Molecular basis for resistance to the anticancer drug cisplatin in *Dictyostelium*

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The efficacy of the widely used chemotherapeutic drug cisplatin is limited by the occurrence of drug-resistant tumour cells. To fully exploit the potential of this drug in cancer therapy, it is imperative to understand the molecular basis of cisplatin resistance. Using an insertional mutagenesis technique in cells of *Dictyostelium discoideum*, we have identified six genes which are involved in cisplatin resistance. None of these genes has been previously linked to resistance to this drug. Several of these genes encode proteins that are involved in signal transduction pathways which regulate cell death, cell proliferation or gene regulation. The resistance of these mutant strains is specific for cisplatin, since deletion of these genes does not confer resistance to other DNA-damaging agents. Significantly, the disruption of three of these genes, encoding the sphingosine-1-phosphate lyase, the RegA cAMP phosphodiesterase and a phosphatidylinositol-4-phosphate 5-kinase, also results in abnormalities in the multicellular development of this organism, although there is no change in the rate of mitotic cell growth. This study has identified previously unsuspected molecular pathways which function in the cellular response to cisplatin and are required for normal morphogenesis, and underscores the complexity of the cellular response to cisplatin. These pathways provide potential targets for modulating the response to this important drug.

Keywords: ceramide, sphingosine 1-phosphate, chemotherapy, drug-resistance, PKA

INTRODUCTION

All species have mechanisms for repairing DNA damage. These mechanisms are highly specific and allow the cells to recognize the type of damage and mount the appropriate response (Friedberg *et al.*, 1995). Failure to repair damage to DNA can lead to mutations or development of cancerous cells. For the treatment of cancer, a variety of radio- and chemotherapeutic regimens exist, which themselves are often based on causing DNA damage to the cancerous cells. However, when tumour cells develop resistance to a chemo-

therapeutic agent, it presents a significant obstacle to therapy (Scagliotti *et al.*, 1999).

An important example is illustrated in the case of cisplatin [*cis*-diamminedichloroplatinum(II)] and its derivatives, which are widely used anti-cancer drugs (Chu, 1994; Eastman, 1986; Lippard, 1982). Cisplatin is used alone or in combination with other drugs for many types of human malignancies, including testicular, ovarian, bladder, cervical, head and neck, oesophageal and some lung cancers (Loehrer & Einhorn, 1984). There is general agreement that the effect of cisplatin is due to its ability to form intrastrand cross-links between adjacent purines in DNA (Sherman & Lippard, 1987; Takahara *et al.*, 1995). However, its therapeutic efficacy is frequently limited by the development of drug-resistant tumour cell populations. In addition, cisplatin has minimal effect against common tumours such as colorectal and pancreatic (Perez, 1998).

Numerous mechanisms have been suggested for the

Abbreviations: MMS, methyl methanesulfonate; PIP5K, phosphatidylinositol-4-phosphate 5-kinase; PKA, protein kinase A; REMI, restriction-enzyme-mediated integration; S-1-P, sphingosine 1-phosphate.

The GenBank accession numbers for the sequences reported in this paper are AF233610 (S-1-P lyase), AF233612 (PIP5K), AF233611 (P2Y purine receptor 1), AF233613 (CAAX prenyl protease) and AF233614 (unidentified gene).

development of cellular resistance to cisplatin. Some include changes in the cellular drug concentration resulting from decreased accumulation, increased efflux or increased inactivation of the drug (Andrews & Howell, 1990; Chu, 1994). Other studies have focused on proteins that affect the recognition of cisplatin damage, such as the high mobility group proteins (Kohn, 1999; Ohndorf *et al.*, 1999) or proteins that affect mismatch repair such as the Mut family of proteins (Drummond *et al.*, 1996; Fink *et al.*, 1997, 1998). Specific molecules have been implicated in cisplatin-induced cell death or resistance, such as p53 and p73, and the tyrosine kinase c-Abl, which has been related to the p73 response to cisplatin (Agami *et al.*, 1999; Gong *et al.*, 1999; Yuan *et al.*, 1999). All of these studies were focused on genes and mechanisms which were *a priori* suspected to modulate the cellular response to the drug.

In this report we used a direct genetic approach, employing insertional mutagenesis, to specifically identify novel genetic pathways that are involved in the cellular response and resistance to cisplatin in *Dictyostelium discoideum*. This relatively simple multicellular organism has strong evolutionary conservation with higher eukaryotes (Baldauf & Doolittle, 1997; Kessin, 1997). It is widely used in studies on cell and developmental biology because of its multicellularity (Alexander & Rossomando, 1992; Loomis, 1982) and the availability of powerful molecular genetic approaches (Kuspa & Loomis, 1992; Spudich, 1987).

Interestingly, the genes that we identified as responsible for cisplatin resistance are not directly involved in drug import or efflux, in DNA repair or in multi-drug resistance. Some define signal transduction pathways that regulate cell death, cell proliferation or gene regulation in other systems. Importantly, some of the mutants exhibit abnormal developmental phenotypes, demonstrating that the genes that are involved in responding to DNA damage also function in normal development. The power of this genetic approach is that it identifies individual genes and pathways previously unsuspected of having a direct link to cisplatin resistance and offers possible ways to manipulate the cellular response to the drug.

METHODS

Strains and conditions for growth and development. *D. discoideum* strain Ax4 was the parent for insertional mutagenesis. Cultures were started from stored desiccated spores on a monthly basis and were grown in HL-5 medium (Cocucci & Sussman, 1970). The cells were used or passed when they reached mid-exponential phase (3×10^6 cells ml⁻¹). Multicellular development was initiated by depositing 10^8 cells, washed free of medium, on 42 mm black paper filters saturated with LPS buffer (Soll, 1987; Sussman, 1987). Under these conditions the cells aggregate via chemotaxis to form roughly 1000 identical multicellular assemblies of 10^5 cells each, which proceed through morphogenesis in synchrony.

Restriction-enzyme-mediated integration (REMI) mutagenesis. Cells were mutagenized by electroporation with the pUCBsrABam plasmid (a gift from K. Saxe, Emory University

School of Medicine) (Bear *et al.*, 1998), which contains the gene for blasticidin S resistance (*bsr*) (Sutoh, 1993), using established methods (Kuspa & Loomis, 1992). Based on our experimental determination of transformation efficiency, we estimate that we examined about 5000–6000 individual insertional mutants in this experiment. Based on current estimates of the total number of genes in *Dictyostelium*, and the frequency of obtaining developmental mutants (Shaulsky *et al.*, 1996), this represents coverage of about 10–15% of the genome.

Transformed cells were selected for growth in HL-5 medium containing 10 µg blasticidin ml⁻¹ (ICN). The blasticidin-resistant transformants were then selected for growth in 300 µM cisplatin, the maximum concentration that can be added to the cells (Sigma). Surviving cells were plated for single colonies on SM agar plates (Sussman, 1987) and clones were picked and re-tested for their ability to grow in the presence of 300 µM cisplatin. The disruption vector and the flanking fragments of the disrupted genes were then excised, ligated, cloned into bacteria and subsequently used for homologous recombination to recapitulate the mutation.

Sequence identification. Both genomic fragments flanking the disruption vector were sequenced, using vector-specific primers. The sequences were then checked against sequences in the GenBank database using the BLAST program, as well as against the sequence data generated by the *Dictyostelium* cDNA and genomic sequencing project (Morio *et al.*, 1998; http://dicty.cmb.nwu.edu/Dicty/dictyostelium_genomics.htm). In most cases we were able to assemble the majority of the gene.

Cell survival assays. Cell survival after exposure to damaging agents was determined by plating aliquots of serially diluted cells along with *Klebsiella aerogenes* on SM plates (Sussman, 1987). Each surviving cell gives rise to a single plaque on the bacterial lawn. Survival was tested after treatments with cisplatin, the oxidizing agent H₂O₂ and the alkylating agent methyl methanesulfonate (MMS) (all from Sigma), as well as after treatment with UV light. For cisplatin, stock solutions were made by dissolving cisplatin in Pt buffer (3 mM NaCl, 1 mM sodium phosphate, pH 7.4), to a final concentration of 3.3 mM and verified by checking the absorbance at 220 nm (based on an extinction coefficient at 220 nm of 1.957 mM⁻¹ cm⁻¹). H₂O₂ was used for 1 h at the indicated concentrations, based on an extinction coefficient at 240 nm of 43.6 M⁻¹ cm⁻¹ (Garcia *et al.*, 2000). MMS was directly diluted into medium from an 11.9 M stock solution, supplied by the distributor, and cells were incubated for 1 h. UV exposure was performed in LPS/5 mM EDTA at indicated fluences, adjusted according to Morowitz (Morowitz, 1950; Yu *et al.*, 1998).

Molecular biology techniques. Standard techniques for molecular cloning, Southern and Northern analyses and PCR were used (Lee *et al.*, 1997; Sambrook *et al.*, 1989).

RESULTS

Identification of genes associated with cisplatin resistance

To isolate cisplatin-resistant mutants, cells were randomly mutagenized using the REMI technique (Kuspa & Loomis, 1992), which results in a transformed population of cells, each containing a single random insertion containing the *bsr* gene. We obtained seven cisplatin-resistant clones after further selection on 300 µM cisplatin. To ensure that the cisplatin resistance

Table 1. Cisplatin-resistant mutant strains

Strain	Homology/identified gene	Function	Cisplatin resistance (-fold)*
1	S-1-P lyase	Degradation of S-1-P to hexadecanal and phosphoethanolamine	25
2	RegA (cAMP phosphodiesterase)	Degradation of cAMP to 5'-AMP; involved in PKA-dependent differentiation	4
3	Golgesin	Golgi- and vesicle-associated protein	5
4	PIP5K	Synthesis of phosphatidylinositol 4,5-bisphosphate (PIP2)	9
5	P2Y purine receptor 1	G-protein-coupled nucleotide receptor	13
6	CAAX prenyl protease	Prenylation of proteins that contain COOH-terminal CAAX motif	11
7		The disruption vector inserted into AT-rich intergenic region; no specific gene has been found	8

* Resistance was determined by assaying survival of the mutant versus the wild-type parent (300 μ M cisplatin, 24 h exposure).

in each of these strains was due to the single identified insertion, each gene was re-disrupted by homologous recombination, using the original excised fragment as the disruption vector. The disruptions were confirmed by Southern analysis, to demonstrate that a single insertion resulted in an altered restriction pattern, or by PCR, using one primer from the *bsr* gene and the other primer from a region of the gene outside the disruption vector (data not shown).

The excised fragments were sequenced and we were able to identify the affected genes in six of the seven mutant strains (Table 1).

Gene 1. The *Dictyostelium* homologue of the sphingosine-1-phosphate (S-1-P) lyase gene. It is 65 % similar to the yeast BST1 gene (Saba *et al.*, 1997) and 67 % similar to the human S-1-P lyase (GenBank accession no. AAD44755).

Gene 2. *regA* encodes a bipartite cAMP phosphodiesterase in *Dictyostelium* (Shaulsky *et al.*, 1996), which is composed of a regulatory response domain and a phosphodiesterase domain that is 49 % similar to the *Drosophila dunce* gene (Davis & Kiger, 1981; Walter & Kiger, 1984).

Gene 3. Golgesin is a novel, previously identified *Dictyostelium* gene. Its gene product associates with the Golgi apparatus and intracellular vesicles (GenBank accession no. AAC16756).

Gene 4. This gene is 45 % similar to the kinase domain within a putative phosphatidylinositol-4-phosphate 5-kinase (PIP5K) from *Arabidopsis* (accession no. AAC78530) and may represent a novel member of this family of kinases, which are involved in the synthesis of phosphatidylinositol 4,5-bisphosphate (Loijens *et al.*, 1996).

Gene 5. The gene has 52 % similarity to the human P2Y purine receptor 1 (Leon *et al.*, 1996) within the first third of the gene for which we have sequence.

Gene 6. The disruption in this strain occurred 280 bp upstream of the gene which is 51 % similar to the human CAAX prenyl protease gene (Tam *et al.*, 1998).

Gene 7. The insertion in this strain occurred in an AT-rich intergenic region, which we have been unable to assign to a specific gene.

Cisplatin resistance

The resistance of these mutant strains to cisplatin was quantified for both the original mutant clones and the homologous recombinants. We determined viability after cells were exposed to a single dose of 300 μ M cisplatin for increasing periods of time up to 24 h and the results are presented in Fig. 1(a) and Table 1. Increasing the time of exposure to cisplatin does not result in further cell death or the selection of heritable mutants (Yu *et al.*, 1998). The resistance of the strains produced by homologous recombination was virtually identical to the resistance of the original mutant clones obtained directly from the REMI mutagenesis (data not shown). In addition, we tested independent disruption mutants for the golgesin and *regA* genes (GenBank accession no. AAC16756; Shaulsky *et al.*, 1996). Although the disruptions in these strains were in different domains from the disruptions we identified, these mutant strains had identical levels of increased resistance when compared to their wild-type parents (data not shown). All the mutants showed increasing resistance to cisplatin, ranging from 4- to 25-fold. These levels of resistance are equal to or higher than the levels observed in cisplatin-resistant animal cell lines (Katabami *et al.*, 1992; Kuppen *et al.*, 1988).

Fig. 1. Cisplatin resistance and growth characteristics of wild-type and mutant strains. (a) Survival curves of parental Ax4 cells and seven cisplatin-resistant mutant strains following 24 h treatment with 300 µM cisplatin. Percentage survival was determined by comparing colony formation relative to cells at time zero. ◆, Strain Ax4; ■, S-1-P lyase⁻; △, RegA⁻; ●, golgesin⁻; ○, PIP5K⁻; □, P2Y purine receptor⁻; ▲, CAAX prenyl protease⁻; ◇, strain 7. (b) Response of the parental Ax4 cells and the three resistant mutant strains to a 10 h exposure to increasing concentrations of cisplatin. ◆, Strain Ax4; ■, S-1-P lyase⁻; △, RegA⁻; ○, PIP5K⁻. (c) Growth curves of Ax4 cells and cisplatin-resistant mutant strains. The cells were grown in HL-5 medium and were counted with a haemocytometer. ◆, Strain Ax4; ■, S-1-P lyase⁻; △, RegA⁻; ○, PIP5K⁻.

Because of their associated developmental phenotypes (see below), three of the strains were further examined for cisplatin resistance. We re-established the resistance to cisplatin by exposing the cells to increasing doses of cisplatin for 10 h (Fig. 1b). The same relative order of resistance was observed as in the previous experiment (Fig. 1a).

The resistance of these cells to cisplatin is not due to a fundamental change in the growth rate of the cells. The growth rate of each of these mutant strains in normal medium was identical to that of the wild-type parent (Fig. 1c).

Developmental abnormalities

Interestingly, three of the mutants had immediately obvious defects in development. The normal size and proportioning of wild-type fruiting bodies are shown in Fig. 2(a), where a mass of spores rests on top of the cellular stalk. The S-1-P lyase null mutant produced short fruiting bodies with a dramatically thicker stalk and a drastically reduced number of spores (Fig. 2b). The *regA* null mutant produced fruiting bodies with spore masses that cannot rise up the stalk during morphogenesis (Fig. 2c). It is identical in phenotype to the *regA* mutant that was produced by Shaulsky *et al.* (1998). The PIP5K null mutant produced fruiting bodies with apparently altered cell-type proportions. It has abnormally long stalks and a spore mass of reduced size (Fig. 2d).

Response of the wild-type genes to cisplatin

The response to cisplatin exposure of these genes in wild-type cells was evaluated by Northern analysis. In contrast to the NER helicase genes *repB* and *repD* (Fig. 3a; Yu *et al.*, 1998), which have been shown to up-regulate their expression in response to cisplatin exposure, there was virtually no change (less than 30%) in the steady-state levels of S-1-P lyase, PIP5K and *regA* mRNAs, as is shown in Fig. 3(b, c, d). Thus the response of the parent strain to cisplatin does not include an abrupt up-regulation of these genes and the resistance of these mutant strains to cisplatin does not result from a lack of up-regulation of the genes in response to cisplatin. This implies that these genes are constitutively expressed at functional levels and that their absence prevents the subsequent activation of other genes required for cell death. This is in contrast to the induction of the p73 by c-Abl (Gong *et al.*, 1999), which is required for cisplatin-induced cell death in mammalian cells.

Mutant cells are specifically resistant to cisplatin

Earlier work on *Dictyostelium* mutants which were isolated based on their sensitivity to γ -irradiation revealed that they were often sensitive to DNA-damaging chemicals as well (Bronner *et al.*, 1992; Podgorski

Fig. 2. Developmental morphologies of wild-type and cisplatin-resistant mutant strains. Cells were grown axenically in HL-5 medium and plated on black filters for development as described in Methods. The fruiting bodies were photographed after 29 h when development was complete (magnification $\times 64$). (a) Wild-type Ax4 cells, (b) S-1-P lyase⁻ cells, (c) RegA⁻ cells, (d) PIP5K⁻ cells.

Fig. 3. Gene expression following treatments with cisplatin. Expression of *repD* (a), S-1-P lyase (b), *regA* (c) and PIP5K (d) genes following cisplatin treatment. Ax4 cells were grown to a density of 2×10^6 cells ml⁻¹ and exposed for 10 h to varying concentrations of cisplatin. Total RNA was analysed for gene expression. mRNA levels are expressed as a ratio of treated to untreated cells.

& Deering, 1980). Therefore, we wished to determine if the cisplatin-resistant mutants we identified showed any cross-resistance to other DNA-damaging agents. We tested mutant cell resistance to UV, the oxidizing agent H₂O₂ and the alkylating agent MMS. Fig. 4 shows the survival curves for mutant and wild-type cells treated with these three agents. We found that for the three mutants tested, there was no increased resistance to the other DNA-damaging agents which was comparable to the increased resistance observed with cisplatin.

DISCUSSION

The development of drug-resistant tumour cells significantly limits the effectiveness of chemotherapeutic approaches to cancer treatment. Although cisplatin is

widely used and is highly effective against many types of tumours, the selection of drug-resistant cells limits its usefulness. Considerable attention has been paid to the underlying mechanisms of resistance to this drug (Andrews & Howell, 1990; Chu, 1994; Perez, 1998). Many of the studies have focused on cultured cisplatin-resistant tumour cells. These studies suffer from the possibility that multiple mutations could cause the drug resistance phenotype in a particular cell line. These systems did not afford the possibility of identifying the specific genes responsible for the drug resistance, although they did associate specific phenotypes with these cell lines, including changes in drug transport, accumulation or inactivation. Other studies have been performed on cultured cells that were selected for resistance to cisplatin by growth in increasing concen-

Fig. 4. Resistance of mutant strains is specific for cisplatin. Survival curves of wild-type Ax4 (◆), S-1-P lyase⁻ (■), RegA⁻ (△) and PIP5K⁻ (○) cells following treatment with various doses of (a) H₂O₂, (b) MMS and (c) UV light.

trations of the drug for many generations, and in some studies it was not clear whether the resistance was indeed a heritable change, rather than a physiological adaptation. Recent studies, using DNA microarray technology, have identified a number of genes that were up- or down-regulated in cisplatin-sensitive versus -resistant cells. However, it is not clear which, if any, of these genes represents a primary response to the drug and it is not known if alteration in the expression of these genes results in drug resistance (Los *et al.*, 1998).

We have taken a direct genetic approach to identifying biochemical pathways that are involved directly in cisplatin resistance by selecting mutants that are resistant to the drug. This approach has the advantage of being unbiased and indeed has resulted in the identification of six genes previously unsuspected of having a role in the cellular response to this drug. Since the mutant screen was not saturating, we expect that additional 'cisplatin resistance' genes will be identified in the future. Indeed, a multi-drug-resistant transporter has been recently identified in *Dictyostelium* (Good & Kuspa, 2000) and it will be interesting to test whether it functions in modulating cisplatin sensitivity. Two of the genes we have identified in this study immediately suggest testable mechanisms that can explain the increased sensitivity to cisplatin.

In the case of S-1-P lyase, complex sphingolipids are known to play a role in cell proliferation and survival (Merrill *et al.*, 1993). Recently, the products of sphingomyelin catabolism (ceramide, sphingosine and S-1-P) have been implicated in signal transduction processes in animal cells, presumably acting as second intracellular messengers. S-1-P lyase catalyses the conversion of S-1-P to hexadecanal and phosphoethanolamine. It has been suggested that the pathway that begins with the degradation of sphingomyelin to ceramide is involved in cell death and that the modulation of ceramide and S-1-P levels acts as a rheostat, maintaining a balance between

proliferation and cell death functions (Spiegel, 1999). The work described herein is the first to demonstrate a relationship between cisplatin resistance and the cellular levels of S-1-P. It will be important to demonstrate that the other members of this pathway can influence resistance to cisplatin. In this regard we have already identified the sphingosine kinase gene and are working to make a disruption in this gene. We expect it will have increased sensitivity to cisplatin due to the decrease in S-1-P level. Overall, this suggests that manipulating the levels of S-1-P in the cells could be an important therapeutic avenue by potentiating tumour cells to be more sensitive to cisplatin or other drugs.

In the case of *regA*, the gene product was recently identified as a central component in the pathway for spore differentiation in *Dictyostelium* (Shaulsky *et al.*, 1996). It has a *Drosophila* homologue, the *dunce* gene, which has been related to learning and memory in this organism (Byers *et al.*, 1981; Davis & Kiger, 1981). The RegA protein is a cAMP phosphodiesterase, regulating the cAMP level in prespore cells, which in turn regulates protein kinase A (PKA). The *Dictyostelium* PKA exists as a dimer of one regulatory and one catalytic subunit, while the mammalian enzyme consists of two subunits of each. The binding of cAMP to the regulatory subunit (PKA-R) releases and activates the catalytic subunit (PKA-C). The developmental analysis of *regA* has been extensive and it is known that the expression of the RegA-, PKA-C- and PKA-R-encoding genes increases during development. There is a low level of expression of all three genes in growing cells, but none is required for growth (Loomis, 1998). The discovery of this gene in our selection was important because it suggests that cisplatin resistance may be linked to PKA signalling pathways. The role of PKA in response to cisplatin can be easily tested because of the wide availability of mutants altered in PKA activity in *Dictyostelium* (Loomis, 1998). In recent work PKA has been linked to cisplatin resistance in CHO cells (Cvijic *et al.*, 1998a),

but has been ruled out as having a role in response to cisplatin in yeast (Cvijic *et al.*, 1998b). Additionally, an elevated level of cAMP has been shown to cause attenuation of the response to cisplatin in macrophages (von Knethen *et al.*, 1998).

There is an increasing number of examples of molecular cross-talk in DNA repair, including the sharing of enzymic components between nucleotide excision repair, base excision repair and mismatch repair mechanisms (Sancar, 1999; Swanson *et al.*, 1999). In *Dictyostelium*, at least eight non-allelic mutants that were isolated based on increased sensitivity to γ -irradiation also showed increased sensitivities to other DNA-damaging drugs (Bronner *et al.*, 1992; Podgorski & Deering, 1980). The results of our mutant selection clearly illustrate that multiple genes and pathways are involved in the response to cisplatin. However, the deletion of S-1-P lyase-, RegA- and PIP5K-encoding genes did not result in resistance to other DNA-damaging agents in addition to cisplatin. The specificity for resistance to cisplatin in *Dictyostelium* is similar to that observed with several cisplatin-resistant tumour cell lines (Teicher *et al.*, 1991; Rabo *et al.*, 1996) and is important because it offers specific potential target pathways for chemotherapeutic agents.

The identification of the developmental phenotypes associated with some of the cisplatin-resistant mutants was an important outcome of this study. Although the role of *regA* in development has been well studied, nothing is known about the other two genes. The severity of the S-1-P lyase mutant is particularly interesting and we have already shown that it produces very few mature spores. Further work is needed to fit the action of these genes into the increasingly well understood gene circuitry that is already known to control development in *Dictyostelium* (Laurence & Firtel, 1999).

Dictyostelium is more often associated with studies of cellular and developmental biology, such as the control of cell chemotaxis and motility (Parent & Devreotes, 1999) and regulated protein secretion (Srinivasan *et al.*, 2000). However, it is clear from the work described here that it can be effectively used to discover genes underlying cellular responses to important pharmacological agents. Moreover, its unique biology allows the immediate identification of those genes that also have a role in multicellular development as well as in the response to the drug.

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