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Identification of inducible damage-recognition proteins that are overexpressed in HeLa cells resistant to *cis*-diamminedichloroplatinum(II)

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Two *cis*-diamminedichloroplatinum(II) (cisplatin)-inducible proteins [b_{130} (~ 130 kDa) and b_{95} (~ 95 kDa)] in HeLa cells that recognize both the cisplatin-modified and u.v.-modified DNA were identified in this study. These damage-recognition proteins were overexpressed in cisplatin-resistant HeLa cells. The results suggest that the damage-recognition proteins are regulated in the cells in response to DNA damage, and they may be important for DNA repair and probably the emergence of cisplatin resistance.

INTRODUCTION

The development of drug resistance in cancer patients causes a major hindrance to cancer treatment [1–4]. Cellular resistance to *cis*-diamminedichloroplatinum(II) (cisplatin), a widely used chemotherapeutic agent, is not an exception, although the mechanism is not clear. To accomplish further understanding of cisplatin resistance, appreciation has been found through studies of cultured cells. The substantial lines of indirect evidence that have accumulated have suggested that the cisplatin-resistant phenotype is usually associated with enhanced DNA repair, although other mechanisms, such as membrane-associated drug efflux, may also be involved [4].

Effective DNA repair in cells depends upon an efficient coupling of the repair enzymes to the target domain of the damaged chromosomal DNA [5]. It is reasonable to say that a similar repair environment can be partly mimicked *in vitro* by using plasmid DNA and isolated cellular proteins. Such a system has recently been established (e.g. [6–9]). To gain a further understanding of cisplatin resistance in human cells, assays involving South-Western blotting [10,11] and gel-mobility shift [12,13] were used. In the present study, we find that cellular factors which recognize cisplatin-modified DNA as well as u.v.-modified DNA were inducible by cisplatin. A cisplatin-resistant cell line, which was also cross-resistant to u.v. [14], showed an overexpression of these damage-recognition proteins (DRPs). The results suggest that the development of cisplatin resistance in human cells may involve a regulated mechanism that is associated with an effective means of lesion recognition.

EXPERIMENTAL

Cell lines and culture conditions

Cisplatin-resistant cells were made by stepwise exposure of HeLa cells to an increasing concentration of cisplatin (Platiamine; Farmitalia Carloerba Ltd., Barcelona, Spain) up to 8 μM [14]. Resistant and parental cells were maintained in monolayer culture in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD, U.S.A.), containing 10% (v/v) foetal-bovine serum, 100 μg of streptomycin/ml and 100 units of penicillin/ml, and incubated at 37 °C in a humidified atmosphere of CO_2/air (1:19). Resistant cells were maintained in medium containing 0.3 μg of cisplatin/ml.

The acquired resistance of cells to cisplatin is defined as the ratio of IC_{50} (drug concentration inhibiting cell survival by 50%) in the resistant cells to that in the parental cells. Cytotoxicity was assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric method [15].

Platination and u.v. irradiation of plasmid DNA

The f130 DNA (see below and Fig. 1*a*) at 100 μg/ml was irradiated in the dark with 1000 J of u.v./m² from a u.v. germicidal lamp at a fluence rate of 25 J/s per m² as described [16]. The f103 DNA (see below) was treated in the dark with cisplatin in 3 mM-NaCl/1 mM-sodium phosphate (pH 7.4), at 37 °C for 18–24 h [17]. The DNA concentration was 100 μg/ml (2×10^{-4} M-nucleotide phosphate). After treatment, the concentration of NaCl was increased to 0.1 M, and the modified DNA was precipitated by standard methods [18], washed once in ethanol/0.4 M-sodium acetate, (5:2, v/v), pH 5.2, and once in 80% (v/v) ethanol to remove the free cisplatin, then resuspended in TE buffer [10 mM-Tris/HCl (pH 7.5)/1 mM-EDTA], and used for transfection. In this experiment, f103 was treated with cisplatin to generate a calculated r_f (the molar ratio of free cisplatin to nucleotide phosphate at equilibrium) of 0.08 [17].

Protein preparation and South-Western blotting

Crude nuclear extracts were prepared from the cells as described by Dignam *et al.* [19]. Cells were lysed with lysis buffer I (0.5% Triton X-100/10 mM-Hepes (pH 8.0)/0.5 M-sucrose/50 mM-NaCl/10 mM-$MgCl_2$/1 mM-dithiothreitol). The nuclei were then collected by centrifugation and lysed with lysis buffer II (lysis buffer I plus 0.5 M-NaCl and 10 mM-spermidine). High-molecular-mass DNA was removed from the lysate by centrifugation, and the supernatant was collected and dialysed against dialysis buffer [50% (v/v) glycerol/10 mM-Hepes (pH 8.0)/50 mM-NaCl/10 mM-$MgCl_2$/1 mM-dithiothreitol]. Quantification of the protein concentration was performed by the Bradford method [20].

Nuclear proteins were separated in an SDS/4–10%-gradient-polyacrylamide gel without prior boiling of the samples. The separated proteins were electrophoretically transferred from the gel to poly(vinylidene difluoride) ('PVDF') filters (Millipore). The filters were soaked in a blocking buffer [10 mM-Hepes (pH 8.0)/5% Carnation skim milk] at room temperature for 1 h, then incubated for 1 h in a binding buffer [10 mM-

Abbreviations used: cisplatin, *cis*-diamminedichloroplatinum(II); DRP, damage-recognition protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMEM, Dulbecco's Modified Eagle's medium.

Hepes (pH 8.0)/50 mM-NaCl/10 mM-$MgCl_2$/1 mM-dithiothreitol/0.25% non-fat milk] containing 5×10^7 c.p.m. of DNA probe. The filters were then washed twice for 1 h each with washing buffer (binding buffer without DNA probe and with 150 mM-NaCl instead of 50 mM). The semi-dried filters were then subjected to Kodak XAR-5 X-ray films for autoradiography. The relative expression of DNA-binding proteins was determined by scanning densitometry of the X-ray film.

DNA probes and gel-mobility-shift assay

The 130 bp *Sph*I–*Bgl*I fragment from plasmid pSVT [21] was ligated to *Sph*I–*Sma*I-opened vector pBS(+) (Stratagene, La Jolla, CA, U.S.A.) at the *Sph*I site. The *Bgl*I site was blunted with Klenow DNA polymerase (New England Biolabs, Beverly, MA, U.S.A.) and ligated to the *Sma*I site. The f130 fragment containing the 17 bp dA/dT-rich region, which is a potential target for u.v. modification, was then generated with *Hin*dIII and *Eco*RI (hereinafter called 'f130'). The DNA fragment f103 [6] was the 103 bp *Stu*I–*Ava*II fragment from recombinant plasmid pCD-α-globin [22], filled, attached to *Eco*RI and *Xba*I linkers respectively, and cloned into pBS(+). The *Hin*dIII–*Eco*RI-cut-out fragment (hereinafter called 'f103') contains a 14 bp string of dG·dC-rich region which is a potential target for cisplatin modification. *Hin*dIII–*Eco*RI-generated f103 or f130 fragments were ^{32}P-labelled ($\sim 3 \times 10^4$ c.p.m./ng of DNA) with Klenow DNA polymerase and spin-column-purified by standard methods [18].

The gel-mobility-shift assay was performed as described [12,13]. Briefly, 10^4 c.p.m. of u.v.-modified f130 (1000 J/m^2) or cisplatin-modified f103 ($r_f = 0.08$), unless otherwise indicated, was incubated with nuclear extracts at 30 °C for 30 min. The reaction mixtures were then separated by 4%-PAGE in low-ionic-strength buffer at 25 °C and 15 mA constant current. The resolved gels were directly exposed to a Kodak XAR-5 X-ray film without further processing. Densitometry was used to quantify the intensity of the binding bands on the autoradiogram.

RESULTS

To identify inducible cellular factors that interact with the damaged forms of DNA, exponentially growing cells were treated with cisplatin. Nuclear proteins were prepared and processed for South-Western blotting, then probed with 5×10^7 c.p.m. each of the unmodified f103 and f130 (Fig. 1*a*) or 5×10^7 c.p.m. of cisplatin-modified f103 (Fig. 1*b*). Two DRPs (b_{130} and b_{95}) were identified, both of which were inducible by treatment with cisplatin for 4 h (compare lanes 1 with 2, and 4 with 5). There is a low constitutive expression of these factors in the parental cells (lane 1). A longer induction period (24 h) did not increase further the level of these binding proteins (cf. lanes 2 with lanes 3). A similar inducible effect was also observed in resistant cells (lanes 4–6; see the Figure legend for details). Scanning densitometry of the X-ray films indicated that resistant cells (lanes 4–6) displayed a 4–5-fold enhancement of b_{95} as compared with parental cells (cf. lanes 1, 2, and 3 with 4, 5 and 6 respectively). Overexpression of b_{130} in resistant cells was also detected (cf. lanes 1 and 4). It became even more dramatic after induction (cf. lanes 2 and 5). A ~60 kDa factor (b_{60}) that was overexpressed in resistant cells was also identified. However, binding of b_{60} was less consistent and it was able to interact with the unmodified DNA probe (also see Fig. 2). Therefore, it should not be considered as a damage-inducible protein. In addition, a ~25 kDa cellular protein (b_{25}) that interacts with damaged as well as undamaged forms of the DNA was monitored in the present study. In contrast with the inducible b_{130} and b_{95}, b_{25} was very sensitive to cisplatin; it was immediately inhibited in response to the treatment (compare lanes 1 and 2). Interestingly, b_{25} was absent in resistant cells. Further studies are needed with regard to the role of b_{25} in the induction of b_{130} and b_{95} and, probably, in the development of cisplatin resistance.

To understand further the nature of the identified cisplatin-inducible DRPs, nuclear extracts from induced (3 μM-cisplatin, 4 h) and non-induced resistant cells were also tested for binding to u.v.-modified DNA. Fig. 2 shows the typical binding patterns seen. Triplicate Western blots were probed separately with 5×

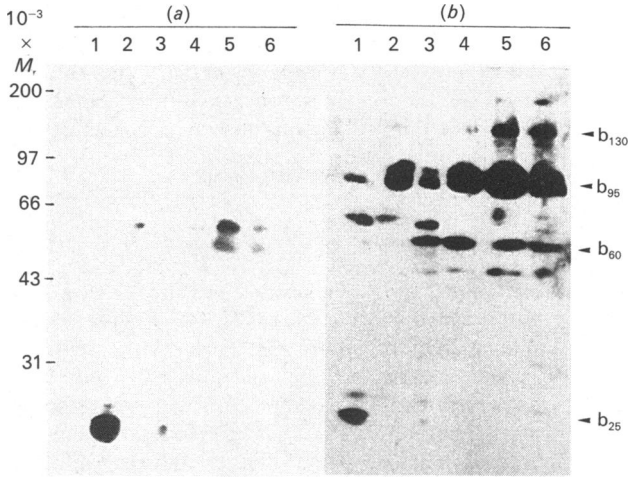

Fig. 1. Induction of DRPs by cisplatin as assayed by South-Western blotting

Cells were treated with 3 μM-cisplatin for various periods of time. A 100 μg portion of nuclear extract was loaded in each lane. Lanes: 1 and 4, untreated cells; 2 and 5, cells treated for 4 h; 3 and 6, 24 h. Lanes 1–3 are parental HeLa cells; lanes 4–6 are cisplatin-resistant cells. Probes: (*a*) unmodified f130 plus f103 (5×10^7 c.p.m. each); (*b*) cisplatin-modified f103 (5×10^7 c.p.m.). M_r values are indicated at the left; binding factors of interest are indicated at the right.

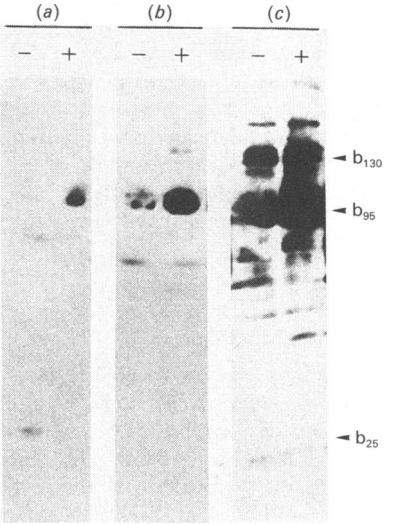

Fig. 2. Cross-interaction of the inducible cisplatin-modified DNA binding factors to u.v.-modified DNA

Cells were untreated (−) or treated (+) with 3 μM-cisplatin for 4 h. A 100 μg portion of nuclear extract was loaded in each lane. (*a*) Unmodified f130 plus f103 (5×10^7 c.p.m. each); (*b*) u.v.-modified f130 (5×10^7 c.p.m.); (*c*) cisplatin-modified f103 (5×10^7 c.p.m.). Binding factors are indicated on the right.

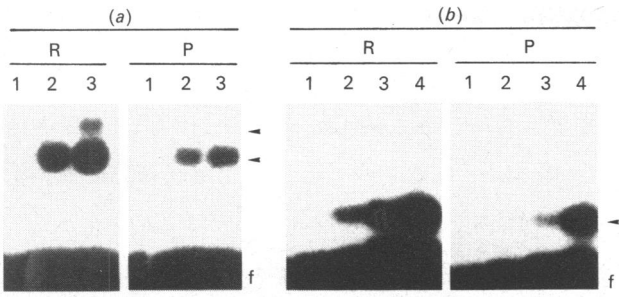

Fig. 3. Overexpression of DRPs in resistant cells as assayed by gel-mobility shifts

Cellular extracts from resistant (R) or parental (P) cells were used for binding to u.v.-modified DNA (*a*) or cisplatin-modified DNA (*b*). (*a*) Lanes: 1, f130 irradiated with u.v. at 0 J/m^2; 2, 3000 J/m^2; 3, 6000 J/m^2. (*b*) Lanes: 1, f103 treated with cisplatin to r_f 0; 2, 0.02; 3, 0.04; 4, 0.08. Symbols: f, free probe; arrowheads, bound probe.

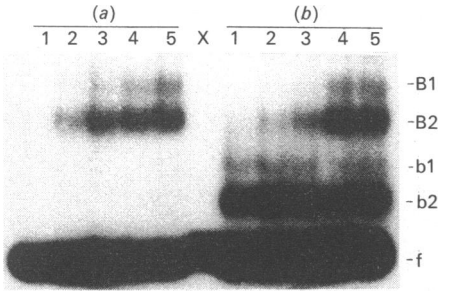

Fig. 4. Lack of competition for cisplatin-modified-DNA-binding factors by u.v.-modified DNA

(*a*) f130 (0.3 ng) was irradiated with various amounts of u.v. and used as a probe to bind with parental nuclear extract for the gel-mobility-shift assay. (*b*) Cisplatin-modified f103 (0.3 ng, r_f 0.08) was also included in the binding reaction as in (*a*), followed by electrophoresis as in Fig. 3. Lanes: 1, 0 kJ/m^2; 2, 1 kJ/m^2; 3, 3 kJ/m^2; 4, 6 kJ/m^2; 5, 9 kJ/m^2; X, without nuclear extract; f, free probe; B1 and B2, bound u.v.-modified DNA; b1 and b2, bound cisplatin-modified DNA.

10^7 c.p.m. each of unmodified f130 and f103 (Fig. 2*a*), 5×10^7 c.p.m. of u.v.-modified f130 (Fig. 2*b*) or cisplatin-modified f103 (Fig. 2*c*). As shown, b_{95} from either induced (+) or non-induced (−) cells also binds to u.v.-modified DNA, although less intensely than to cisplatin-modified DNA. b_{130} also interacts with u.v.-modified DNA, but binds preferentially to the cisplatin-modified DNA. It should be noted that the amount of probes used for the control experiment (Fig. 2*a*) was doubled. Similar results were obtained using parental-cell nuclear extracts (results not shown). Therefore the inducible binding of b_{95} and b_{130} to u.v.-modified DNA should be significant.

An alternative to South-Western blotting for the detection of DNA–protein interaction is using gel-mobility shift. Fig. 3 shows the typical results. A 2 μg portion of crude nuclear extracts from resistant (R) or parental (P) cells were incubated with u.v.-modified f130 (Fig. 3*a*) or cisplatin-modified f103 (Fig. 3*b*) probe. The level of both binding proteins (indicated with arrowheads) was dependent upon the severity of the DNA lesion (see the legend to Fig. 3 for details). It is apparent that the DRPs were overexpressed in resistant cells.

To demonstrate further the independence of the identified cisplatin-modified and u.v.-modified DNA-binding proteins in gel-shift studies, competition assays were carried out. With increasing damage to the u.v.-modified DNA probe (Fig. 4, panel *a*; lanes 2–5), the u.v.-modified DNA-binding activities from parental extracts (indicated by B1 and B2) were increasingly enhanced. When a fixed amount of cisplatin-modified DNA probe was included in the reaction mixture, the increased u.v.-modified DNA binding did not significantly affect the cisplatin-modified-DNA binding activities (indicated by b1 and b2 in Fig. 3*b*). Extracts from resistant cells gave similar binding patterns (results not shown). The results indicate the different mobilities of u.v.-modified-DNA and cisplatin-modified-DNA binding complexes.

DISCUSSION

We have applied South-Western blotting to study DNA–protein binding and identified two damage-inducible cellular proteins that bind to damaged DNA. These DRPs were overexpressed in cisplatin-resistant cells. Studies using gel-mobility shifts also showed enhanced DRPs in resistant cells. Competition analysis suggests that cisplatin-modified-DNA-binding protein is independent of u.v.-modified-DNA-binding protein. However, South-Western blotting indicates that b_{95} and b_{130} also interacts with u.v.-modified DNA, suggesting that the u.v.-modified-DNA-binding activities identified by two methods are different. Alternatively, these results also implicate that a common domain may exist in u.v.-modified- and cisplatin-modified-DNA-binding proteins. This finding is not surprising, because only a small portion of the treated DNA molecule carries a DNA lesion, leaving a large part of the molecule unmodified (Chao, C. C.-K., unpublished work). The cross-interaction of the cisplatin-modified-DNA-binding protein b_{95} to u.v.-modified-DNA (see Fig. 2) may be interpreted as due to the unmodified DNA-binding domain of the molecule.

In the present study we have also identified b_{25}, a binding protein suppressible by cisplatin and absent in resistant cells. This factor is also inhibited by u.v. (results not shown). It is reasonable to believe that cisplatin can elicit the expression of DRPs, presumably because these inducible molecules are required for DNA repair, and especially so because these cisplatin-inducible DRPs are overexpressed in resistant cells. In addition, resistant cells also showed cross-resistance to u.v. [14]. Therefore it is not surprising that u.v.-modified-DNA-binding proteins were also overexpressed in resistant cells [9,23]. However, it is difficult to imagine why b_{25} is not inducible and is absent from resistant cells. One possibility is that b_{25} plays a role in cisplatin resistance through negative regulation. Absence of this molecule mediates the induction of damaged-DNA binding proteins. However, this is purely hypothetical, and more studies are required to test this hypothesis.

Additionally, we have demonstrated that the inducible DRPs are constitutively overexpressed in cisplatin-resistant cells. By contrast, the suppressible binding protein for unmodified DNA (i.e. b_{25}) is down-regulated in resistant cells. Nevertheless, it is not yet clear as to what is the connection between the DRPs identified by South-Western blotting and those identified by gel-mobility shifts. It seems possible that the 95 kDa protein identified here could not be the 91 kDa protein described by Donahue *et al.* [7], because the mRNA corresponding to the 91 kDa protein in resistant cells remains the same as the parental cells (Chao, C. C.-K., unpublished work). Although the two studies differ with regard to u.v.-irradiated DNA, this could reflect differences in assay sensitivities and substrates used. Our results indicate that the DRPs are inducible in human cells. The results also suggest the DRPs might be potential indicators of cisplatin resistance in human cancer.

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