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Phosphorylation of human oxoguanine DNA glycosylase (α-OGG1) modulates its function

Jingping Hu, Syed Z. Imam, Kazunari Hashiguchi, Nadja C. de Souza-Pinto and Vilhelm A. Bohr*

Laboratory of Molecular Gerontology, National Institute on Aging, NIH, Baltimore, MD 21224, USA

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ABSTRACT

Oxoguanine DNA glycosylase (OGG1) initiates the repair of 8-oxoguanine (8-oxoG), a major oxidative DNA base modification that has been directly implicated in cancer and aging. OGG1 functions in the base excision repair pathway, for which a molecular hand-off mechanism has been proposed. To date, only one functional and a few physical protein interactions have been reported for OGG1. Using the yeast two-hybrid system and a protein array membrane, we identified two novel protein interactions of OGG1, with two different protein kinases: Cdk4, a serine-threonine kinase, and c-Abl, a tyrosine kinase. We confirmed these interactions *in vitro* using recombinant proteins and *in vivo* by co-immunoprecipitation from whole cell extracts. OGG1 is phosphorylated *in vitro* by Cdk4, resulting in a 2.5-fold increase in the 8-oxoG/C incision activity of OGG1. C-Abl tyrosine phosphorylates OGG1 *in vitro*; however, this phosphorylation event does not affect OGG1 8-oxoG/C incision activity. These results provide the first evidence that a post-translational modification of OGG1 can affect its catalytic activity. The distinct functional outcomes from serine/threonine or tyrosine phosphorylation may indicate that activation of different signal transduction pathways modulate OGG1 activity in different ways.

INTRODUCTION

8-oxoguanine (8-oxoG) is one of the most abundant and well-characterized DNA lesions generated by oxidative stress [reviewed in (1)]. It has been estimated that ~180 guanines are oxidized to 8-oxoG per mammalian cell per day (2). 8-oxoG is a miscoding lesion that can cause G:C to T:A or T:A to G:C transversion mutations (3). This lesion accumulates in DNA with age, particularly in the mitochondrial genome, and it has been causally linked to several cancers and neurodegenerative diseases, such as Alzheimer's and Parkinson's (4).

8-oxoG is removed from DNA mostly by the base excision repair (BER) pathway (5). BER is initiated by a DNA glycosylase that recognizes and hydrolyses the modified base. In mammals, oxidized guanines are repaired by oxoguanine DNA glycosylase (OGG1) and MYH (MutY glycosylase); OGG1 removes the mutagenic 8-oxoG lesion situated opposite cytosine, and MYH removes adenine from 8-oxoG/A mismatches with its adenine glycosylase activity [reviewed in (6)]. The biological importance of the OGG1 enzyme in mammals is attested by several lines of evidence: (i) polymorphisms of OGG1 are found to be associated with an increased risk of lung and prostate cancers in human populations (7,8); (ii) concomitant disruption of the *Ogg1* and *Myh1* genes in mice predisposes 66% of the mice to tumors, predominantly lung and ovarian tumors and lymphomas (9); and (iii) lower OGG1 activity in peripheral blood lymphocytes is strongly associated with increased risk of developing lung cancer in humans (10).

The OGG1 protein catalyzes damaged base removal through its glycosylase activity, followed by β-elimination on the resulting abasic site causing strand cleavage 3′ to the original damaged base. However, under physiological conditions, it is hypothesized that OGG1 does not effectively catalyze strand cleavage, since its apurinic/apyrimidinic (AP) lyase activity is significantly lower than its glycosylase activity (11,12). Instead, the observation that AP endonuclease 1 (APE1) stimulated OGG1-specific activity on an 8-oxoG/C substrate by preventing its re-association with the AP/C product (13) suggested cooperative functions of OGG1 and APE1 in initiating BER of oxidative damage. This is consistent with the proposed 'passing the baton' mechanism of BER, in which molecular hand-offs between one enzyme and its successor coordinate the sequential steps of BER to prevent the

*To whom correspondence should be addressed. Tel: +1 410 558 8162; Fax: +1 410 558 8157; Email: vbohr@nih.gov
Present address:
Kazunari Hashiguchi, Department of Molecular Genetics, IDAC, Tohoku University, Japan

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formation of potentially dangerous intermediates (14). However, stable protein–protein interactions between various BER players have not been clearly demonstrated. In fact, OGG1 does not stably interact with APE1; so far only a stable interaction with the scaffolding protein XRCC1 has been found (15).

In an attempt to identify protein partners of OGG1, we utilized yeast two-hybrid screening with OGG1 as the bait protein and a protein array membrane with several DNA repair proteins. Using these approaches, we identified strong protein interactions with two protein kinases, Cdk4 and c-Abl.

Phosphorylation and other post-translational modifications modulate various aspects of the DNA damage response. Various DNA repair proteins are phosphorylated after DNA damage by the activation of specific kinases, such as ATM, ATR and DNA–PK, and these modifications alter their intracellular localization, protein–protein interactions and catalytic properties (16,17). Despite much work in this area, the impact of post-translational modifications on BER enzymes is still poorly understood (18). Phosphorylation of the DNA glycosylases UDG (19), MYH (20) and OGG1 (21) has recently been detected, as well as acetylation of APE1 (22), NEIL1 (23) and TDG (24). However, the functional consequences of these modifications are only partially characterized. Thus, we investigated the physical and functional interactions of OGG1 with Cdk4 and c-Abl. Cdk4 is a cyclin D-dependent serine/threonine kinase that is involved in cell cycle regulation, controlling the progression from G_1 to S phase (25); its expression and activity are tightly regulated during the cell cycle (26). c-Abl is a tyrosine kinase activated in response to various stimuli, including genotoxic stress (27) that plays a prominent role in the DNA damage response (28). These two kinases participate in different signaling pathways and have distinct biological roles. Here, we show that OGG1 interacts with and is phosphorylated *in vitro* and *in vivo* by both kinases. While serine/threonine phosphorylation of OGG1 by Cdk4 increases its 8-oxoG incision activity, tyrosine phosphorylation by c-Abl has no effect on its glycosylase activity. Our results suggest that OGG1 phosphorylation may represent an important regulatory event, involving the functional modulation of its biochemical properties, since modification of different residues by different kinases appears to have alternative functional outcomes.

MATERIALS AND METHODS

Yeast two-hybrid screen

A yeast two-hybrid screening was performed using the Matchmaker™ Gal4 Two-hybrid system 3 (Clontech) to identify OGG1 interacting proteins. In brief, a human whole brain cDNA library, pre-transformed into the yeast strain Y187, was purchased from Clontech. DNA encoding a fragment of OGG1-α (29-315) was used as the bait and cloned into pGBKT7 vector (pGBKT7-OGG1-α). After transformation of the bait vector into the yeast strain AH109, the bait strain was combined with the pre-transformed cDNA library strain and incubated for 24 h for mating. Diploid yeast cells were grown on high stringency selection plates (SD/-Ade/-His/-Leu/-Trp/X-α-Gal). Plasmids in the positive yeast colonies were isolated with EZ Yeast™ plasmid mini-preparation kit (Geno Technology Inc.) and co-transformed into strain AH109 with the bait plasmid pGBKT7-OGG1-α for verification. cDNA inserts in the retested positive colonies were analyzed by DNA sequencing and alignment with NCBI databases.

Plasmid construction

The OGG1-α expressing plasmid pCMV-Tag2-OGG1-α is an N-terminal FLAG-tagged OGG1-α construct. The PCR for OGG1-α coding region was performed with 5′-primer (5′-CGGGCGGATCCCATGCCTGCCCGCGCGCTT-3′) and 3′-primer (5′-TATCGAATTCTAGCCTTCCGGCCCTTT-3′). The PCR product with a BamH1 site added at the 5′end and an EcoRI site at the 3′ end was subcloned into pCMV-Tag-2A vector (Stratagene). The PCR was carried out with pfuUltra™ Hotstart DNA polymerase (Stratagene). This construct was verified by DNA sequencing. His-tagged OGG1 (OGG1-6His) was cloned, expressed and purified as described previously (29).

Cell culture and immunoprecipitation

Human embryonic kidney (HEK) 293 cells from ATCC were grown in DMEM supplemented with 10% fetal bovine serum (Gibco BRL). An aliquot of 800 μg/ml of Geneticin (Invitrogen) was added to the medium in the case of 293 cells harboring OGG1-α-FLAG plasmid or empty vector. The cells were incubated at 37°C under an atmosphere of 5% of CO_2. For the immunoprecipitation experiments, cells were grown until confluence, washed twice with phosphate-buffered saline (PBS) and lysed at 4°C in lyses buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, complete protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Sigma). After centrifugation at 20 000 *g* for 20 min at 4°C, the supernatant was pre-cleared with Protein A-agarose for 2 h. A monoclonal antibody, anti-FLAG M2-agarose (Sigma), was used to immunoprecipitate FLAG-tagged OGG1. The immunoprecipitates were resolved on Tris–Glycine gels (Invitrogen) followed by electro-blotting to PVDF membrane. The immunoblots were analyzed with the following antibodies: monoclonal anti-OGG1-α antibody (Assay Designs, Inc.), polyclonal anti-Cdk4 antibody (clone H-22, Santa Cruz Biotechnology, Inc.), mixed mouse monoclonal anti-phospho-Serine/Threonine antibody (Upstate Biotechnology), and visualized with ECL Plus (Amersham) or LumiGLO™ Reagent (Cell Signaling Technology Inc.).

Far western analysis

Far western analysis was performed as described previously (30). Briefly, purified Cdk4-GST protein (purchased from Santa Cruz Biotechnology, Inc.) and OGG1-α-6his (as the positive control) were run on two identical gels and electro-transferred to PVDF membranes. After overnight blocking in 5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBST), one membrane was incubated with 1 μg/ml purified OGG1-α-6his and the other with the non-specific control protein BSA for 1 h. The experiment was processed as described previously. Anti-OGG1-α monoclonal antibody (Assay Designs, Inc.) and ECL Plus were used to detect the presence of OGG1-α on each membrane.

Protein array

DiscoverLight™ Protein-Array membranes (Pierce) were spotted with 25–100 ng of the following proteins: Cdk4, c-Abl, XRCC1 (31), Werner Syndrome protein (WRN) and OGG1, according to the manufacture's instructions. The membrane was blocked with 10% BSA in PBST and incubated with whole cell lysate of HEK293 cells expressing OGG1-α-FLAG for 1 h at room temperature. The membrane was then developed with anti-FLAG polyclonal antibody (Santa Cruz Biotechnology) and visualized with ECL Detection Kit (Amersham).

In vitro phosphorylation of OGG1 and 8-oxoG base release/strand cleavage assay

For the *in vitro* kinase assays, Cdk4 was immunoprecipitated from HEK293 cell extracts with agarose-anti-Cdk4, as described above. An aliquot of 10 ng of purified OGG1-6his were incubated with immunoprecipitated Cdk4 beads in kinase buffer containing 20 mM Tris–HCl, pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$ and 0.5 mM DTT. The kinase reaction was carried out at 30°C for 90 min and stopped by the addition of equal volume of incision buffer (50 mM Tris–HCl, pH 7.4, 50 mM NaCl and 0.1 μg/μl BSA) supplemented with 10 mM EDTA. For c-Abl phosphorylation, 0.5–12 ng of OGG1-6his was incubated with 100 ng active or heat-inactivated full-length c-Abl (Upstate Biotechnology Company) in kinase buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT and 5 mM EDTA) for 15 min at 30°C. For PKC kinase assays, serial dilutions of OGG1-6his were incubated with or without 5 ng PKC (Promega) in a reaction buffer containing 20 mM HEPES, pH 7.4, 1 mM DTT, 10 mM $MgCl_2$, 1.7 mM $CaCl_2$, 5 mM ATP and 0.6 μg/μl phosphatidyl serine (Sigma), at 37°C for 10 min. Phosphorylated proteins were separated by SDS–PAGE and analyzed by immuno-blotting with the antibodies specified in the figure legends. OGG1 incision activity was assayed as described previously (29), with serial dilutions of phosphorylated OGG1-α-6his and 150 fmol of 30mer 8-oxoG/C containing oligonucleotide at 37°C for 20 min. To measure strand cleavage (DNA glycosylase + AP lyase) activity, reactions were terminated by adding an equal volume of formamide loading dye and substrate and product DNAs were resolved in a 20% acrylamide/7 M urea gel. To measure 8-oxoG base release (DNA glycosylase activity), reactions were performed as above and terminated by adding 4 μl of 0.5 M putrescine–HCl, pH 8.0; the reaction mixture was heated at 95° C for 5 min and then mixed with formamide loading dye followed by heating for 1 min at 95°C. The substrate and products were then resolved in a 20% acrylamide/7 M urea gel. The gels were visualized using a PhosphoImager and analyzed with the ImageQuant software.

GST fusion proteins and GST pull-down assay

Full-length GST-Abl, GST-Abl (K-R), GST-SH3 and GST-SH2 were kind gifts from Dr Donald Kufe. The GST-tagged proteins (500 ng) were incubated with 500 ng OGG1-α-6his in 1× binding buffer (PBS, 0.01% Tween-20 and 3% BSA). The bound proteins were precipitated with glutathione–sepharose beads and the adsorbents analyzed by SDS–PAGE. The membranes were then analyzed by immuno-blotting with monoclonal anti-OGG1 or c-Abl antibodies.

Protein phosphatase treatment

When stated, the dephosphorylation treatment was performed before the kinase assay. For that, purified OGG1 was combined with equal molar concentration of protein phosphatase 1 [(PP1) New England Biolabs Inc.] in 1× PP1 buffer with 1 mM $MnCl_2$, as described by the manufacturer. The reaction was carried out for 30 min at 30°C and stopped by adding 1 μl of phosphatase inhibitor cocktail (Sigma) followed by incubation for 5 min at 30°C.

RESULTS

OGG1 interacts with Cdk4 *in vitro* and *in vivo*

In order to identify protein partners of OGG1-α we carried out a yeast-two-hybrid screening using a fragment of OGG1-α (29-315) fused to the Gal4 DNA binding domain (DNA-BD) as bait protein. The use of the OGG1 fragment lacking the N-terminal mitochondrial targeting sequence ensured that the bait protein did not translocate to mitochondria, which could compromise our analysis since the prey proteins would most likely be localized in the nucleus. A pre-transformed human brain cDNA library fused to the Gal4 activation domain (AD) was used in the screening. After yeast mating, 29 putative positive colonies were obtained from $\sim 1 \times 10^6$ screened clones. The sequences of 7 out of 29 (24%) positive colonies matched $p34^{SEI-1}$ as revealed by a BLAST search. This result was independently confirmed by co-transformation with OGG1 and $p34^{SEI-1}$ clones (Figure 1A). $p34^{SEI-1}$ is involved in controlling the cell cycle progression though its regulatory activity on the Cdk4/cyclin D protein kinase. It forms a stable quaternary complex with Cdk4, Cyclin D and $p16^{INK4a}$ and affects Cdk4 kinase activity in a concentration-dependent manner (32). Since $p34^{SEI-1}$ is almost always found in complex with Cdk4, we investigated whether OGG1 directly interacted with Cdk4. Far western analysis with purified recombinant proteins indicated that OGG1 and Cdk4 associate *in vitro* (Figure 1B). After blocking, membranes containing Cdk4 and OGG1 were incubated in buffer containing OGG1 or excess amounts of a non-specific control protein, BSA. Blotting with an anti-OGG1-α antibody detected a 58 kDa band corresponding to the size of the Cdk4-GST fusion protein in the immunoblot incubated with OGG1, but not on the membrane incubated with non-specific BSA. This result demonstrates a direct protein–protein interaction between the recombinant OGG1-α and Cdk4 *in vitro* and suggested that these two proteins may exist as a complex in the cell.

The association between OGG1 and Cdk4 was confirmed to exist *in vivo* by immunoprecipitation. Extracts from HEK293 stably expressing FLAG-tagged OGG1 were incubated with an anti-FLAG monoclonal antibody. Cdk4 was co-immunoprecipitated with OGG1-FLAG (Figure 2, lane 1) but not with the empty vector control (Figure 2, lane 2). These results suggest that Cdk4 and OGG1 associated in a stable complex *in vivo*. Moreover, the western blot with anti-phosphoserine/threonine-specific antibody revealed that

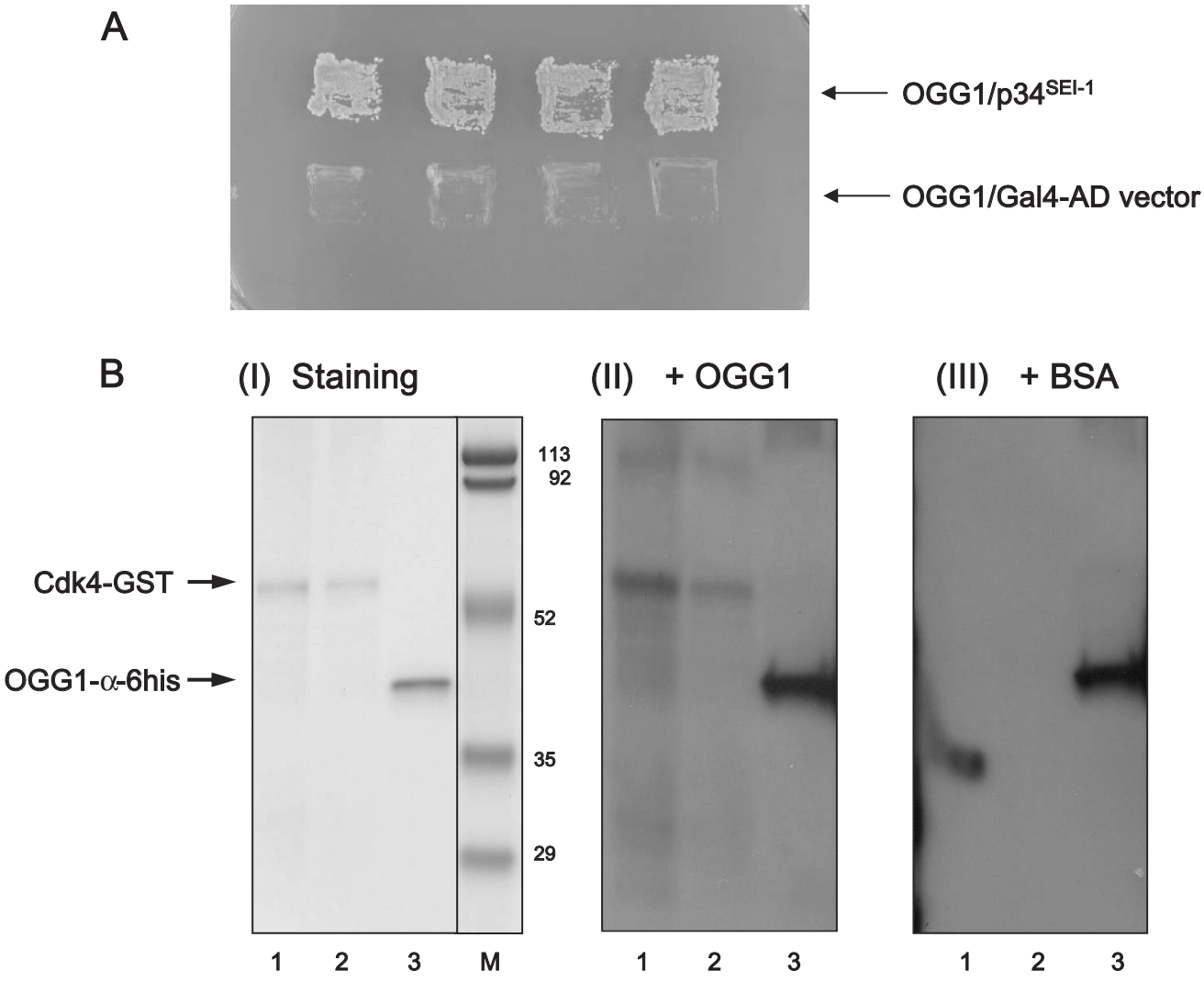

Figure 1. Physical interaction between OGG1 and Cdk4. (**A**) Yeast strain AH109 [Matchmaker™ Gal4 Two-hybrid system 3 (Clontech)] was transformed with pGBKT7-*OGG1*-α and p34^{SEI-1} or empty Gal4-AD vector. The yeast were cultured in high-stringency selective meduim and allowed to grow for 1 week. (**B**) Recombinant his-tagged OGG1 (OGG1-6his) and GST-tagged Cdk4 (Santa Cruz Biotech.) were separated on 12% Tris–glycine polyacrylamide gels. One gel was stained with Coomassie blue and the others were electro-transferred to PVDF membranes. The membranes were then incubated with either purified OGG1-6his (+OGG1) or BSA (+BSA) and probed with anti-OGG1 antibody, as described in Materials and Methods. Lanes 1 and 2 contain 800 and 400 ng Cdk4-GST protein; lane 3 contains 450 ng OGG1-6his in the Coomassie gel and 10 ng in the immunoblots.

a portion of OGG1 co-precipitating with Cdk4 was phosphorylated on its serine/threonine residues (Figure 2, lower panel). Thus, it is likely that Cdk4 might be one of the kinases responsible for this phosphorylation event *in vivo*.

OGG1-α is phosphorylated by Cdk4 *in vitro*

Cdks 1, 2, 4 and 6 belong to the proline-directed kinase family, which phosphorylate serine or threonine residues ahead of proline residues in the minimal consensus sequence [S/T*-P] or in the optimal consensus sequence [S/T*-P-X-K/R] (where S/T* represents the target residue and X represents any amino acid) (33). Sequence analyses revealed that OGG1 contains up to five potential Cdk4 phosphorylation sites (data not shown). Thus, OGG1 is a putative substrate for Cdk4-mediated phosphorylation. In order to test whether Cdk4 could phosphorylate OGG1 directly, we first determined whether the recombinant OGG1-6his protein was already phosphorylated. OGG1 purified from insect cells (Sf9) was recognized by anti-phosphoserine antibody, but only at very high protein concentrations (Figure 3A), indicating fairly limited phosphorylation. Since insect cells have post-translational modification machinery similar to mammalian cells, this observation also indicates that OGG1 is a target for phosphorylation *in vivo*. Thus, to demonstrate the direct phosphorylation of OGG1 by Cdk4, we utilized radio-labeled ATP to detect new OGG1 phosphorylation. After incubation of recombinant OGG1 with immunoprecipitated Cdk4 beads in the presence of [γ-^{32}P]ATP, radioactive bands were detected corresponding to the molecular weights of OGG1-6his and Cdk4 (Figure 3B, lane 3). The identity of the bands was further confirmed by western blot (left two panels). Heat-inactivated Cdk4 beads failed to generate radioactive bands, and the radioactive signal disappeared if the reactions were incubated with PP1 after the kinase treatment (lanes 2 and 1, respectively).

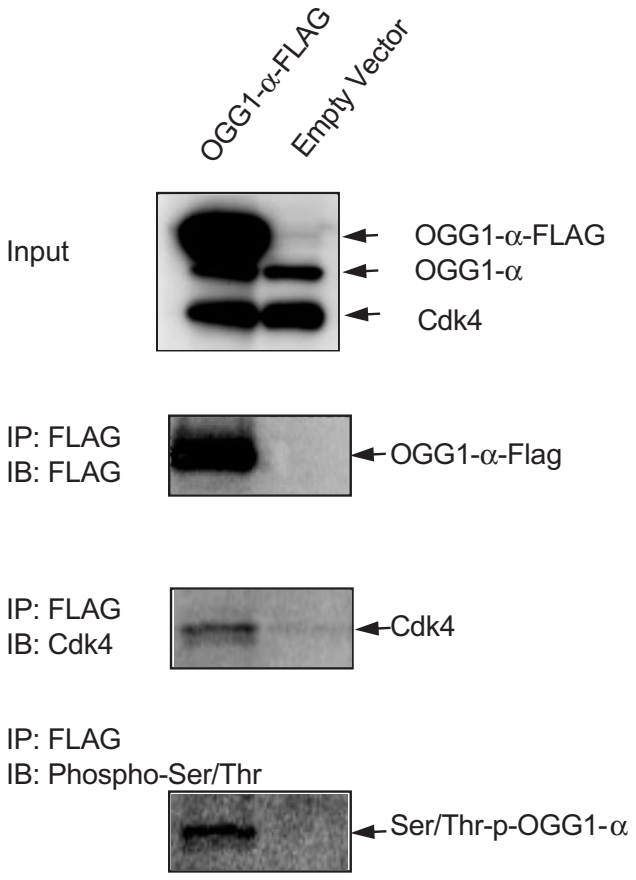

Figure 2. OGG1 and Cdk4 interact *in vivo*. HEK293 cells were transfected with FLAG-tagged OGG1 plasmid or empty vector. After 2 weeks selection with G418 (Invitrogen), whole cell extracts were prepared from the pool of cells as described in Materials and Methods. A monoclonal antibody, anti-FLAG-M2 agarose (Sigma), was used to immunoprecipitate FLAG-tagged OGG1. The immunoprecipitates were separated on 12% Tris-glycine PAGE gels and analyzed by western blot with the indicated antibodies.

We next investigated whether OGG1 phosphorylation by Cdk4 would affect its catalytic activity. Before the kinase reaction OGG1 was subjected to PP1 treatment to ensure the complete removal of any phosphate group introduced during protein expression. OGG1 was then incubated with active or heat-inactivated Cdk4 beads in the presence of ATP, and OGG activity was determined using an oligonucleotide incision assay with a 30mer substrate containing a single 8-oxoG at position 11. Since OGG1 catalyses two distinct reactions—damaged base release and strand cleavage of the resulting abasic site (11)—we measured both activities in samples incubated with active or heat-inactivated Cdk4 (Figure 4). The strand cleavage activity (Figure 4A) reflects the combined glycosylase/AP lyase activities. OGG1 incision activity significantly increased ($P < 0.05$) after Cdk4-catalyzed serine/threonine phosphorylation, up to 2-fold higher activity than the de-phosphorylated protein. It has been previously shown that OGG1 glycosylase activity is more robust than its associated AP lyase activity (11,12). We confirmed these results in our experiments, where we measured OGG1 DNA glycosylase activity (Figure 4B) to be about two times higher than its incision activity (compare the values between the top and bottom graphs). We also observed that base release by OGG1 was also significantly higher ($P < 0.05$) in Cdk4 phosphorylated samples, although to a lesser degree of ~30–40% increase. These results clearly demonstrate that OGG1 phosphorylation by Cdk4 modulates its enzymatic activity.

OGG1-α phosphorylation by PKC

Previously, Dantzer and co-workers (21) observed that OGG1 was phosphorylated *in vivo* by PKC. They found that this event did not modify 8-oxoG incision activity, but rather modulated the cellular localization of the protein since the phosphorylated form associates with the nuclear matrix. Since PKC also phosphorylates serine residues, although in a different sequence context (S*-F-R, S*-A-R and T*-NK) (21), we investigated whether recombinant OGG1 was phosphorylated by PKC *in vitro* and if this modified its catalytic activity. We incubated OGG1 with or without PKC in the presence of [γ-^{32}P]ATP. The reactions were then resolved by SDS–PAGE. Radioactive bands were detected corresponding to the molecular weights of OGG1-6his and PKC (Figure 5A) in the lanes incubated with PKC (lanes 4 and 5) but not in the lanes incubated without PKC (lanes 2 and 3), indicating that PKC directly phosphorylated OGG1 *in vitro*. The identity of the bands was also confirmed by western blot analysis (Figure 5A, bottom panel). We then compared the incision activity of the non-phosphorylated and PKC-phosphorylated OGG1 and found no difference (Figure 5B), similar to what was observed previously (21). Altogether, our results indicate that serine/threonine phosphorylation of OGG1 may have very distinct functional consequences, depending on the identity of the protein kinase and on the residues phosphorylated.

C-Abl interacts with and phosphorylates OGG1 *in vitro*

The c-Abl kinase is activated in response to genotoxic stresses. Cellular responses to DNA damage involve interactions of c-Abl with various DNA repair proteins, and tyrosine phosphorylation by c-Abl plays important regulatory roles in the DNA damage response through modulation of many DNA repair activities. We used a high-throughput screening protein array membrane to identify protein partners of c-Abl and found a direct protein–protein interaction with OGG1 (data not shown). This interaction was then further investigated by the reverse experiment, in which c-Abl, along with Cdk4, XRCC1 (a known protein partner of OGG1) (15), WRN and OGG1 itself were spotted in the membrane and incubated with whole cell extracts from 293-FLAG-α-OGG1 cells (Figure 6A). This experiment confirmed the direct physical interaction of OGG1 with c-Abl, as well as Cdk4 and the positive control XRCC1. Interestingly, we observed a strong interaction between OGG1-FLAG in the extract and recombinant OGG1 on the membrane, suggesting that OGG1 may self-associate *in vivo*. Furthermore, we confirmed the physical interaction between OGG1 and c-Abl with a GST pull-down assay. Purified OGG1-α-6his was incubated with GST-Abl fusion protein or GST alone (Figure 6B). While OGG1 did not precipitate with GST alone, both active and inactive GST-Abl fusion proteins precipitated OGG1, confirming a direct association between these two proteins independent of c-Abl catalytic activity. We then investigated whether c-Abl could phosphorylate OGG1 *in vitro*.

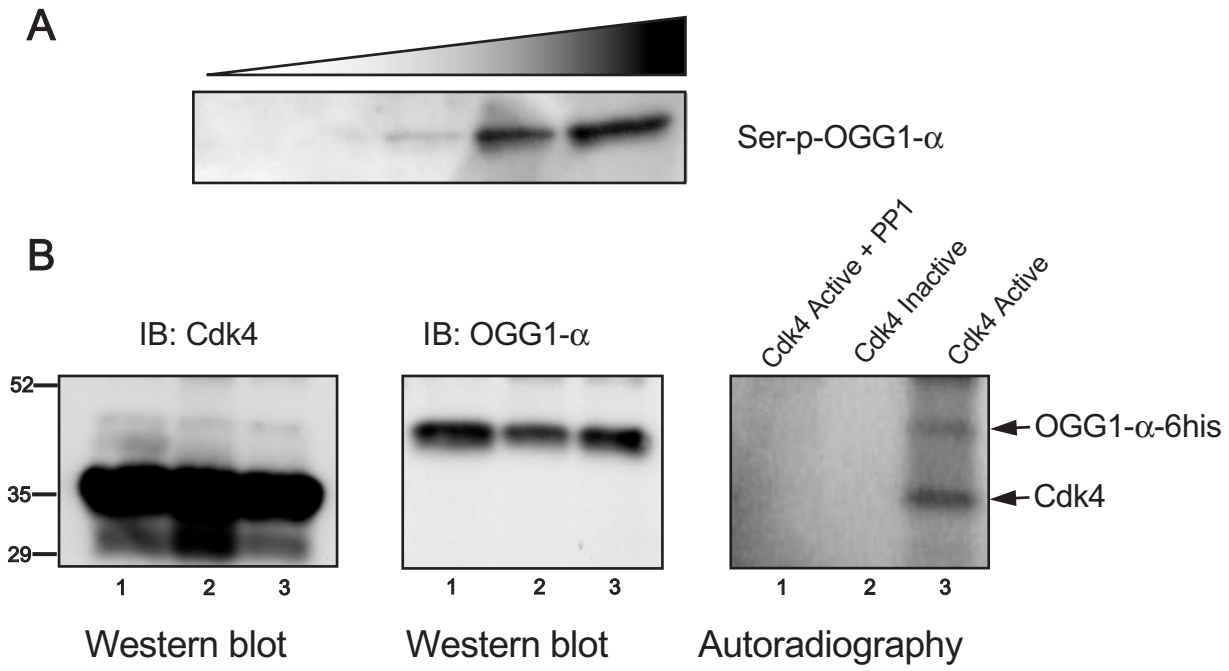

Figure 3. *In vitro* phosphorylation of OGG1 by Cdk4. (**A**) Recombinant OGG1-6his (62, 125, 250, 500 and 1000 ng), purified from insect cell (Sf9), was immunobloted with an anti-phosphoserine antibody. (**B**) An aliquot of 100 ng of purified OGG1 was incubated with either active immunoprecipitated Cdk4 beads (lanes 1 and 3) or heat-inactivated immunoprecipitated Cdk4 beads (lane 2) in the presence of [γ-^{32}P]ATP. One sample (lane 1) was then subjected to PP1 treatment after Cdk4 phosphorylation. The phosphorylated proteins were analyzed by autoradiography, followed by electro-blotting of the same gel to PVDF membrane, and western blot analysis with the indicated antibodies.

The incubation of OGG1 with full-length c-Abl in the presence of ATP yielded the appearance of bands reactive to tyrosine-phosphate antibody at the molecular weight corresponding to OGG1-α-6his (Figure 6C, middle panel, lanes 3 and 4). No tyrosine-phosphate signal was detected in the absence of OGG1 (lane 2) or ATP (lanes 5–7). The presence of OGG1 and equal amounts of c-Abl were confirmed by western blot.

To analyze the effect of OGG1 phosphorylation by c-Abl on its catalytic activity, we assayed 8-oxoG incision of OGG1 incubated with active (Figure 7B, lanes 3–8) or heat-inactivated (lanes 9–14) c-Abl. Tyrosine phosphorylation of OGG1 under these conditions was again confirmed by western blot (Figure 7A, lanes 2–4). In contrast to our observations with serine/threonine phosphorylation by Cdk4, tyrosine phosphorylation of OGG1 by c-Abl did not change incision activity on an 8-oxoG-containing substrate. Since OGG1 recognizes and cleaves other substrates, such as other oxidized purines (34) and abasic sites (11), we cannot exclude the possibility that tyrosine phosphorylation of OGG1 affects other catalytic activities of the enzyme.

DISCUSSION

Post-translational modifications of proteins play a central role in cellular homeostasis by modulating a plethora of biological functions. Such modifications can regulate catalytic activities, change protein–protein interactions, target proteins to different cellular compartments and to degradation. Here, we provide the first evidence that a post-translational modification of OGG1 can modulate its 8-oxoG repair activity. We show that OGG1 physically interacts with Cdk4 and c-Abl kinases *in vitro* and *in vivo*, and is phosphorylated by both proteins. Furthermore, we confirmed the previous observation by Dantzer *et al*. (21) that PKC also phosphorylates OGG1 *in vitro*. However, we find that different phosphorylation events have distinct functional consequences. We found that OGG1 serine/threonine phosphorylation by Cdk4 stimulates 8-oxoG incision 2-fold, while serine/threonine phosphorylation by PKC has no effect on incision activity. Each one of these two kinases phosphorylates serine/threonine residues in different sequence contexts. Dantzer *et al*. (21) identified three potential PKC phosphorylation sites at S44, S211 and T295. Using the NetPhos prediction software (http://www.cbs.dtu.dk/services/NetPhos) (35), we predicted five potential Cdk4 phosphorylation sites, T19, S51, T89, S292 and T295. All these residues are highly conserved between human, mouse and rat OGG1s, although the crystal structure of OGG1 does not directly implicate any of those residues in DNA binding or catalysis in the human protein (36). However, we have recently shown that mutation of V317 abolishes 8-oxoG incision activity (29), even though this residue does not make direct contact with the substrate or the DNA backbone. Thus, it is possible that modifications in one of the putative Cdk4 phosphorylation sites, but not in others, slightly alter the structure of the protein, also modifying its catalytic properties.

Interestingly, we observed that the base release activity of OGG1 was stimulated by Cdk4 to a lesser extent than the combined DNA glycosylase + AP lyase activity (strand cleavage). It has been previously demonstrated that these two activities are uncoupled in the OGG1 protein, with the base release proceeding much faster than the strand cleavage step. It has

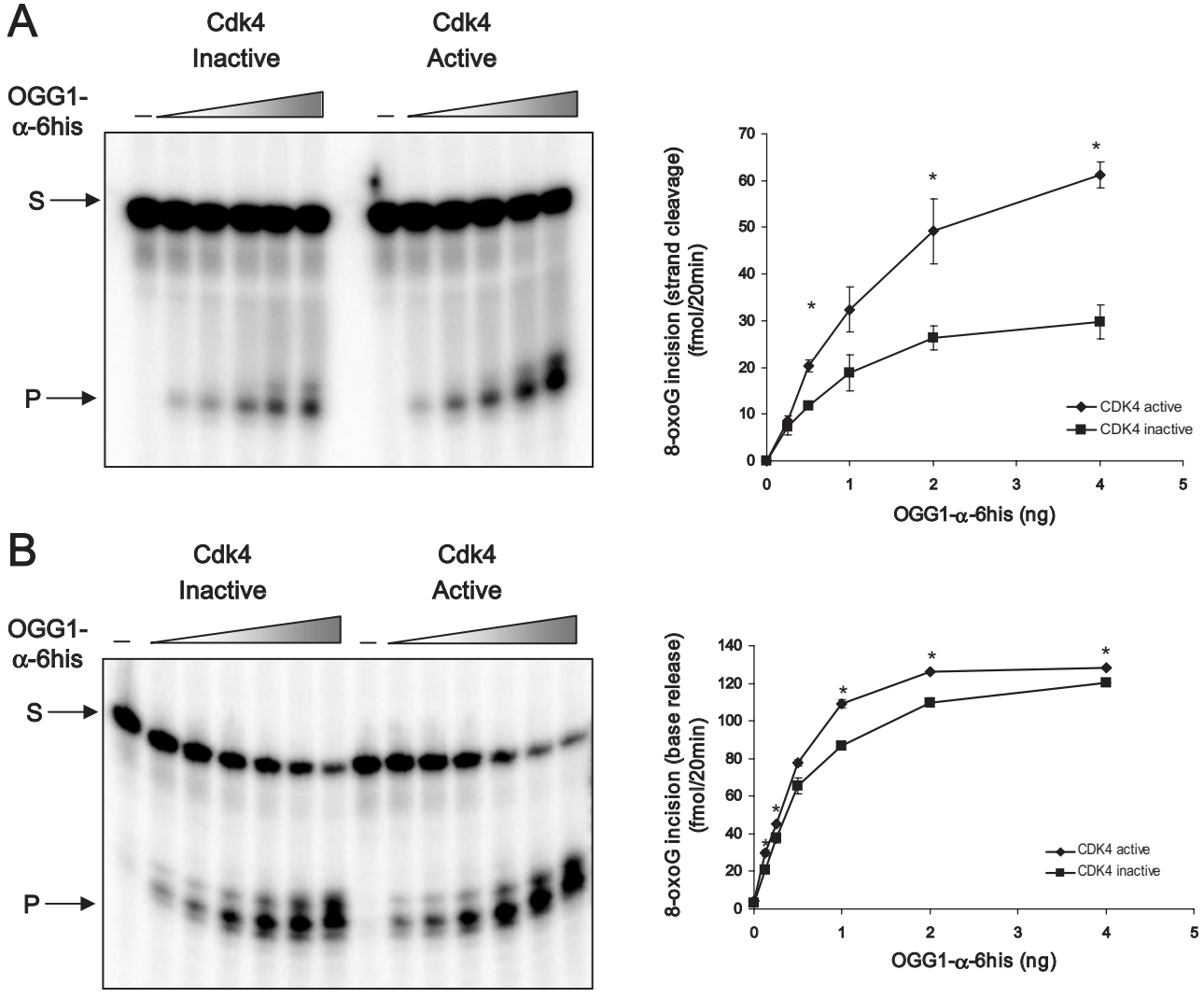

Figure 4. OGG1 phosphorylation by Cdk4 increases 8-oxoG strand cleavage and base release activities. 10 ng of purified OGG1-α-6his was first incubated with PP1 to ensure complete dephosphorylation. The proteins were then incubated with immunoprecipitated Cdk4, or heat-inactivated Cdk4 as a negative control. (**A**) Serial dilutions of OGG1-α-6his (0.25, 0.5, 1, 2 and 4 ng) were analyzed for 8-oxoG strand cleavage activity measurement, as described in Materials and Methods. (**B**) Similar OGG1-α-6his concentrations were analyzed for base release activity. The graphs on the right side of each panel present the average ±SEM of at least two independent replicates. Asterisks, significantly higher than inactive Cdk4 at $P < 0.05$.

been suggested that OGG1's AP lyase activity is not physiologically significant because APE1 has a much higher affinity for the abasic site and displaces OGG1 before the lyase reaction takes place (13). Our observation suggests that Cdk4 phosphorylation preferentially affects the AP lyase activity of OGG1, enhancing the coupling between the base release and strand cleavage steps. Thus, it is possible that the very low rates of AP lyase measured so far for OGG1 *in vitro* are due in part to the non-phosphorylated state of recombinant proteins generated in bacteria. These results may also indicate that OGG1's phosphorylation state may change its turnover rate, in a similar fashion to how APE1 increases OGG1 turnover by displacing it from the abasic site.

Cyclin-dependent kinases 2, 4 and 6 control progression through the G_1–S checkpoint. After DNA damage, cells arrest in late G_1 to allow time to repair damaged DNA before starting DNA replication. Cell cycle arrest is believed to be mediated by p16/p21^{Waf1} inhibition of Cdk activity (37). Two distinct reports have provided evidence that cell cycle arrest of human diploid fibroblasts (38) and vascular smooth muscle cells (39) after H_2O_2 treatment is associated with decreases in mRNA levels and activity of Cdk2 but no changes in Cdk4. Frippiat *et al*. (38) also found no significant changes in Cyclin D1, which associates specifically with Cdk4, levels after H_2O_2. Thus, it is possible that upon DNA damage the Cyclin D1/Cdk4 complex is still active and available to phosphorylate OGG1 in order to up-regulate DNA repair.

OGG1 strongly associates with p34^{SEI-1} (Figure 1, our yeast-two hybrid results), a protein that was cloned base on its ability to antagonize p16 inhibitory effect on Cdks 4 and 6 (32). Addition of p34^{SEI-1} to Cyclin D1/Cdk4 *in vitro* renders the complex resistant to inhibition by p16, and the ectopic expression of this protein allows fibroblasts to escape cell cycle control and proliferate even in very low concentration of serum (32). It is possible that the presence of p34^{SEI-1} in the Cdk4/OGG1 complex overrides the inhibition

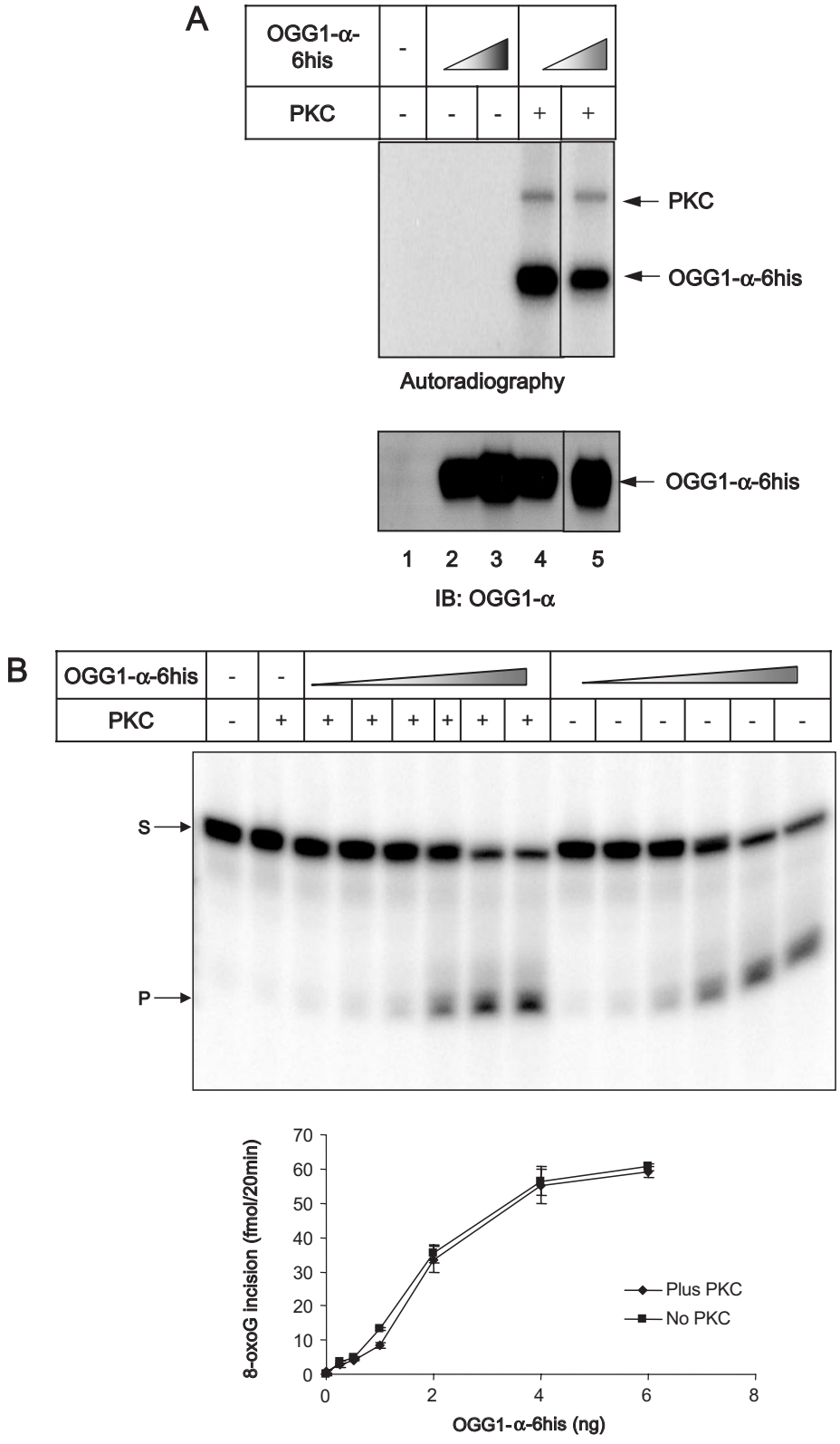

Figure 5. PKC phosphorylates OGG1 *in vitro* but does not change its incision activity. (**A**) An aliquot of 50–100 ng of recombinant OGG1-6his was incubated with or without 5 ng PKC (Promega) in the presence of [γ-^{32}P]ATP. The reactions were separated by SDS–PAGE and analyzed by autoradiography (top panel) and western blotting (bottom panel). (**B**) An aliquot of 0.25–6 ng of purified OGG1-α-6his was incubated with or without 5 ng PKC and 8-oxoG incision activity was measured as described in Materials and Methods. The graph presents the average ±SEM of two independent replicates.

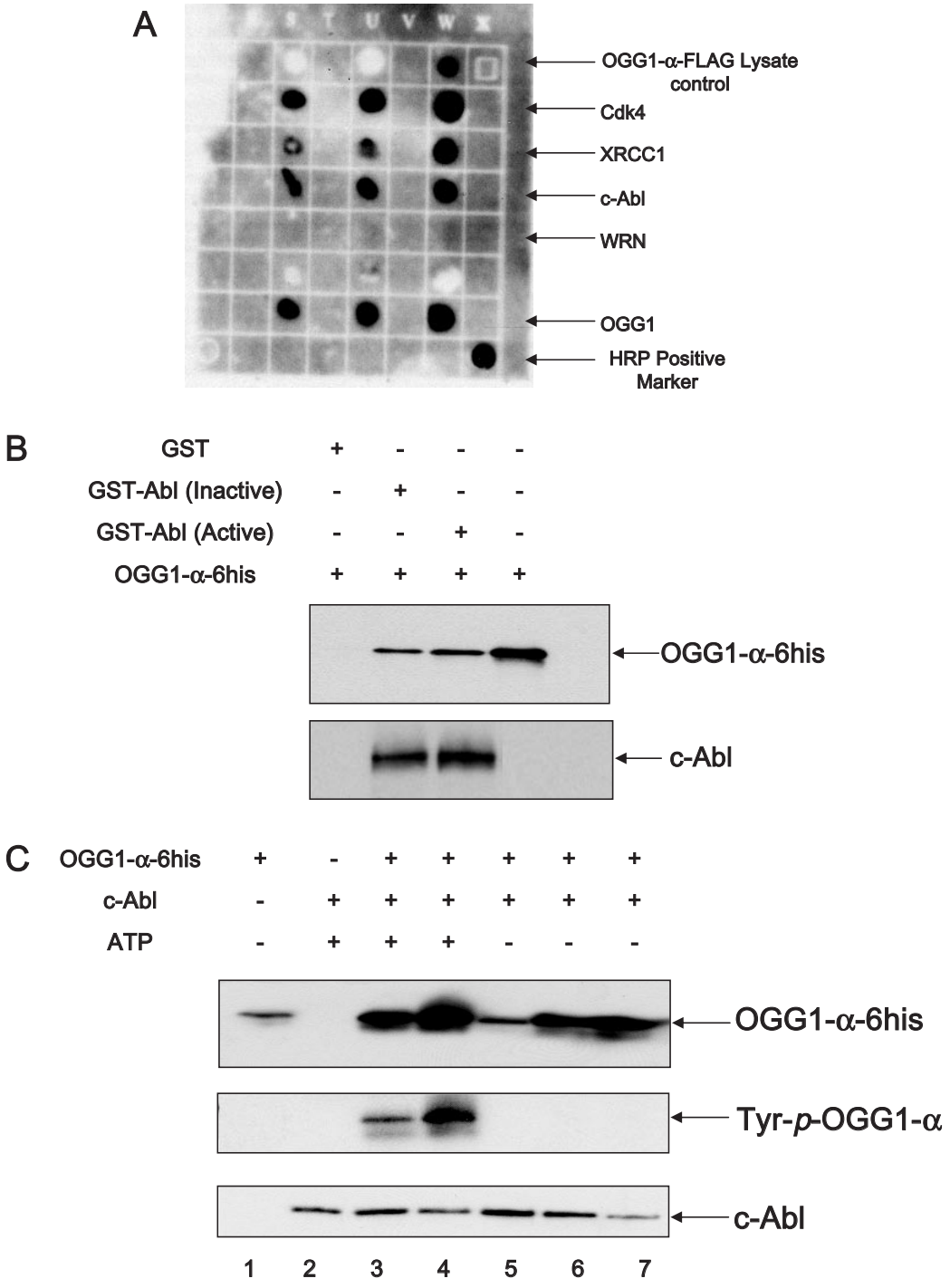

Figure 6. c-Abl physically interacts with and phosphorylates OGG1 *in vitro*. (**A**) An aliquot of 25, 50 and 100 ng of Cdk4, XRCC1, c-Abl, WRN and hOGG1 were applied onto the DiscoverLight™ Protein-array membrane. The membrane was processed and developed as described in the Materials and Methods. As positive controls, we blotted 1 µl of 293-FLAG-OGG1 lysate and a horseradish peroxidase control provided by the manufacturer. (**B**) Purified OGG1-α-6his was incubated with either GST (lane 1) or GST-c-Abl fusion proteins (inactivated in lane 2, active in lane 3). GST-pull down was performed as described in Materials and Methods. The proteins were then detected by immunoblot. OGG1-α-6his was loaded in lane 4 as a positive control. (**C**) Purified OGG1-6his was incubated with 100 ng full-length c-Abl either in the presence (lanes 2–4) or absence of ATP (lanes 5–7) to look at tyrosine phosphorylation. The proteins were analyzed by immunoblot with anti-phosphotyrosine (middle panel) followed by anti-OGG1 (top panel) and anti-c-Abl (bottom panel) antibodies.

by p16 after DNA damage, allowing Cdk4 to still phosphorylate OGG1.

Tyrosine phosphorylation by c-Abl does not change 8-oxoG incision. However, it was previously shown that OGG1 phosphorylation by PKC modulates its intracellular localization, and so it is possible that c-Abl-directed phosphorylation modulates another aspect of the biological function of OGG1, such as protein–protein interactions, intracellular localization or targeting to degradation.

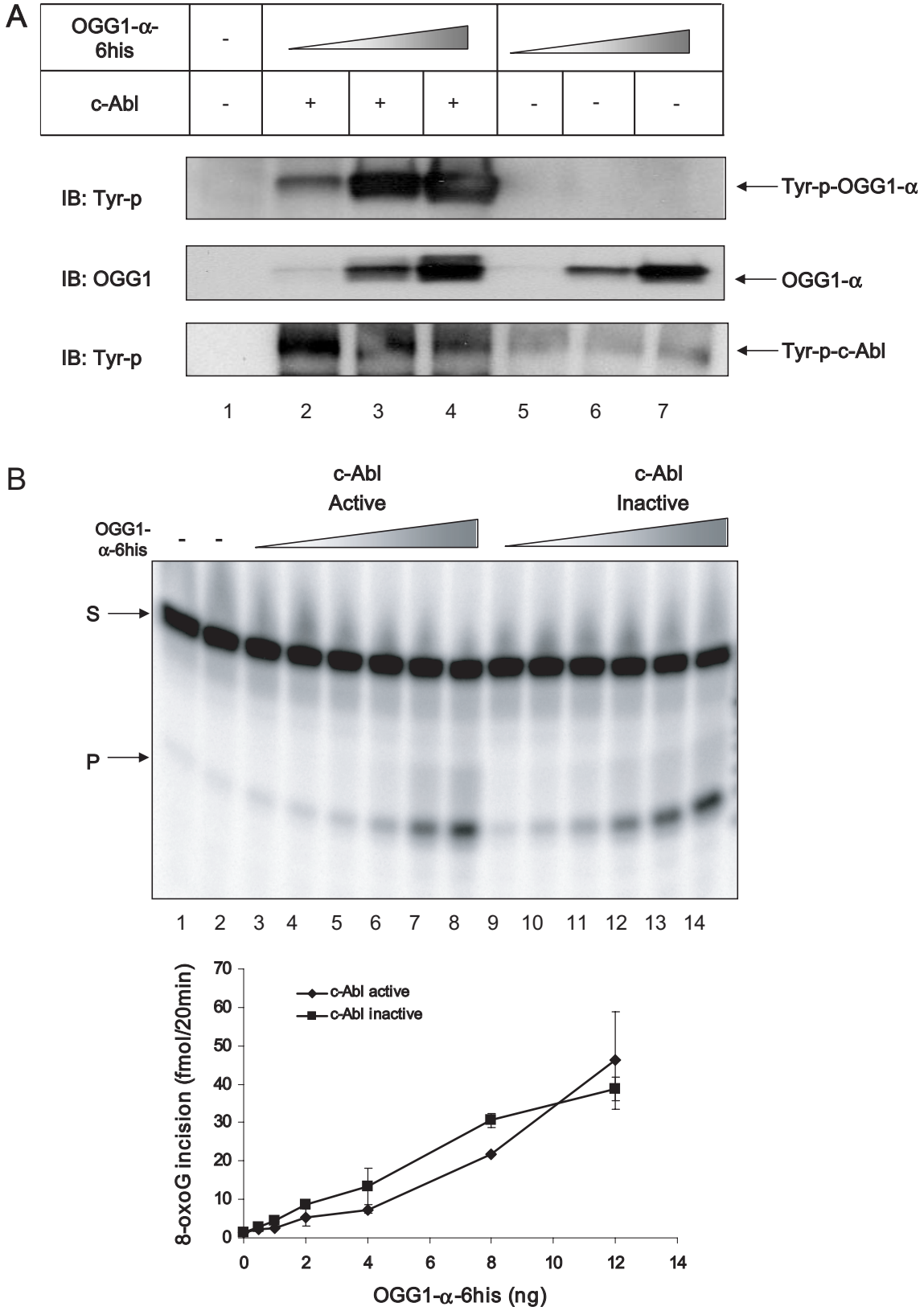

Figure 7. c-Abl phosphorylation of OGG1 does not change 8-oxoG incision. (**A**) Purified OGG1-6his protein (12.5, 25 and 50 ng) was incubated with full-length c-Abl (100 ng in each reaction), either active (lanes 2–4, allowing tyrosine phosphorylation) or heat-inactivated (lanes 5–7, unphosphorylated). The proteins were then analyzed by western blotting with the specified antibodies. (**B**) Purified OGG1-6his (0.25–12 ng) was incubated with 100 ng active (lanes 3–8) or heat-inactivated (lanes 9–14) c-Abl and then used for an 8-oxoG incision activity assay, as described in Materials and Methods. The graph presented shows the average ±SEM of at least two independent replicates.

Altogether, our results suggest that it is likely that phosphorylation, and possibly other post-translational modifications, play significant roles in controlling not only OGG1 activity but its localization and stability. These conclusions may have significant implications for the cellular regulation of repair of oxidative DNA damage because they suggest that the activation of different signaling pathways, and the consequent activation of specific protein kinases, would result in modifications of different residues in OGG1, thus differentially regulating BER efficiency.

Although we failed to see changes in incision activity after c-Abl and PKC-directed phosphorylation of OGG1, the possibility exists that those events modulate OGG1 catalytic activities on other substrates. Two recent reports (20,40) suggest that MYH glycosylase activity is enhanced by phosphorylation. Interestingly, dephosphorylation of native MYH reduces its glycosylase activity on A/G much more extensively than on A/8-oxoG mismatches (20). This observation suggests that substrate specificity of DNA repair enzymes may be altered by post-translational modifications, thus 'tailoring' the repair activity to a particular type of DNA damage. Moreover, Lu and co-workers (19) recently showed that UDG dephosphorylation by the p53-regulated phosphatase PPM1D reduced UDG activity, suggesting that PPM1D may inhibit BER by dephosphorylating UDG to direct its inactivation after completion of repair.

Another aspect that should be considered is the possibility that phosphorylation at particular residues targets the protein for degradation. Fischer *et al*. (41) recently reported that degradation of UDG is cell cycle dependent and is prevented when cells are incubated with the cyclin-dependent kinase inhibitor roscovitine. These results suggest that UDG phosphorylation participates in the regulation of protein levels via targeting the protein for ubiquitination. The studies published so far on changes in the level of OGG1 after DNA damage are highly contradictory. While some studies report varying degrees of up-regulation of protein (42,43) and mRNA levels (44) after cell exposure to several oxidants, another study reports unchanged protein levels after gamma-irradiation of human cells in culture (45). These studies, however, did not investigate the phosphorylation status of OGG1. Thus, it is possible that the activation of different kinases in each model system used in these studies resulted in the phosphorylation of OGG1 at different sites. This could, in turn, result in changes in sub-cellular localization and/or protein stability, and consequently affect protein levels detected in each study.

Polymorphisms in the *Ogg1* gene, along with lower 8-oxoG incision activity, have recently been associated with increased risk for cancers (7,8,10,46), underscoring the possibility that unregulated BER plays a causal role in carcinogenesis. The pRB-cyclin D-Cdk4 pathway has been proposed to be the molecular link between cellular senescence and malignant transformation (47,48). Also, expression of the Cdk4 inhibitor p16INK4a has recently been shown to increase dramatically in old mice and this protein has been proposed to be a new biomarker of aging (49). Thus, regulation of OGG1 activity through phosphorylation by Cdk4 may provide an additional link between changes in BER capacity and tumorogenesis and aging. It remains unclear, however, which functional consequences other post-translational modifications have on OGG1 activity and overall BER efficiency.

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