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Reversible protein phosphorylation modulates nucleotide excision repair of damaged DNA by human cell extracts

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ABSTRACT

Nucleotide excision repair of DNA in mammalian cells uses more than 20 polypeptides to remove DNA lesions caused by UV light and other mutagens. To investigate whether reversible protein phosphorylation can significantly modulate this repair mechanism we studied the effect of specific inhibitors of Ser/Thr protein phosphatases. The ability of HeLa cell extracts to carry out nucleotide excision repair *in vitro* was highly sensitive to three toxins (okadaic acid, microcystin-LR and tautomycin), which block PP1- and PP2A-type phosphatases. Repair was more sensitive to okadaic acid than to tautomycin, suggesting the involvement of a PP2A-type enzyme, and was insensitive to inhibitor-2, which exclusively inhibits PP1-type enzymes. In a repair synthesis assay the toxins gave 70% inhibition of activity. Full activity could be restored to toxin-inhibited extracts by addition of purified PP2A, but not PP1. The p34 subunit of replication protein A was hyperphosphorylated in cell extracts in the presence of phosphatase inhibitors, but we found no evidence that this affected repair. In a coupled incision/synthesis repair assay okadaic acid decreased the production of incision intermediates in the repair reaction. The formation of 25–30mer oligonucleotides by dual incision during repair was also inhibited by okadaic acid and inhibition could be reversed with PP2A. Thus Ser/Thr-specific protein phosphorylation plays an important role in the modulation of nucleotide excision repair *in vitro*.

INTRODUCTION

The reversible phosphorylation of proteins plays a major role in the regulation of diverse cellular processes. These include metabolism, contractility, membrane transport, transcription, translation and progression through the cell cycle. The level of phosphorylation of any protein depends on the relative activities of protein kinases (PKs) and protein phosphatases (PPs). Although less well studied than the protein kinases, knowledge of the structure and functions of the protein phosphatases has increased enormously in the past few years. This has led to the appreciation that PPs are not just passive counterparts of PKs, but are regulated in a sophisticated manner and play an active and essential role in the regulation of cellular processes (reviewed in 1,2).

Proteins can be phosphorylated on either serine, threonine or tyrosine residues, but the vast majority of phosphorylation events in mammalian cells involve serine and threonine (3). The Ser/Thr-specific protein phosphatases that dephosphorylate these residues show broad and overlapping substrate specificities *in vitro* and their classification requires the use of specific inhibitors and activators (4,5). In most cases the native forms of Ser/Thr-specific protein phosphatases are comprised of one of four different types of catalytic subunit (PP1, PP2A, PP2B or PP2C) together with additional proteins that modulate their activity and/or substrate specificity and intracellular localization (5,6).

Type 1 phosphatases (PP1) specifically dephosphorylate the β subunit of phosphorylase kinase and are inhibited by the thermostable proteins inhibitor-1 and inhibitor-2. Conversely, type 2 (PP2) phosphatases preferentially dephosphorylate the α subunit of phosphorylase kinase and are unaffected by these inhibitors (5). In addition, protein phosphatases containing the PP1 or PP2A catalytic subunit can be selectively inhibited by specific toxins, such as the polyketal fatty acids okadaic acid and tautomycin and the cyclic heptapeptide microcystin-LR (7–9). These toxins are now being widely employed to investigate the involvement of Ser/Thr protein phosphatases in a variety of cellular processes (10–12). The concentration-dependent selectivity of these toxins allows an assessment of the relative contribution of different protein phosphatases to a particular cellular process (4,7,9).

The aim of this work was to investigate whether nucleotide excision repair (NER) of DNA in mammalian cells is subject to regulation by reversible protein phosphorylation. NER is used by mammalian cells to remove a large variety of DNA lesions, including the major photoproducts induced by UV light in DNA (13,14). Several human inherited syndromes, including xeroderma pigmentosum, are associated with mutations in NER genes (15). The NER process can be broadly divided into two stages. The first stage involves recognition of the damage by a multiprotein system and enzymatic cleavage to produce two

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incisions flanking the lesion. In the second stage an oligomer carrying the damage is excised, DNA synthesis forms a repair patch using the opposite strand as a template and repair is completed by a DNA ligase. More than 20 polypeptides are known to participate in the whole process in mammalian cells, including proteins that also have roles in DNA replication and transcription (16).

NER can be carried out by mammalian cell extracts, thus allowing its biochemical study and manipulation *in vitro* (17,18). Repair synthesis can be monitored by incubating damaged circular DNA with cell extracts and then quantifying the incorporation of deoxynucleotides. In this study we report that Ser/Thr-specific protein phosphorylation can modulate NER of damaged DNA *in vitro*. In particular, we show that a PP2A-type phosphatase activity is required for maximum efficiency of the first stage (recognition/incision) in the repair process.

MATERIALS AND METHODS

Protein phosphatases and phosphatase inhibitors

The catalytic subunit of PP2A (PP2Ac) was purified to homogeneity from bovine heart (19) and was kindly provided by Dr R.W.MacKintosh (University of Dundee). Human PP1γ (20) was expressed and purified in *Escherichia coli* as described by Alessi *et al.* (21) and was kindly provided by Dr P.W.T.Cohen (University of Dundee). Okadaic acid, microcystin-LR and tautomycin were purchased from Calbiochem (Nottingham, UK), dissolved in DMSO and stored at –20°C. Working solutions were prepared in distilled water immediately before use. Recombinant inhibitor-2 (250 µM) was kindly provided by Dr P.T.W.Cohen.

PP1 and PP2A were assayed by the dephosphorylation of 10 µM glycogen phosphorylase (22,23). ^{32}P-Labelled glycogen phosphorylase was kindly provided by Dr R.W.MacKintosh. One unit of activity catalyses the dephosphorylation of 1 µmol glycogen phosphorylase in 1 min. Diluted extracts and inhibitors were preincubated for 15 min at 30°C prior to initiating the reaction by addition of substrate. For this assay extracts were diluted 50- to 200-fold in 50 mM Tris–HCl, pH 7.5, 0.1% (v/v) 2-mercaptoethanol and 1 mg/ml bovine serum albumin. Under these conditions 1 nM okadaic acid is sufficient to inhibit PP2A in the diluted extracts.

Preparation and fractionation of whole cell extracts

HeLa cells were grown in suspension in RPMI 1640 medium containing 5% foetal calf serum to a density of ~8×10^5 cells/ml, washed in phosphate-buffered saline (PBS) and collected by centrifugation. A whole cell extract was prepared as described (24). Whole cell extract protein was loaded onto a phosphocellulose column (Whatman P11) equilibrated in buffer A (25 mM HEPES–KOH, pH 7.8, 1 mM EDTA, 0.01% NP40, 10% glycerol, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride) containing 0.15 M KCl. Fractions were collected at a flow rate of 90 ml/h. After collection of flow-through fractions bound protein was eluted with PBS containing 1.0 M KCl. The peak protein fractions from the flow-through (CFI) and the 1.0 M KCl elution (CFII) were used. CFI was fractionated on a DEAE Biogel column (BioRad) that had been equilibrated in PBS containing 0.15 M KCl. The flow-through was collected at 90 ml/h and bound protein was eluted from the column in PBS containing 1.0 M KCl. Peak fractions from the flow-through (CFIA, containing RPA) and the 1.0 M KCl elution (CFIB, containing PCNA) were used. CFII, CFIA and CFIB were dialysed against 25 mM HEPES–KOH, pH 7.9, 1 mM EDTA, 17% glycerol, 1 mM dithiothreitol, 12 mM $MgCl_2$ and 0.1 M KCl. Aliquots of the whole cell extract, CFII, CFIA and CFIB were frozen at –80°C. RPA was purified to homogeneity from HeLa cells as previously described (25).

Repair synthesis assay

The plasmids used were the 3.0 kb pBluescript KS(+) and the 3.7 kb pHM14. pBluescript KS(+) was UV irradiated (450 J/m^2). Both plasmids were treated with *E.coli* Nth protein and closed circular DNA was isolated from caesium chloride and sucrose gradients (24). Reaction mixtures (50 µl) contained 250 ng irradiated pBluescript KS(+) and 250 ng non-irradiated pHM14, 45 mM HEPES–KOH, pH 7.8, 70 mM KCl, 7.4 mM $MgCl_2$, 0.9 mM dithiothreitol, 0.4 mM EDTA, 20 µM each dGTP, dCTP and TTP, 8 µM dATP, 74 kBq [α-^{32}P]dATP (110 TBq/mmol), 2 mM ATP, 22 mM phosphocreatine (di-Tris salt), 2.5 µg creatine phosphokinase, 3.4% glycerol, 18 µg bovine serum albumin and cell extract, fractions and/or repair proteins as indicated. Reactions were incubated at 30°C for 3 h or as specified. Plasmid DNA was purified from reaction mixtures, linearized (when appropriate) and separated by electrophoresis overnight on a 1% agarose gel containing ~0.3 µg/ml ethidium bromide. Data were collected by autoradiography or by use of a phosphorimager and calibrated by scintillation counting of the excised bands. Densitometry of photographic negatives took into account the 1.6-fold greater fluorescence of nicked circular DNA over closed circular DNA.

Dual incision assay

The analysis of damaged oligonucleotides formed by the dual incision reaction has been described in detail elsewhere (44). Briefly, 300 ng M13mp18GTG duplex closed circular DNA containing a single 1,3-intrastrand d(GpTpG) cisplatin crosslink (26,27) was incubated under the same conditions as the DNA repair synthesis reactions described above, except that [α-^{32}P]dATP was omitted. DNA was phenol extracted, precipitated with ethanol, resuspended in 8 µl buffer (10 mM Tris–HCl, pH 8, 1 mM EDTA) and then subjected to electrophoresis in a denaturing 12% polyacrylamide gel. After transfer to a nylon membrane, oligonucleotides produced by dual incision were detected by hybridization to a 5′-^{32}P-labelled 27mer oligonucleotide (5′-GAAGAGTGCACAGAAGAAGAG-GCCTGG-3′) complementary to the sequence containing the platinum lesion.

SDS–PAGE and immunoblotting

For immunoblotting 50 µl repair reactions containing 10 µl HeLa whole cell extract (200 µg protein) were used. Reactions were stopped by the addition of Laemmli sample buffer, boiled for 5 min and proteins separated on 15% SDS–polyacrylamide gels as described (28). Proteins were transferred to Immobilon/polyvinylidene difluoride membranes (Millipore) using an electrophoretic cell (BioRad) following the manufacturer's recommendations. The blots were washed briefly with PBS-T (PBS containing 0.1% Tween-20) and blocked with 20% dry milk in the same solution. After three washes blots were probed for the 34 kDa subunit of RPA using a 1:150 dilution of monoclonal antibody SSB34A raised against this subunit (25). Blots were then washed three times

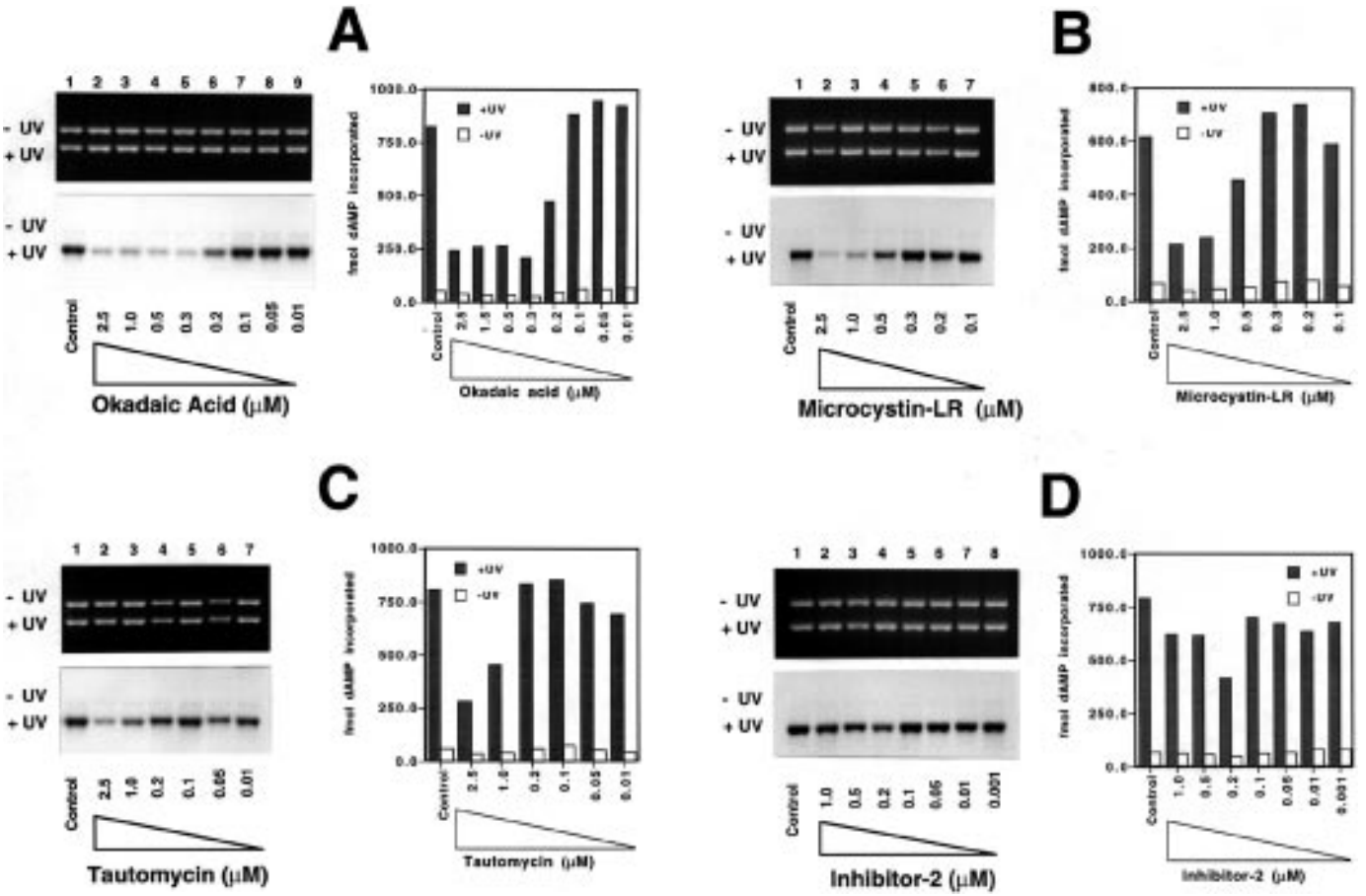

Figure 1. Effect of protein phosphatase inhibitors on nucleotide excision repair by cell extracts. HeLa whole cell extract (200μg protein) was incubated in reaction buffer for 20 min at 30°C in the presence of different amounts of okadaic acid (**A**), microcystin-LR (**B**), tautomycin (**C**) or inhibitor-2 (**D**). UV-irradiated and non-irradiated plasmids (250 ng each) were then added and the incubation proceeded for 3 h at 30°C. DNA was extracted, linearized with *Bam*HI and separated by electrophoresis on a 1% agarose gel (top panels) before autoradiography to show repair synthesis (bottom panels).

with PBS-T, incubated with horseradish peroxidase-conjugated goat anti-mouse IgG second antibody (Sigma) and visualized by the ECL detection method (Amersham).

RESULTS

Nucleotide excision repair is sensitive to inhibitors of Ser/Thr-specific phosphatases

The Ser/Thr-specific protein phosphatase inhibitors okadaic acid, tautomycin and microcystin-LR were used to investigate whether modulation of protein phosphorylation affected nucleotide excision repair by HeLa whole cell extracts. It is known that the toxins inhibit PP1 and PP2A with different affinities and that the concentrations used have negligible effects on PP2B and PP2C. Okadaic acid inhibits PP2A more strongly than PP1, tautomycin inhibits PP1 more strongly than PP2A and microcystin-LR inhibits both PP1 and PP2A at similar concentrations (4,7,9). All three toxins were assayed over the range 10 nM to 2.5 μM (Fig. 1A–C). In these experiments the cell extracts were preincubated for 20 min with the toxin in repair buffer prior to initiating the reaction by addition of the mixture of damaged and undamaged DNA. All three phosphatase inhibitors decreased incorporation of radioactivity into the UV-damaged plasmid, suggesting that Ser/Thr-specific protein phosphatase activity is required for efficient nucleotide excision repair *in vitro*. Okadaic acid inhibited the repair reaction at lower concentrations than microcystin-LR or tautomycin. Only 300 nM okadaic acid was required to obtain ~70% inhibition of repair (Fig. 1A). In comparison, 1.0 μM microcystin-LR and 2.5 μM tautomycin were required to attain a similar effect (Fig. 1B and C). This extreme sensitivity to inhibition by okadaic acid and relative insensitivity to inhibition by tautomycin strongly implicates a PP2A-type phosphatase as the target for these drugs. This conclusion is supported by the results of additional experiments in which inhibitor-2 was added to repair reactions (Fig. 1D). This small heat-stable protein specifically inhibits PP1-type phosphatases at nanomolar concentrations, but does not affect PP2A-type enzymes. Repair reactions were completely insensitive to addition of inhibitor-2 even at a concentration (1 μM) well above that known to abolish PP1 activity (4).

Purified PP2A phosphatase restores repair to inhibited extracts

The above results strongly suggested that Ser/Thr dephosphorylation events mediated by a PP2A-type activity are required for NER *in vitro*. To test this suggestion we attempted to reverse the inhibition by addition of purified catalytic subunits of PP1 and PP2A (Fig. 2). The repair activity of HeLa whole cell extracts was

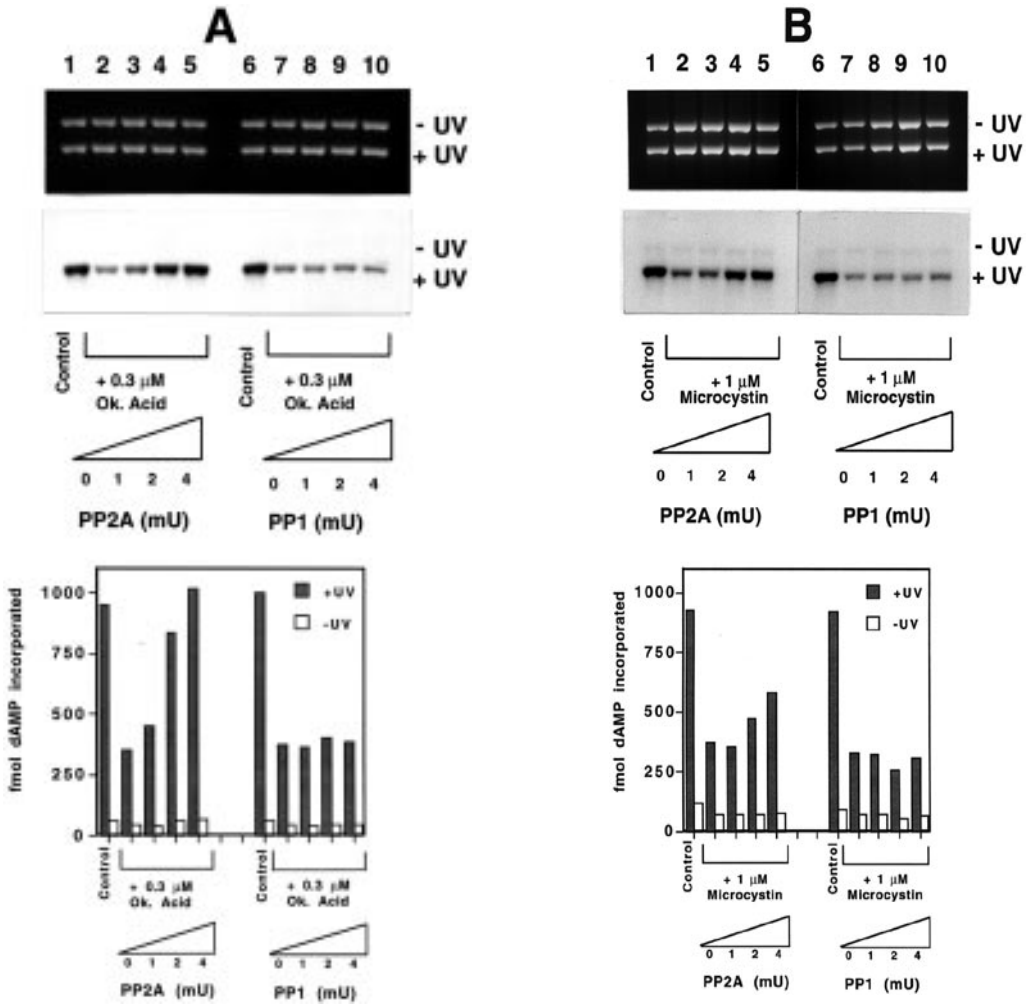

Figure 2. Purified PP2Ac restores repair activity to toxin-inhibited extracts. HeLa whole cell extracts (200µg protein) were incubated in reaction buffer for 20 min at 30°C in the presence of 0.3 µM okadaic acid (**A**) or 1 µM microcystin-LR (**B**). UV-irradiated and non-irradiated plasmids (250 ng each) were then added together with different amounts of purified PP2Ac or PP1γ and the incubation proceeded for 3 h at 30°C. DNA was extracted, linearized with *Bam*HI and separated by electrophoresis on a 1% agarose gel (top panels) before autoradiography to show repair synthesis (bottom panels).

determined either in the absence of inhibitors (Fig. 2A and B, lanes 1 and 6) or in the presence of okadaic acid (Fig. 2A, lanes 2–5 and 7–10) or microcystin-LR (Fig. 2B, lanes 2–5 and 7–10). As observed previously, both okadaic acid and microcystin-LR potently inhibited repair (Fig. 2A and B, lanes 2 and 7). Addition of purified PP1γ to the okadaic acid- or microcystin-LR-inhibited extracts did not have any effect (Fig. 2A and B, lanes 8–10). However, addition of PP2Ac was able to rescue the repair reaction from the inhibition exerted by both toxins (Fig. 2A and B, lanes 3–5). Addition of 4 mU PP2Ac fully restored repair activity in the okadaic acid-inhibited extract to the level seen in the uninhibited control extract (Fig. 2A).

Effect of okadaic acid on a coupled incision/synthesis repair reaction

The previous results demonstrated that a PP2A-type Ser/Thr-specific protein phosphatase is required for optimal repair capacity of human cell extracts *in vitro*. To investigate the stage(s) of repair where the phosphatase acts a cell fractionation scheme was employed. During the first stage of NER a lesion is recognized and incised in a reaction that requires human replication protein A (RPA) (29–32). During the second stage the repair patch is synthesized and ligated in a reaction that requires PCNA protein as part of the eukaryotic DNA polymerase holoenzyme (30). Separating RPA and PCNA from whole cell extracts allows dissection of NER *in vitro* into these two stages (30). A simple phosphocellulose fractionation yields a flow-through fraction CFI (containing RPA and PCNA) and a bound fraction CFII (containing the remaining proteins required for nucleotide excision repair). Further fractionation of CFI by DEAE Biogel column chromatography gives CFIA (containing RPA) and CFIB (containing PCNA). Levels of PP1 and PP2A activity were followed during this fractionation. Dephosphorylation of the standard substrate glycogen phosphorylase was measured in the absence and presence of phosphatase inhibitors. The results showed that significant amounts of both PP1- and PP2A-type activities were present in all fractions, with PP1 activity most concentrated in CFII and PP2A activity most concentrated in CFIB (Table 1).

Table 1. Levels of PP1 and PP2A in HeLa cell extracts and fractions

Extract or fraction	Specific activity (mU/mg protein)		
	Total	PP1	PP2A
	(no inhibitor)	(+ 1 nM okadaic acid)	(+ 0.2 μM inhibitor-2)
Whole cell extract	9.45	Not done	1.78
CFII	9.18	9.42	0.28
CFIA	2.17	0.88	0.25
CFIB	5.62	0.49	3.64

To determine if the phosphatase inhibitors affected the first stage of the reaction we used an assay that monitors the amount of incised DNA created during repair. UV-irradiated and non-irradiated plasmids were first incubated with fraction CFII and purified human RPA in the absence of PCNA. Under these conditions dual incisions occur and incised repair intermediates accumulate in damaged DNA (30). Exonuclease-free *E.coli* DNA polymerase I was then added with [α-^{32}P]dATP. Exonuclease-free polymerase I can fill the NER gaps independently of the human polymerase system and thus can be used to measure the formation of incised intermediates (31,33). Without exonuclease-free polymerase I damage-dependent labelling was almost undetectable (Fig. 3A, lanes 1–8). Addition of exonuclease-free polymerase I resulted in significant damage-dependent labelling in nicked circular and closed circular DNA (Fig. 3A, lanes 9–16). Damage-dependent incorporation by exonuclease-free polymerase I depended on the presence of RPA, as expected (Fig. 3A, lanes 9 and 11), since RPA is needed to form the incised repair intermediates during the first stage. In these experiments fraction CFII and RPA were preincubated for 1 h in repair buffer in the presence of different amounts of okadaic acid prior to initiating the reaction by addition of the mixture of damaged and undamaged DNA. The presence of okadaic acid potently inhibited radiolabel incorporation in nicked circular damaged DNA (Fig. 3A, lanes 11–15), by 70% at the highest dose tested (2.5 μM). A somewhat higher concentration of okadaic acid was required than in crude extracts, presumably because there is a different balance of phosphatases and kinases in the CFII fraction. The same extent of inhibition was seen when RPA was added either during the 1 h preincubation with okadaic acid (lane 15) or with the DNA at the beginning of the repair reaction (lane 16). In reactions with no exonuclease-free polymerase I a low background of repair synthesis took place that was carried out by human polymerase(s). This background synthesis showed a pattern of okadaic acid inhibition similar to the exonuclease-free polymerase I-dependent synthesis (Fig. 3A, bottom panel). This suggests that the inhibitory effect was independent of the specific enzymatic mechanism responsible for gap filling.

The previous results suggested that the okadaic acid-dependent inhibition of repair synthesis was due to an effect on the production of incision intermediates, but it was still possible that the exonuclease-free polymerase I enzyme was being affected by hyperphosphorylation or by direct interaction with the toxin. To check these possibilities the experiment shown in Figure 3B was performed. The polymerization step was carried out either with fresh exonuclease-free polymerase I (lanes 1–3) or with enzyme which had been previously incubated for 45 min with the CFII extract in the absence (lanes 4–6) or presence of okadaic acid (lanes 7–9). Preincubation of the polymerase had no effect on its activity, in either control or toxin-inhibited reactions. In summary, the okadaic acid inhibition observed in a coupled incision/synthesis repair reaction strongly suggests that a Ser/Thr protein phosphatase activity is required for the generation of incised intermediates during the first stage of NER.

Effect of okadaic acid on the production of oligonucleotides by the dual incision reaction

The suppression of NER by Ser/Thr protein phosphatase inhibitors was further analyzed by assessing the effect of okadaic acid on an assay that measures the formation of oligonucleotides carrying a lesion from a plasmid containing a single 1,3-intrastrand d(GpTpG) platinum crosslink (44). The oligonucleotides are separated by electrophoresis, transferred to a filter and detected by hybridisation with a ^{32}P-labeled 27mer probe complementary to the sequence containing the platinum lesion. In these experiments whole cell extracts were preincubated for 20 min with the toxin in repair buffer prior to initiating the reaction by addition of damaged or undamaged DNA (Fig. 4A). The presence of okadaic acid severely inhibited the dual incision repair reaction as measured by the production of 25–30mer oligonucleotides. As found in the repair synthesis assay (Fig. 1A), the effect was concentration-dependent and maximum inhibition (70–90%) was obtained with 0.3–1.0 μM toxin. To determine if exogenous PP2Ac could restore dual incision activity HeLa whole cell extracts were assayed either in the absence of inhibitor (Fig. 4B, lanes 3 and 6) or in the presence of 0.3 (lanes 4 and 7) or 1.0 μM (lanes 5 and 8) okadaic acid. Addition of 4 mU pure PP2Ac was able to rescue the dual incision reaction from the inhibition exerted by 0.3 μM okadaic acid (lanes 4 and 7) and restored some activity to the 1.0 μM reaction (lanes 5 and 8).

Hyperphosphorylation of the RPA p34 subunit in okadaic acid-inhibited HeLa cell extracts

The inhibitory effect of Ser/Thr-specific protein phosphatases on NER most probably results from an unbalanced phosphorylation state of one or more proteins required for this repair pathway. An obvious repair component to examine was RPA. This single-stranded DNA binding protein is composed of three subunits of 70, 34 and 14 kDa. Cell cycle-related changes in phosphorylation occur on multiple serines in the 34 kDa subunit in human cells (34) and this subunit becomes hyperphosphorylated when cells are UV irradiated (35). It has been suggested that this phosphorylation may regulate the activity of RPA in the cell and, indirectly, some of the processes in which it functions, such as DNA replication (34,36,37). It is not yet certain which kinase(s) is responsible for phosphorylation of RPA *in vivo*, but RPA is a

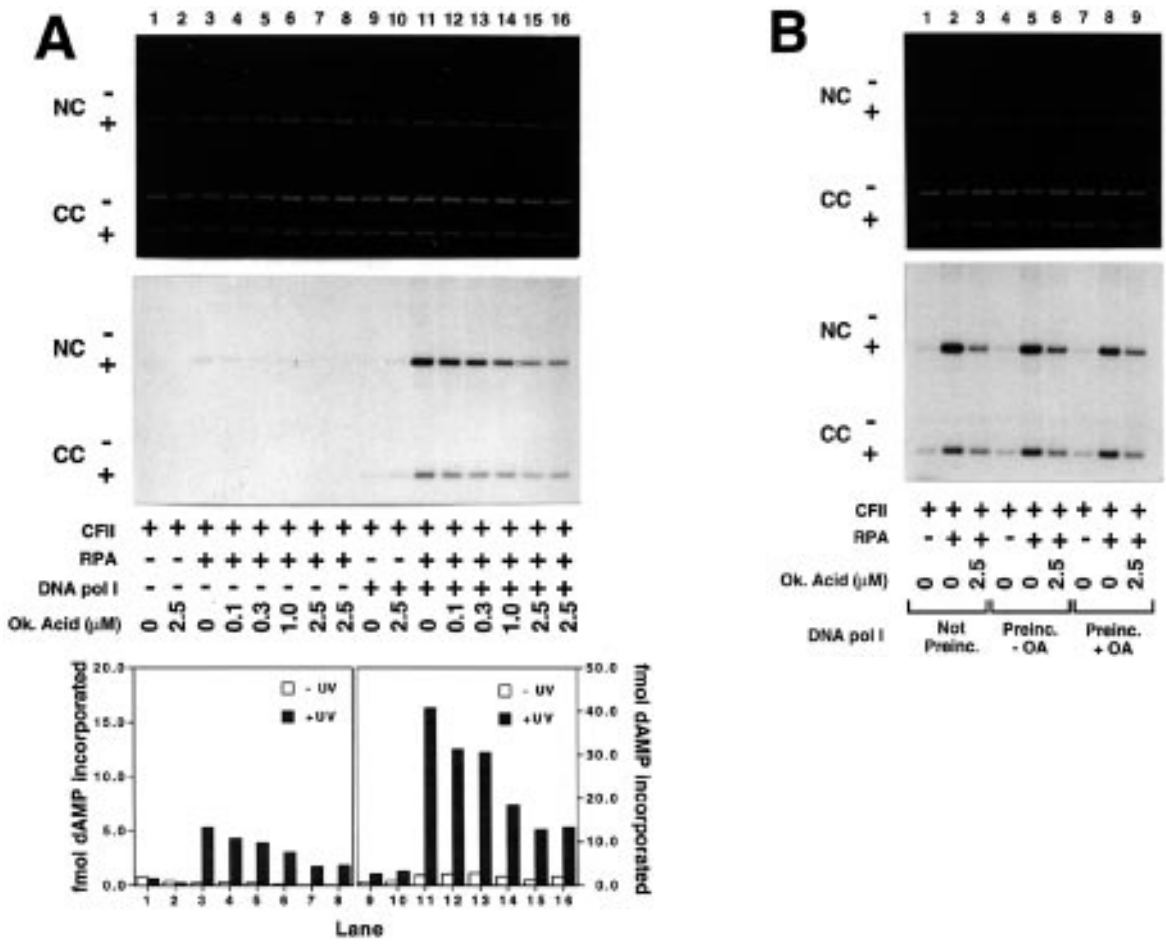

Figure 3. Effect of okadaic acid on a coupled incision/synthesis reaction. (**A**) A mixture of 75 μg CFII protein and 750 ng human RPA was incubated in reaction buffer in the absence or presence of different amounts of okadaic acid for 1 h at 30°C. UV-irradiated and non-irradiated plasmids (250 ng each) were then added and the incubation proceeded for 1 h at 30°C. Exonuclease-free polymerase I (1 U) (lanes 9–16) and 2 μCi [α-^{32}P]dATP (lanes 1–16) were added and the reaction was further incubated for 30 min. DNA was purified and separated without restriction enzyme treatment on a 1% agarose gel (top panel) before autoradiography to show repair synthesis (middle panel).The amount of dAMP incorporated per reaction in the nicked circular DNA was quantified (bottom panel; note the different scales in the two graphs). In lanes 8 and 16, RPA was absent during preincubation with the toxin and was added together with the DNA. (**B**) Reactions were carried out as described above but exonuclease-free polymerase I (1 U) was added either fresh (lanes 1–3) or after 45 min preincubation (1 U/μl, 50 μl final volume) in repair buffer containing 75 μg CFII in the absence (lanes 4–6) or presence (lanes 7–9) of okadaic acid (2.5 μM). This mixture had been previously preincubated for 2 h.

substrate for p34cdc2 kinases and DNA-dependent protein kinase (DNA-PK) *in vitro* (36–39).

Since phosphorylated forms of RPA p34 show reduced mobility on SDS–polyacrylamide gels, we examined the phosphorylation state of RPA in okadaic acid-inhibited cell extracts by immunoblotting with a specific monoclonal antibody against the p34 subunit (Fig. 5). Repair reactions containing 200 μg HeLa whole cell extract were incubated for 3 h at 30°C in the presence of different amounts of okadaic acid. After incubation proteins were separated by gel electrophoresis and the 34 kDa subunit of RPA was detected by immunoblotting. All the p34 protein present in a non-incubated extract had a mobility corresponding to the unphosphorylated form of the subunit (Fig. 5, lane 1). Cell extracts incubated for 3 h at 30°C in repair buffer showed slower migrating bands (Fig. 5, lane 2). The addition of increasing amounts of okadaic acid increased the relative intensity of these slow mobility forms (Fig. 5, lanes 3–6) and in the presence of 2.5 μM almost all the 34 kDa subunit showed the slowest mobility (Fig. 5, lane 6). However, we found no evidence that the phosphorylation state of RPA actually affected its function in DNA repair. Addition of pure unphosphorylated RPA (up to 360 μM) to toxin-inhibited cell extracts did not have any effect on the extent of inhibition (data not shown).

DISCUSSION

In this study we have presented evidence that reversible protein phosphorylation plays an important role in NER *in vitro*. The suppression exerted by Ser/Thr protein phosphatase inhibitors on NER carried out by human cell extracts suggests that a phosphatase activity present in such extracts is required for optimal efficiency of the repair process. It is inferred that one or more protein kinases in the HeLa cell extracts and fractions used in these experiments phosphorylate at least one protein factor required for repair. During the incubation in repair buffer the phosphorylation state of proteins in the HeLa repair extracts and fractions is dynamic, with phosphate groups being continuously added and removed by competing kinase and phosphatase

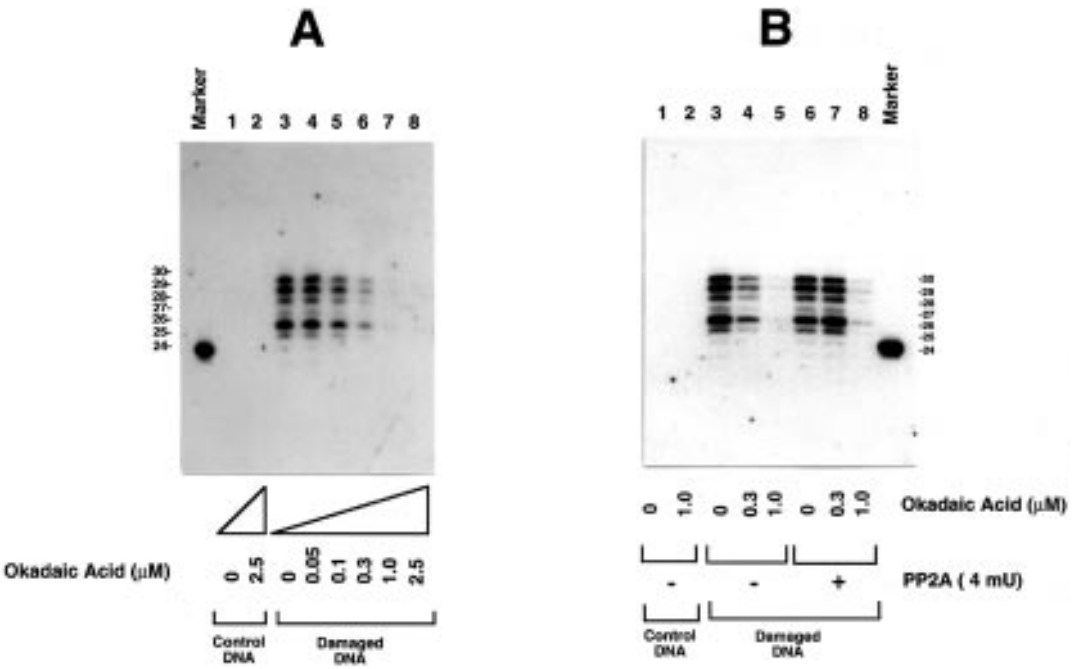

Figure 4. Okadaic acid inhibits dual incision activity. (**A**) HeLa whole cell extract protein (200 μg) was incubated in reaction buffer for 20 min at 30°C in the presence of different amounts of okadaic acid before adding 300 ng control DNA or DNA containing a 1,3-intrastrand d(GpTpG) platinum crosslink. The incubation continued for 30 min and the DNA was purified and loaded onto a denaturing polyacrylamide gel. After transfer to a membrane oligonucleotides produced by dual incision were detected by hybridization to a labelled probe complementary to the sequence containing the platinum lesion. The marker was a 24mer oligonucleotide 5′-TCTTCTTCTGTGCACTCTTCTTCT-3′ containing a single 1,3-cisplatin crosslink at the GTG sequence. (**B**) Repair reactions were carried out as in (A) except that after the 20 min preincubation different amounts of PP2Ac were added together with the DNA.

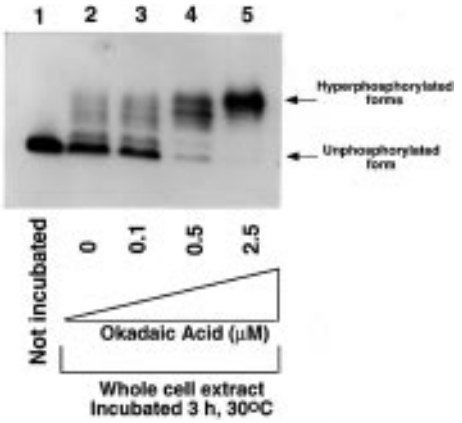

Figure 5. Electrophoretic mobility of human RPA p34 in okadaic acid-inhibited HeLa whole cell extracts. Extracts (200 μg) were incubated (final volume 50 μl) in repair reaction buffer for 3 h at 30°C in the presence of different amounts of okadaic acid (lane 2–5). An aliquot of the reaction (10 μl) was then analysed by 15% SDS–PAGE. The p34 subunit was visualized by immunoblotting. Lane 1 contained an aliquot of the same cell extract which was not incubated.

activities. When phosphatase inhibitors are added to the extract the balance between phosphorylation and dephosphorylation activities changes and specific proteins become phosphorylated to a degree that inhibits repair.

The relative efficiencies of various inhibitors in arresting nucleotide excision repair suggested that the protein phosphatase involved is a PP2A type. Consistent with this, highly purified PP2Ac was able to restore repair capacity to okadaic acid- and microcystin-LR-inhibited extracts, while the addition of PP1γ did not have any effect. Direct assays showed that both PP1 and PP2A activities are indeed present in HeLa cell extracts and in the fractions used in the repair assays.

We tested the idea that the inhibition exerted by okadaic acid might affect the first stage of the repair process. A coupled incision/synthesis repair assay was first used, in which exonuclease-free DNA polymerase I from *E.coli* incorporates deoxynucleotides after creation of incisions by human proteins. DNA damage-dependent repair synthesis required the presence of RPA during the first stage of the reaction. Addition of okadaic acid was found to strongly inhibit the formation of incised intermediates. More direct evidence that PP2A inhibition affects the first stage of the repair process was obtained by use of an assay to detect the oligonucleotides generated during the incision/excision stage of repair. The results do not exclude the possibility that post-incision steps are also affected by the phosphorylation state of repair factors.

Our data indicate that one or more protein factors whose activity is required for the first stage of the repair reactions are subject to regulation by the reversible phosphorylation of Ser or Thr residues. Such proteins could be either direct participants in the repair process or extrinsic components which act to modulate the activity of repair factors. One repair protein which is known to be phosphorylated *in vivo* is the 34 kDa subunit of RPA, which undergoes cell cycle-dependent phosphorylation (34) and shows increased phosphorylation after cells are irradiated (35). We found that endogenous p34 RPA in HeLa whole cell extracts

becomes hyperphosphorylated under conditions in which repair is inhibited by okadaic acid. However, three lines of evidence suggest that RPA is not the main target for the arrest exerted by Ser/Thr protein phosphatase inhibitors on nucleotide excision repair. First, the addition of pure unphosphorylated RPA to toxin-inhibited cell extracts had no effect on the extent of inhibition (data not shown). Second, the absence of RPA during preincubation of the CFII fraction in the presence of okadaic acid did not affect the intensity of inhibition (Fig. 3A, compare lanes 15 and 16). Third, a mutant RPA with a deletion of residues 2–29 from the p34 subunit, thus lacking the two consensus p34cdc2 kinase phosphorylation sites, is still able to support nucleotide excision repair. Further, a repair reaction carried out in the presence of this mutant RPA is still inhibited by okadaic acid (S.-H.Lee, R.R.A. and R.D.W., unpublished data). This is in agreement with studies of others who have found no direct evidence that the phosphorylation state of RPA actually affects its function in DNA repair (40). The critical target(s) for the reversible phosphorylation remains to be identified.

The demonstration that a Ser/Thr-specific protein phosphatase activity is involved in nucleotide excision repair *in vitro* raises interesting questions about its role *in vivo*. Although inhibitors such as okadaic acid are extremely useful tools *in vitro*, their use in cells has significant drawbacks, because of their pleiotropic effects. In the case of okadaic acid these include cell cycle arrest (41) and cytotoxicity, including initiation of apoptosis (42). Indeed, in attempts to detect any effect of okadaic acid on DNA repair *in vivo* by measuring unscheduled DNA synthesis after UV light irradiation we found that even relatively low concentrations of the inhibitor (50–300 nM) substantially affected cell survival and morphology (data not shown). To ascertain the precise role of reversible phosphorylation on DNA repair *in vivo* it will probably be necessary to identify and purify the phosphatase(s) and kinases(s) which modulate the repair process *in vitro*.

How can exogenous PP2Ac affect the activity of specific repair factors? Although the catalytic subunits of protein phosphatases are known to have inherent substrate specificities (5), studies on cytoplasmic and nuclear forms of PP1 and PP2A have shown that additional regulation of substrate specificity can be conferred upon native enzymes through the presence of targeting subunits (6,43). It is possible, therefore, that exogenous PP2Ac interacts with free targeting subunits in the cell extract to dephosphorylate a specific repair factor(s). A major goal for future studies will be isolation of the native protein phosphatase acting on the repair pathway and establishment of whether PP2A itself or another protein phosphatase with overlapping substrate specificity acts upon the nucleotide excision repair factors *in vivo*.

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