6.8), 0.002% SDS, and 114 mM β-mercaptoethanol at 56°C for 45 min and reincubated as described above with a 1:5000 dilution of mouse monoclonal anti-β-actin (Sigma-Aldrich) as first antibody.

Results and Discussion

Disruption of the *SKY1* Gene in Yeast Induces Cisplatin Resistance. In this study, we used the budding yeast *S. cerevisiae* as a model system to search for novel genes that, upon disruption, confer cellular resistance to the commonly applied anticancer drug cisplatin. A yeast genomic mini-Tn*3::lacZ::LEU2* transposon insertion library (7), referred to as Δ35, was introduced into *S. cerevisiae* cells. Transformation originates from homologous recombination between yeast DNA sequences flanking the transposon and endogenous genomic sequences, leading to replacement of the original genomic copy with the mutagenized version (9). A cisplatin-sensitive yeast strain referred to as MGSC131, which displays a steep dose-response curve to the drug (5, 6), was used as recipient. Untransformed MGSC131 cells seeded on cisplatin-containing YNB medium plates did not yield any colonies, even after prolonged incubation. In contrast, when 3×10^5 transformants of library Δ35 were plated under identical conditions, 31 colonies surviving the one-step drug selection grew within 3–6 days. Upon verification of the resistance phenotypes of the colonies derived from this genome-wide yeast screening approach, we selected nine strains that showed a similar cisplatin resistance level for further characterization. Southern blotting using a transposon-specific probe revealed that each strain contained one single transposon insertion (data not shown). The sites flanking the transposons (*i.e.*, the loci that had been disrupted) could thus be identified directly by means of inverse PCR (8), followed by sequencing of the respective products. Sequences of 50–60 bp on either side of the transposon were obtained, and the insertion sites were defined by comparison with public *S. cerevisiae* databases.

Most prominently, the YMR216C locus corresponding to the *SKY1* gene (14) turned out to be disrupted in at least five transposon-containing cisplatin-resistant yeast strains, referred to as Δ35-2, Δ35P-1, Δ35P-3, Δ35P-4, and Δ35P-5. At least three strains (Δ35-2 or Δ35P-1, Δ35P-3 or Δ35P-4, and Δ35P-5) appeared to contain different insertions in the same gene, as judged by the sizes of the PCR products obtained (Fig. 1*a*). In the remaining transposon-containing cisplatin-resistant yeast strains, other loci had been disrupted, as will be described elsewhere.[4] In a quantitative clonogenic survival assay, strains Δ35-2, Δ35P-1, Δ35P-3, Δ35P-4, and Δ35P-5 were 4-fold cisplatin resistant as compared with parental MGSC131-*SKY1*$^+$ cells. Fig. 1*b* shows a typical cisplatin sensitivity profile of the transposon-derived *sky1*Δ transformant Δ35-2 as compared with untransformed isogenic *SKY1*$^+$ cells.

The observed cisplatin-resistant phenotype of the transposon-containing transformants could, in principle, have arisen from unrelated

[4] Manuscript in preparation.

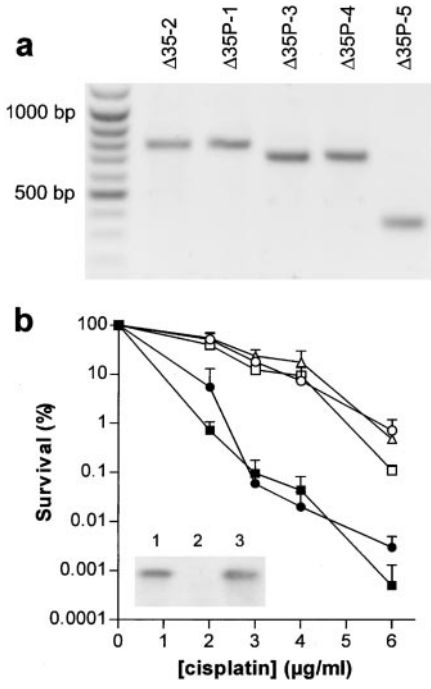


Fig. 1. Disruption of yeast *SKY1* induces resistance to cisplatin. *a*, *S. cerevisiae* cells were transformed with yeast genomic mini-Tn*3*::*lacZ*::*LEU2* transposon insertion library Δ35, and on random disruption and cisplatin selection, genomic DNA was isolated from cisplatin-resistant yeast strains. Inverse PCR was then performed using transposon-specific outward-directed primers. An agarose gel loaded with the respective PCR products derived from strains Δ35-2, Δ35P-1, Δ35P-3, Δ35P-4, and Δ35P-5 is shown. The product size for strains Δ35-2 and Δ35P-1 was ~750 bp, the product size for strains Δ35P-3 and Δ35P-4 was ~650 bp, and the product size for strain Δ35P-5 was ~350 bp (as judged by the molecular masses of the marker in the *left lane*), indicating that the transposon resided in at least three different positions. Sequencing of these PCR products revealed that the *SKY1* gene had been disrupted. *b*, cisplatin sensitivity profiles of *S. cerevisiae* wild-type MGSC131-*SKY1*$^+$ cells (■), transposon-derived isogenic *sky1*Δ mutant Δ35-2 cells (○), replacement-derived MGSC-*sky1*Δ cells (□), and MGSC-*sky1*Δ cells transformed with the constitutive low-copy yeast expression vector pYCTEF (△) or pYCTEF-*SKY1* (●). Percentage survival (colony formation) at each concentration of cisplatin is expressed relative to untreated control cells (100%). Means of three independent experiments performed in triplicate are shown; *bars* , SD. Bars are sometimes masked by the data point symbols. Northern blotting (*inset*) using an NH_2-terminal *SKY1* probe confirmed that whereas MGSC131-*SKY1*$^+$ cells do express the *SKY1* gene (*Lane 1*), *SKY1* mRNA was absent from MGSC-*sky1*Δ cells (*Lane 2*). The same procedure confirmed the expression of *SKY1* on introduction of pYCTEF-*SKY1* into MGSC-*sky1*Δ cells (*Lane 3*).

mutations acquired during the screening procedure (*i.e.*, cisplatin-induced mutations leading to resistance) rather than library-derived gene disruption. To address this issue, PCR-generated one-step gene replacement constructs (9) were used to disrupt the *SKY1* gene from the *S. cerevisiae* genome independently. Successful disruption was confirmed by PCR (data not shown) and Northern blotting (Fig. 1*b*, *inset*), and possible cisplatin resistance of the replacement-derived *sky1*Δ mutant (MGSC-*sky1*Δ) was monitored. Indeed, the cisplatin sensitivity profile appeared to be similar to that of the original transposon-containing *sky1*Δ strains (Fig. 1*b*), *i.e.*, MGSC-*sky1*Δ cells were 4-fold cisplatin-resistant.

Subsequently, we reintroduced the *SKY1* gene into the *sky1*Δ deletion mutant cells. For this purpose, the *SKY1* open reading frame was cloned into yeast expression vector pYCTEF, and the resulting plasmid was transformed into MGSC-*sky1*Δ cells. The transformants became as sensitive to cisplatin as the original MGSC131-*SKY1*$^+$ strain, whereas transformation with the empty vector alone left the resistance of the MGSC-*sky1*Δ cells completely unaffected (Fig. 1*b*). Our findings clearly demonstrate that the cisplatin resistance, which was observed originally, was linked to disruption of the *SKY1* gene. Moreover, the data suggest that Sky1p is involved in the cytotoxicity of cisplatin, *i.e.*, that *SKY1* might be regarded as a cisplatin sensitivity gene.

Heterologous Expression of the Human Homologue SRPK1 Restores Cisplatin Sensitivity in sky1Δ Mutant Yeast. Database analysis revealed that *SKY1* has a human homologue, which encodes the SR protein-specific kinase SRPK1. SR protein-specific kinases and the SR proteins that they phosphorylate are thought to be key regulators of RNA processing and, in mammalian cells, alternative splicing through multiple mechanisms (14, 15). The predicted polypeptide sequence encoded by the human *SRPK1* cDNA shares 35% identity over 644 amino acids to Sky1p. Notably, SRPK1 and Sky1p also share structural homology: their kinase domains are interrupted by a unique spacer sequence. Because several data suggest strongly that Sky1p and SRPK1 perform homologous functions *in vivo* (14, 16), we tested *SRPK1* for its ability to complement the cisplatin resistance phenotype of *S. cerevisiae sky1*Δ cells. *SRPK1* was cloned into yeast expression vector pYCTEF and transformed into MGSC-*sky1*Δ mutant cells. Heterologous RNA expression was confirmed by Northern blotting (Fig. 2, *inset*) using a PCR-generated probe specific for the spacer domain of *SRPK1* (14). In MGSC-*sky1*Δ yeast cells the cisplatin resistance phenotype appeared to be largely reversed by introduction of the human *SRPK1* gene, as was the case when the yeast *SKY1* gene was reintroduced. In contrast, MGSC-*sky1*Δ cells transformed with a construct encoding a well-established dominant-negative kinase-inactive mutant (K109M) of SRPK1 (dn*SRPK1*; Refs. 12 and 16) were still cisplatin resistant (Fig. 2). Apparently, *SRPK1* can functionally substitute *SKY1* in *S. cerevisiae* cells and can therefore be considered a cisplatin sensitivity gene as well. To our knowledge, *SKY1* and *SRPK1* have not been previously associated with cisplatin sensitivity or resistance. The observation that dn*SRPK1* (encoding a kinase-inactive protein) could not substitute *SKY1* suggests that the kinase function of SR protein-specific kinases is essential for the cytotoxicity of cisplatin in yeast.

Antisense Experiments. Because *SRPK1* was able to act as a cisplatin sensitivity gene in *S. cerevisiae* cells, we assessed whether it also functions as a gene that is necessary for proper cisplatin-induced cell kill in human cells. Therefore, we designed experiments in which we could monitor the effects of SRPK1 inactivation by antisense ODNs on cisplatin sensitivity in human cells. First, we established the effect of AS-SRPK1 on SRPK1 protein expression levels in ovarian carcinoma A2780 cells by immunofluorescence. In untreated A2780 cells, we found SRPK1 expression in both the cytoplasm and nuclear speckles (Fig. 3, *a* and *b*), as demonstrated previously (17). Treatment

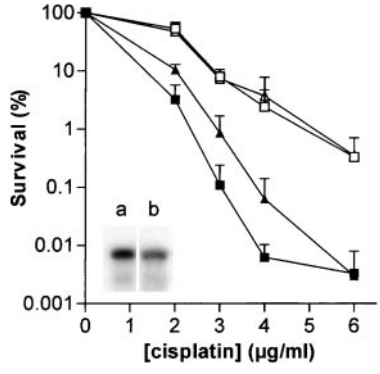


Fig. 2. Restoration of cisplatin sensitivity in *sky1*Δ deletion mutant yeast by the human homologue *SRPK1*. Cisplatin sensitivity profiles of wild-type MGSC131-*SKY1*$^+$ (■), deletion mutant MGSC-*sky1*Δ (□), and MGSC-*sky1*Δ transformed with pYCTEF-*SRPK1* (▲) or pYCTEF-dn*SRPK1* (△) are shown. Percentage survival (colony formation) at each concentration of cisplatin is expressed relative to untreated control cells (100%). Means of at least two independent experiments performed in triplicate are shown; *bars* , SD. *Inset*, RNA was isolated from *S. cerevisiae* MGSC-*sky1*Δ cells transformed with pYCTEF-*SRPK1* or pYCTEF-dn*SRPK1*, and Northern blotting using an *SRPK1*-specific probe was used to confirm the heterologous expression of the human *SKY1* homologue *SRPK1* in its wild-type and dominant-negative form (*Lanes a* and *b*, respectively).

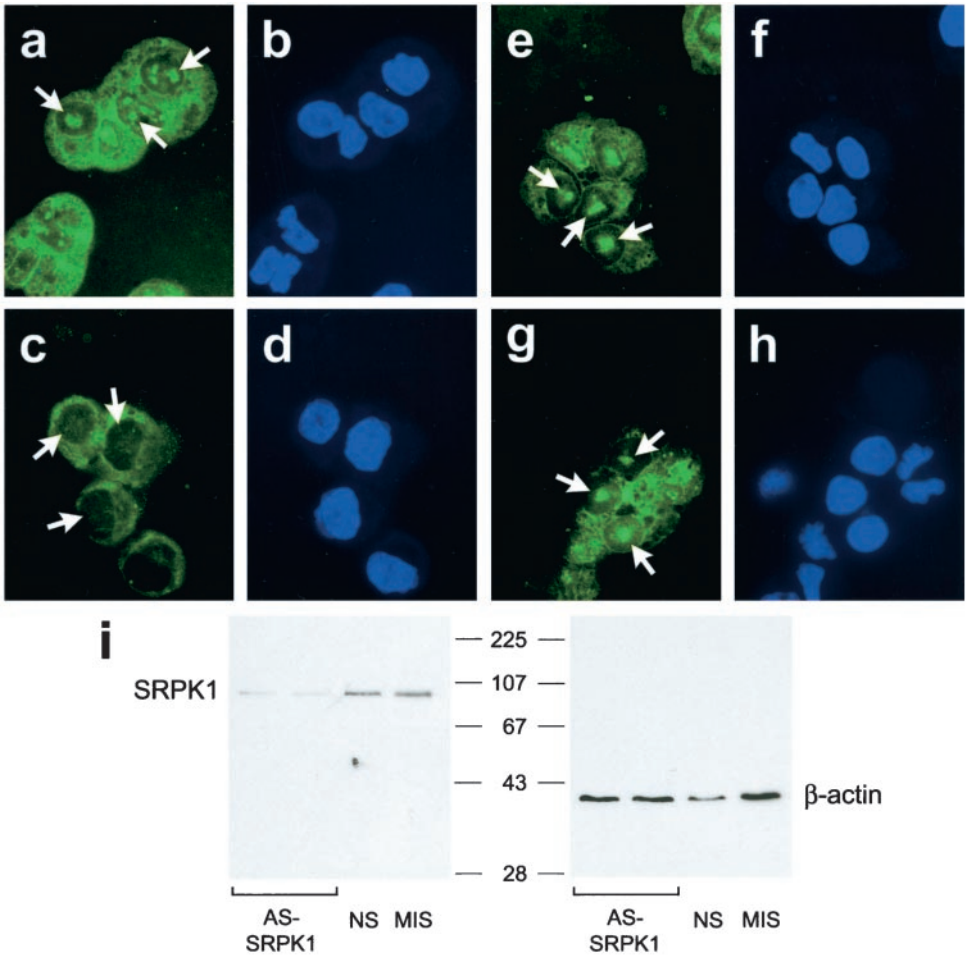
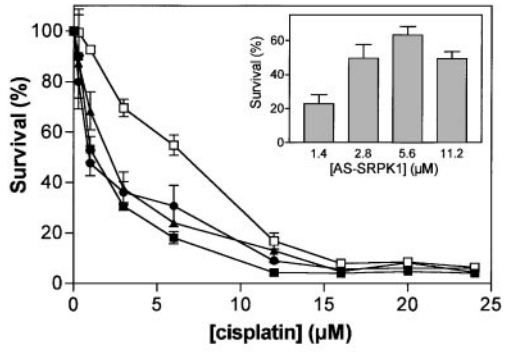


Fig. 3. Effect of antisense ODN treatment on SRPK1 protein expression, and specificity of the antibody used. Human A2780 cells were either left untreated (*a* and *b*) or treated with specific AS-SRPK1 (*c* and *d*), NS (*e* and *f*), or MIS (*g* and *h*). Expression and localization of SRPK1 were visualized by indirect immunofluorescence staining of cytospins using a mouse monoclonal anti-SRPK1 antibody followed by FITC-conjugated secondary antibody (*a*, *c*, *e*, and *g*). The slides were also stained with 4′,6-diamidino-2-phenylindole to visualize the nuclei (*b*, *d*, *f*, and *h*). *Arrows* indicate nuclear speckles, which were clearly discernible in slides obtained from untreated or control-treated cells (*a*, *e*, and *g*). In cytospins from cells treated with specific AS-SRPK1 (*c*), FITC staining was virtually absent from the nucleus. *i*, Western blot analysis of total protein preparations. A2780 cells were treated with specific AS-SRPK1, NS, or MIS, as indicated *below* the lanes, and 6×10^3 cell equivalents/lane were loaded. SRPK1 was visualized using primary mouse monoclonal anti-SRPK1, secondary horseradish peroxidase-linked goat anti-mouse IgG antibodies, and chemiluminescence staining (*left panel*). To confirm that equivalent amounts of protein were present in each lane, the blot was stripped and reincubated with mouse monoclonal anti-β-actin as first antibody (*right panel*). Apparent molecular masses of the marker are indicated in kDa. SRPK1 and β-actin migrated at ~92 and ~42 kDa, respectively.

of A2780 cells with control antisense ODNs, NS, or MIS had no detectable effect on SRPK1 protein expression levels or subcellular localization (Fig. 3, *e–h*). In contrast, expression of the SRPK1 protein was largely decreased after treatment of A2780 cells with specific AS-SRPK1 (Fig. 3, *c* and *d*). Incubation with AS-SRPK1 almost completely abolished the nuclear staining of SRPK1 protein (Fig. 3, *c* and *d*) as compared with NS or MIS (Fig. 3, *e–h*), whereas cytoplasmic staining was reduced to the background level detected using a nonspecific antibody (data not shown). Notably, the anti-SRPK1 monoclonal antibody used in these experiments is highly specific: on Western blots, it recognized a single band of ~92 kDa which is in agreement with the relative molecular mass of the SRPK1 protein, as published by Gui *et al.* (15). Furthermore, Western blotting experiments confirmed the specific down-regulation of SRPK1 expression by AS-SRPK1: there was a marked decrease in the level of SRPK1 protein detected on incubation of A2780 cells with AS-SRPK1 as compared with NS or MIS (Fig. 3*i*).

Now that we had shown that AS-SRPK1 was able to specifically down-regulate SRPK1 protein expression, we tested it for its effects on cisplatin sensitivity. First, we determined the optimum dose for AS-SRPK1 treatment. A2780 cells were incubated with increasing concentrations of AS-SRPK1 (1.4, 2.8, 5.6, and 11.2 μM) at a fixed dose of cisplatin (6 μM), and survival was estimated by monitoring cell proliferation in MTT assays (Fig. 4, *inset*). For the ODN dose range tested, the optimum concentration for reduction of cisplatin-induced growth inhibition appeared to be 5.6 μM AS-SRPK1 (Fig. 4, *inset*). Subsequently, we determined the shift in EC_{50} value (*i.e.*, the cisplatin concentration predicted to result in half-maximal cytotoxic-



Treatment	EC_{50} value (mean ± SD)	*P* value
NS	1.5 ± 0.19 μM	$P < 0.0001$
MIS	2.2 ± 0.63 μM	$P < 0.0001$
delivery agent	2.2 ± 0.30 μM	$P < 0.0001$
AS-SRPK1	6.3 ± 0.70 μM	

Fig. 4. Down-regulation of SRPK1 by antisense ODN treatment induces resistance to cisplatin in human cells. Survival was determined by monitoring the proliferation of A2780 cells on ODN treatment and cisplatin incubation, using MTT assay. The optimum AS-SRPK1 concentration for reduction of cisplatin-induced cytotoxicity was determined using increasing concentrations of specific AS-SRPK1 at 6 μM cisplatin (*inset*). Cisplatin sensitivity profiles of cells treated with NS (■), MIS (●), or delivery agent alone (▲) *versus* cells treated with specific AS-SRPK1 (□) were then determined at the optimal dose of 5.6 μM ODNs. Percentage survival at each concentration of cisplatin is expressed relative to control cells that had been incubated in the absence of cisplatin (100%). A typical experiment (of three performed) carried out in triplicate is shown; *bars*, SE. The cisplatin concentrations predicted to result in half-maximal cytotoxicity (EC_{50}s), based on the modified Hill equation fitted to the data, are indicated *below* the curves. *P*s indicate the significance of the differences in EC_{50}s between AS-SRPK1 treatment and the appropriate controls.

ity) at a fixed dose of 5.6 μM AS-SRPK1, as compared with the same dose of NS or MIS, by MTT assay. Repeatedly, AS-SRPK1 induced a 3- to 4-fold cisplatin resistance in A2780 cells, as evaluated against incubation with NS or MIS. Notably, the growth rate of A2780 cells in the absence of cisplatin was not decreased after treatment with AS-SRPK1, as compared with NS or MIS (data not shown). A typical experiment (of three performed) is shown in Fig. 4; whereas incubation with NS, MIS, or delivery agent alone resulted in similar EC_{50} values (1.5 $\pm$ 0.19, 2.2 $\pm$ 0.63, and 2.2 $\pm$ 0.30 μM cisplatin, respectively), AS-SRPK1 treatment led to a 3- to 4-fold increase in EC_{50} (6.3 $\pm$ 0.70 μM cisplatin). Statistical analysis comparing NS, MIS, or delivery agent only *versus* AS-SRPK1 treatment indicated that the differences in EC_{50} were significant at $P < 0.0001$, as estimated by Student's *t* test. It can thus be concluded that down-regulation of SRPK1 protein by specific antisense ODNs rendered A2780 cells resistant to cisplatin, *i.e.*, *SRPK1* may function as a cisplatin sensitivity gene in this human ovarian carcinoma cell line.

Mechanistic Aspects and Implications for Clinical Studies. To characterize the specificity and underlying mechanisms of the observed resistance of *sky1*Δ yeast cells, other DNA-damaging agents, including platinum-based drugs, will be used in future studies. Because, in our hands, *sky1*Δ strains showed normal growth characteristics,[5] and the growth rate of human A2780 cells was not decreased by incubation with specific ODNs, the cisplatin-resistant phenotypes were obviously not caused simply by distorted growth. Because we did not detect altered levels of yeast *SKY1* RNA on cisplatin treatment,[5] the gene is probably not regulated by cisplatin at the transcriptional level. Sky1p (and SRPK1) activity could be regulated instead by autophosphorylation or posttranslational modification by upstream components.

As stated above, our data suggest that the kinase function is essential to the involvement of SR protein-specific kinases in the cytotoxicity of cisplatin. The *S. cerevisiae* SR protein encoded by *NPL3* has been shown (14) to be phosphorylated by Sky1p both *in vitro* and *in vivo* and shuttles between the nucleus and the cytoplasm to deliver mRNA and/or proteins. It has been demonstrated (18–21) that phosphorylation of Npl3p and mammalian SR proteins specifically modulates their protein-protein and protein-RNA interactions and localization. Sky1p and SRPK1 may thus be key players in pathways determining cisplatin sensitivity via mRNA and/or protein delivery by Npl3p or mammalian SR proteins, respectively.

In future studies, we will assess the relative contribution of pathways using SRPK1 to clinical cisplatin sensitivity and resistance. Low levels of cisplatin resistance are generally believed to be sufficient to cause lack of clinical responsiveness. Changes of <2-fold may account for treatment failure in human ovarian carcinoma xenografts (22). Interestingly, *SRPK1* is highly expressed in testis (23), whereas, at the same time, testicular germ cell tumors are extremely sensitive to cisplatin-containing chemotherapy (1). Indeed, constitutive expression of *SKY1* from a high-copy vector clearly made *sky1*Δ yeast cells more sensitive to cisplatin, as compared with an appropriate *SKY1*$^+$ control strain,[5, 6] which suggests that SR protein-specific kinases are cisplatin sensitivity genes in the sense that they are actively involved in the cytotoxicity of cisplatin. In addition, we found that down-regulation of SRPK1 with AS-SRPK1 also made NT2 testicular cancer cells less sensitive to cisplatin, as compared with cells treated with control MIS.[5] Given these observations, we will monitor samples derived from both ovarian and testicular cancers, which are generally treated with cisplatin-containing regimens in the clinic (1), for SRPK1 expression and correlation with clinical responsiveness.

[5] P. W. Schenk, K. Nooter, unpublished data.

[6] J. A. Brandsma and J. Bronwer, unpublished data.

Acknowledgments

We thank Dr. Petra Ross-Macdonald for providing us with the random mini-Tn*3*::*lacZ*::*LEU2* insertion libraries and Drs. Xiang-Dong Fu, Huan-You Wang, and Jian-Hua Ding for the *SRPK1* and dn*SRPK1* constructs. We thank Mariël Brok for expert technical assistance, Dr. Alex Sparreboom for performing statistical analysis and valuable comments, and Dr. John Foekens for critical reading of the manuscript.

References

1. Einhorn, L. H. Testicular cancer: an oncological success story. Clin. Cancer Res., *3:* 2630–2632, 1997.
2. Johnson, S. W., Ferry, K. V., and Hamilton, T. C. Recent insights into platinum drug resistance in cancer. Drug Resist. Updates, *1:* 243–254, 1998.
3. Gately, D. P., and Howell, S. B. Cellular accumulation of the anticancer agent cisplatin: a review. Br. J. Cancer, *67:* 1171–1176, 1993.
4. Crul, M., Schellens, J. H. M., Beijnen, J. H., and Maliepaard, M. Cisplatin resistance and DNA repair. Cancer Treat. Rev., *23:* 341–366, 1997.
5. Burger, H., Capello, A., Schenk, P. W., Stoter, G., Brouwer, J., and Nooter, K. A genome-wide screening in *Saccharomyces cerevisiae* for genes that confer resistance to the anticancer agent cisplatin. Biochem. Biophys. Res. Commun., *269:* 767–774, 2000.
6. Verhage, R. A., Van de Putte, P., and Brouwer, J. Repair of rDNA in *Saccharomyces cerevisiae*: *RAD4*-independent strand-specific nucleotide excision repair of RNA polymerase I transcribed genes. Nucleic Acids Res., *24:* 1020–1025, 1996.
7. Burns, N., Grimwade, B., Ross-Macdonald, P. B., Choi, E-Y., Finberg, K., Roeder, G. S., and Snyder, M. Large-scale analysis of gene expression, protein localization, and gene disruption in *Saccharomyces cerevisiae*. Genes Dev., *8:* 1087–1105, 1994.
8. Ochman, H., Gerber, A. S., and Hartl, D. L. Genetic applications of an inverse polymerase chain reaction. Genetics, *120:* 621–623, 1988.
9. Rothstein, R. Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. Methods Enzymol., *194:* 281–301, 1991.
10. Gietz, R. D., and Sugino, A. New yeast-*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. Gene (Amst.), *74:* 527–534, 1988.
11. Mumberg, D., Müller, R., and Funk, M. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene (Amst.), *156:* 119–122, 1995.
12. Colwill, K., Feng, L. L., Yeakley, J. M., Gish, G. D., Cáceres, J. F., Pawson, T., and Fu, X-D. SRPK1 and Clk/Sty protein kinases show distinct substrate specificities for serine/arginine-rich splicing factors. J. Biol. Chem., *271:* 24569–24575, 1996.
13. Perez, R. P., Godwin, A. K., Handel, L. M., and Hamilton, T. C. A comparison of clonogenic, microtetrazolium and sulforhodamine B assays for determination of cisplatin cytotoxicity in human ovarian carcinoma cell lines. Eur. J. Cancer, *29A:* 395–399, 1993.
14. Siebel, C. W., Feng, L., Guthrie, C., and Fu, X-D. Conservation in budding yeast of a kinase specific for SR splicing factors. Proc. Natl. Acad. Sci. USA, *96:* 5440–5445, 1999.
15. Gui, J-F., Lane, W. S., and Fu, X-D. A serine kinase regulates intracellular localization of splicing factors in the cell cycle. Nature (Lond.), *369:* 678–682, 1994.
16. Yeakley, J. M., Tronchère, H., Olesen, J., Dyck, J. A., Wang, H-Y., and Fu, X-D. Phosphorylation regulates *in vivo* interaction and molecular targeting of serine/arginine-rich pre-mRNA splicing factors. J. Cell Biol., *145:* 447–455, 1999.
17. Wang, H-Y., Lin, W., Dyck, J. A., Yeakley, J. M., Songyang, Z., Cantley, L. C., and Fu, X-D. SRPK2: a differentially expressed SR protein-specific kinase involved in mediating the interaction and localization of pre-mRNA splicing factors in mammalian cells. J. Cell Biol., *140:* 737–750, 1998.
18. Yun, C. Y., and Fu, X-D. Conserved SR protein kinase functions in nuclear import and its action is counteracted by arginine methylation in *Saccharomyces cerevisiae*. J. Cell Biol., *150:* 707–717, 2000.
19. Gilbert, W., Siebel, C. W., and Guthrie, C. Phosphorylation by Sky1p promotes Npl3p shuttling and mRNA dissociation. RNA (N. Y.), *7:* 302–313, 2001.
20. Xiao, S-H., and Manley, J. L. Phosphorylation of the ASF/SF2 RS domain affects both protein-protein and protein-RNA interactions and is necessary for splicing. Genes Dev., *11:* 334–344, 1997.
21. Koizumi, J., Okamoto, Y., Onogi, H., Mayeda, A., Krainer, A. R., and Hagiwara, M. The subcellular localization of SF2/ASF is regulated by direct interaction with SR protein kinases (SRPKs). J. Biol. Chem., *274:* 11125–11131, 1999.
22. Andrews, P. A., Jones, J. A., Varki, N. M., and Howell, S. B. Rapid emergence of acquired *cis*- diamminedichloroplatinum(II) resistance in an *in vivo* model of human ovarian carcinoma. Cancer Commun., *2:* 93–100, 1990.
23. Papoutsopoulou, S., Nikolakaki, E., Chalepakis, G., Kruft, V., Chevaillier, P., and Giannakouros, T. SR protein-specific kinase 1 is highly expressed in testis and phosphorylates protamine 1. Nucleic Acids Res., *27:* 2972–2980, 1999.