# IN VITRO BINDING OF BASE EXCISION REPAIR GLYCOSYLASES TO POLY(ADP-RIBOSE)

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The members of the Committee appointed to examine the thesis of JOSEPH A. NICHOLS find it satisfactory and recommend that it be accepted.

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Chair

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IN VITRO BINDING OF BASE EXCISION REPAIR GLYCOSYLASES TO POLY(ADP-RIBOSE)

Abstract

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After genomic DNA is damaged, one of the earliest eukaryotic cellular events is the initiation of Poly(ADP-ribose)polymerase-1 (PARP-1) enzymatic activity. PARP-1 converts $NAD^+$ to poly(ADP-ribose) polymers (PAR) which covalently modify several nuclear proteins, most notably histone proteins and PARP-1 itself. PARP-1 and its enzymatic activity are known to be critical for the proper functioning of Base Excision Repair (BER), the primary cellular pathway involved in the repair of DNA single strand breaks and damaged nucleobase products. However, the precise roles that PARsylation plays in BER are not currently clear. In addition to being a posttranslational modification of nuclear proteins, PAR also serves as a binding scaffolding for many proteins via non-covalent, highly specific molecular contacts between protein motifs and PAR. Since the BER proteins XRCC1 and DNA Ligase III have previously been shown to associate with PAR, it is thought that PAR-protein interactions play a role in latter stages of BER. In this thesis, the hypothesis was tested that BER

glycosylases, the enzymes involved in the earliest stages of BER, would bind to PAR as well. After the identification of putative PAR-binding sequences in the primary structure of five DNA glycosylases (OGG1, NEIL1, NTH1, MYH, and MPG) slot blot and phenol partitioning assays were carried out on the glycosylase peptides. These assays showed that peptides from OGG1, NTH1, NEIL1, and MPG bind to PAR. The native glycosylase proteins OGG1 and NEIL1 were also observed to bind to PAR, indicating that these glycosylases may interact with PAR in vivo. These PAR-glycosylase interactions have allowed us to propose a new model for the regulation of the earliest stages of BER by PAR. Finally, we have developed a new assay based on formaldehyde crosslinking to assess protein-PAR interactions. This technique is envisioned to facilitate future in vivo studies of PAR-protein interactions in the human cell nucleus following DNA damage.

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# CHAPTER ONE

# INTRODUCTION

## *Postranslational modifications of proteins*

Eukaryotic cells have evolved elaborate communication processes that are employed to regulate all necessary functions. Post-translational modification (PTM) of proteins is one means of cellular communication and PTMs are involved in processes ranging from metabolism to mitosis, and even cell death. Poly(ADP-ribose) (PAR) is the nuclear PTM of interest in this thesis, and the Poly(ADP-ribosyl)ation of proteins has been linked to a variety of cellular processes.(1) Poly(ADP-ribose) polymerases (PARPs) catalyze the transfer of PAR onto acceptor proteins, a process referred to as PARsylation. They can covaletly attach PAR onto themselves (automodification) or other proteins (heteromodification).

## *PARP Overview*

The discovery of PARPs was made over forty years ago in rat liver nuclei as a result of the observed connection between $NAD^+$ depletion and ADP-ribose formation.(2) PARP-1 was the initial isoform isolated and it is credited with forming over 80% of the PAR in cells.(3) Along with being the first isolated protein in the PARP superfamily, currently at 17 members and counting,(4) PARP-1 is also the most abundant at approximately 1-2 million copies per

nucleus.(5) Functional redundancy highlights the importance of PARPs and PARsylation reactions in cells. Knocking out either of the first two isoforms of this protein, PARP-1 and PARP-2, leaves viable cells, but the phenotype has decreased DNA repair rates. These two proteins are responsible for generating approximately 95% of all PAR produced in cells. However, PARP-1 / PARP-2 double knock-out mutants were found to be a lethal combination.(6,7) The other members of the PARP superfamily have been shown to have specific functions within the cell, but PARP-1 and PARP-2 remain the most abundantly expressed and are involved in more cellular processes compared to other PARPs.(8)

The PARP-1 protein is a modular protein with the three distinct domains shown in Figure 1. The amino-terminus contains the nuclear localization signal (NLS) and the DNA binding domain (DBD) comprised of two zinc-fingers that can bind to duplex DNA, DNA strand breaks, and other nuclear proteins in a DNA independent manner.(9-13) The central domain of PARP-1 contains a BRCT (breast cancer C-terminus associated) domain for protein-protein interactions (14) and also has the automodification domain (AMD) where a group of glutamic acid residues serve as covalent attachment points for PAR polymers generated by PARP-1 automodification.(15) Recently, a third zinc-finger was found between the DBD and the AMD. (16) This newly recognized feature was shown to provide allosteric control of the PARP-1 enzymatic activity in a DNA binding dependent manner. The carboxy-terminus of PARP-1 contains the catalytic domain that converts the β-$NAD^+$ substrate into PAR via three distinct enzymatic activities: 1) initial covalent attachment of ADP-ribose moiety onto a glutamic

acid side chain of an acceptor protein; 2) elongation of the PAR polymer backbone via the formation of 1''-2' bonds; 3) the formation of branches at random intervals off of the PAR backbone via the formation of 2''-1''' bonds.(17-19) See Figure 2 for a diagram of PAR and molecular details of the three steps in PAR synthesis. Specific proteins serve as targets for PARsylation, and most of them are associated with chromatin packaging. Included in this group are PARP-1 and the histones H1 and H2B.(20,21) The cellular consequences of the PARsylation of nuclear proteins are discussed in the next section.

*Cellular functions associated with PARPs and PARsylation*

PARP-1 has been associated with numerous cellular processes. The most frequently studied cellular processes involving PARP-1 are mitosis, transcriptional regulation, DNA damage recognition and repair, and cell death pathways.(1,22) The field of PARP research has even grown out of basic research into clinical trials of PARP inhibitors that are being screened as adjuvant therapies for the treatment of critical illnesses, such as cancers and cardiac failure.(23)

PARP-1 has been shown to be associated with cell division. This involvement with the mitotic process found this protein to localize at centromeres and interact with kinetochore proteins CENPA, CENPB, and BUB3.(24,25) The association with mitosis was further strengthened when PAR was found to be important for proper microtubule formation at spindle poles, though this ADP-ribose was credited as coming from PARP family member Tankyrase-1. (26)

Currently, some of the most sought after questions about PARP-1's involvement in cellular functions come from research into its role in transcriptional regulation. Studies have shown both the enzyme and its PAR product to regulate chromatin structure, transcriptional activation, and repression. (27) Some of the earliest work with PARP-1 showed that it can PARsylate the histone proteins responsible for chromatin packaging. It was further shown that once PARsylated, histone proteins lose their affinity for DNA in vitro and this in turn causes the relaxation of packaged chromatin structures like the 30 nm fiber.(28-30) Since the compaction of chromatin back into a more condensed form accompanies the addition of the glycohydrolase responsible for PAR degradation (PARG), the "histone shuttling" model was proposed.(31,32) In this scheme (Figure 3) PARP-1 activity leads to the relaxation of chromatin by disruption of DNA-histone binding, which in turn allows access of various DNA binding proteins, like those needed for transcriptional activation or repression, to their functional sites. After some period of time, the chromatin will be re-packaged once the interfering PAR polymers are degraded by PARG.(31,32)

The ability of PARP-1 activity to relax chromatin could facilitate DNA binding by proteins for processes like transcription. PARP-1 has been shown to interact directly with histones outside of the PARslyation for chromatin relaxation. An interaction between histone variant macroH2A and PARP-1 was found to inhibit the enzymatic activity of PARP-1.(33) This interaction was found to be responsible for silencing the X chromosome in cells through studies with a reporter gene inserted into the X chromosome.(33) In contrast to transcriptional

silencing, chromatin immunoprecipitation coupled to genomic microarrays showed that PARP-1 is associated with approximately 90% of RNA polymerase II expressed promoters.(34) This analysis also showed that histone H1, understood to form condensed, transcriptionally repressed regions of chromatin, is depleted in these regions. Prior to this, PAR was found to be crucial for proper size formation of puff loci on drosophila salivary gland chromosomes.(35) The puff loci are associated with active transcription and one study also found that PAR formation in these regions was crucial for normal levels of heat shock protein 70 (hsp70) gene expression. (35) Along with the association of PARP and its product at promoter regions, a number of immunoprecipitation studies have shown a protein-protein association with numerous transcriptional activators and repressors, such as NF-κB, Sp1, NFAT, Elk1 and YinYang1, further establishing this protein as a transcriptional coregulator. (27)

Because PARP-1 uses $NAD^+$ as a substrate, it has been linked to cellular metabolic states that can be a trigger for cell death. (36) Since PARP-1 can be activated upon binding to DNA strand breaks, cells that undergo heavy DNA damage have been shown to have arrested glycolysis as a direct result of PARP-1 activation and the ensuing depletion of cellular $NAD^+$ pools as shown in astrocyte cultures treated with MNNG (N-methyl-N'-nitro-N-nitrosoguanidine). (37) In normal animal cells, PARP-1 is inhibited by ATP (38), and just recently, PARP-1 was shown to affect the expression of the adenylate kinase gene through its association with Topoisomerase I.(39)

With this association to cell metabolism it follows that PARP-1 activation can also play a role in cell death since the apoptotic pathway is ATP dependent. Accompanying this link, two lines of evidence show that PARP-1 has a direct role in cell death. First, the PARP-1 protein contains a Caspase-3 cleavage site that will inactivate the protein upon induction of apoptosis.(40) Since PARP-1 activation can greatly influence the cellular ATP pool, inactivating this protein will allow ATP-dependent processes, like apoptosis, to proceed via PARP-1's consumption of $NAD^+$ substrate. Second, PAR has been shown to be able to translocate from the nucleus to the cytoplasm where it binds mitochondria and causes the mitochondria to release apoptosis inducing factor (AIF). (41-43) Though this cell death pathway shares some steps with apoptosis, it is a caspase independent form of cell death.(44) More specifically, the over-activation of PARP-1 was initially thought to induce necrosis, but finding that PAR will induce the release of AIF from the mitochondria showed a relation to apoptosis.(44,45) Since this form of cell death is distinct from both necrosis and apoptosis, it was named, "Parthanatos".(45)

One of the earliest cellular functions associated with PARP-1 and PAR is DNA damage recognition and repair. As mentioned previously, the first two zinc-fingers in PARP-1 can bind to strand breaks in the DNA backbone. Perhaps the earliest association between PARP-1 and DNA repair showed that PARP-1 inhibitors prevented resealing of DNA strand breaks caused by dimethyl sulfate. (46) PARP-1 knock-out cells have been shown to have decreased repair rates when treated with DNA damaging agents. (47) PARP-1 has also been found to

associate with proteins involved in DNA repair like XRCC1 (x-ray cross-complementation protein 1) and DNA ligase III (48,49) A more thorough examination of PARRP-1's involvement in DNA damage recognition and repair will be given in the following chapter.

*The covalent PARsylation of proteins versus PAR binding by proteins*

The association between PAR and nuclear proteins occurs in two ways that has given rise to two different branches of research focused on studying this polymer. As a post-translational modification, PAR is covalently attached to acceptor proteins. The other association is through a non-covalent and transient binding of PAR to various proteins, most notably histone proteins. (50,51) This interaction was shown to be able to withstand challenge from various agents. Specifically, PAR – protein binding can withstand strong acids (up to 1 M acetic acid), salt concentrations up to 1 M NaCl, detergents (up to 2% SDS), chaotropic agents (up to 4 M urea), and organic solvents (e.g. chloroform:phenol extraction). (52) The work investigating this interaction between histones and PAR found that all four core histones, H2A, H2B, H3 and H4, and also histone H1 can all bind to PAR with different affinities. (52)

Further research into this phenomenon showed that the PAR-protein interaction occurs at a specific sequence in the primary structure of the protein. This sequence, subsequently named the "PAR-binding motif", was categorized as a 22-amino acid region possessing two distinct patterns. The N-terminus of the PAR motif is lysine and arginine rich, while the C-terminus displays a

hydrophobic/basic residue repeating pattern.(53) A PAR-binding motif was found in many nuclear proteins, including the DNA repair pathway proteins XRCC1 and DNA ligase III. (53) Both of these proteins are involved in the latter stages of the base excision repair pathway (BER). (54)

*PAR binding by BER glycosylases*

Base excision repair is responsible for repairing damaged nucleobases. The pathway is initiated by glycosylase proteins that will remove the damaged nucleobase. (55) A central question to the work in this thesis is how the glycosylases find the damaged bases of DNA. Glycosylases are commonly outnumbered by DNA damage and at times of heavy genomic stress on the order of several magnitudes. (34,55,56) With the offset ratio of BER glycosylases to DNA damage, the involvement of PARP-1 activation in DNA damage recognition, and the fact that PAR binds to later stage BER pathway proteins XRCCI and DNA ligase III, we wanted to see if PAR could bind to BER glycosylases.

# CHAPTER 2

# POLY(ADP-RIBOSE) BINDING TO BASE EXCISION REPAIR GLYCOSYLASES HOGG1 AND NEIL1 IN VITRO AND CROSSLINKING TO HOGG1

## Introduction

While Poly(ADP-ribosyl)ation is perhaps the most dynamic post-translational modification of nuclear proteins, it is currently one of the least understood. Under times of cellular stress, cellular $NAD^+$ levels have been observed to drop up to ~60% within minutes because of their conversion into Poly(ADP-ribose) (PAR).(57) The enzymes responsible for this biochemical transformation belong to a family of 18 proteins collectively known as poly(ADP-ribose) polymerases (PARPs).(15) PARP-1, the first isolated member in the family, is one of the most abundant proteins in the nucleus of higher eukaryotes, with current estimates in the range of 1-2 million copies per cell.(5) This abundance is one reason why PARP1 is responsible for the production of ~80% of the total PAR found in cells.(8) The molecular link between the observed NAD+ decrease and cellular stress was provided by the discovery that DNA strand breaks activate PARP-1 and PARP-2. Furthermore, since PARP1 has now been shown to play a structural role in chromatin(13), it appears to be well-positioned in the genome to play the role of a damage sentinel to facilitate DNA repair and genomic maintenance. Although it is not related to the principal focus of this work, we note that additional studies have expanded the cellular roles attributed to PARP-1 to include involvement in cell cycle maintenance,

transcriptional activation and repression, as well as DNA replication.(4) Just as quickly as PAR is formed in the nucleus, it can be degraded by Poly(ADP-ribose) glycohydrolase (PARG) to create a dynamic, and transient PARsylation spikes in the nucleus.(58)

PAR is a heterogeneous nucleic acid synthesized by PARP1 via its multiple enzymatic activities. In the first step, the nicotinamide group of $NAD^+$ is cleaved and an ester bond is formed between the C1 of the resulting ADP-ribose and a glutamic acid side chain of a PAR-acceptor protein. Examples of nuclear acceptor proteins include PARP1 itself, histones H2B and H1, and Topoisomerase II.(59,60) In the second stage, ADP-ribose units are stitched together through 1'-2'' glycosidic bonds to form chains of PAR up to 200 residues in length. In the third step, PARP1 will create 1'-2' bonds between ADP-ribose and a preexisting PAR chain to form branches. The posttranslational modification of nuclear proteins by PAR can lead to dramatic changes in their function. For instance, PARsylation of the histones leads to the disruption of chromatin structure.(29,30)

Since PAR is a nucleic acid, it is perhaps not surprising that there exist proteins which bind with high affinity to PAR. There is a growing recognition that PAR binding proteins are integral members of nearly all nuclear protein families and that these proteins carry out many vital nuclear functions. The first proteins identified as PAR binders were the four core histones, histone H1 and protamine.(52) The binding of these proteins to PAR occurs with high specificity and affinity since PAR-protein complexes resist the action of strong acids,

detergents, and high salt concentrations.(52) Further studies of nuclear proteins showed that proteins such as MARCKS (myristoylated alanine-rich C kinase substrate), XRCC1, DNA ligase III, and APLF (aprataxin PNK-like factor) and CHFR (checkpoint protein with FHA and RING domains) all bind to PAR as well.(51,53,61) In the case of the MARCKS and BER proteins, as well as the core histones, these proteins were all found to possess a PAR-binding motif in their primary sequence which consists of two domains—a lysine/arginine rich region upstream followed by an alternating pattern of hydrophobic and basic residues downstream (Figure 4). On the other hand, APLF and CHFR were shown to bind to PAR via their Zn-finger domains.(61)

Of all of the cellular processes carried out in the nucleus, one of the strongest links to PARP activity and PAR exists for DNA Repair, and in particular the Base Excision Repair (BER) pathway.(62) For instance, when most eukaryotic cells undergo genotoxic stress, two immediate responses are 1) an increased expression of PARP-1 and PARP-2,(63) and 2) the creation of PAR polymers at areas that contain DNA strand breaks,(64) a DNA damage substrate repaired by BER. Furthermore, PARP1$^{-/-}$ mice show increased sensitivity to DNA damaging agents such as methyl methane sulfate and ionizing radiation whose products are largely repaired by BER. The observation that PARP-1 is found in a complex containing the two BER proteins XRCC1 and Pol $\beta$(65) indicates that PARP-1 can directly recruit enzymes involved in the latter stages of BER to sites of DNA damage. The role of PAR in BER is less clear however. Pleschke et al.(53) showed that the BER proteins XRCC1, DNA ligase III, and Pol $\varepsilon$ all

possess a PAR-binding motif, indicating that PAR may be regulating the latter stages of BER.

Since PAR appears to play a role, albeit currently unknown, in the latter stages of BER, we suspected that PAR might also have a role in the earliest stages of BER as well. In particular, since PAR binding was established for enzymes like XRCC1 and Lig III, we hypothesized that the DNA glycosylases involved in the recognition and excision of DNA nucleobase lesions arising from genotoxic stress would also possess a PAR-binding region. To test this hypothesis, we first identified putative PAR-binding regions in the sequences of the human DNA glycosylases OGG1(8-oxo-guanine glycosylase 1), NTH1 (endonuclease III homologue 1), NEIL1(Nei-Like 1), MPG (methylpurine glycosylase), and MYH(MutY homolog). Using modified versions of two established PAR-binding assays, membrane blotting and phenol partitioning, we established that peptides from OGG1, NEIL1, NTH1, and MPG bind to PAR, while MYH does not. PAR binding was also observed in the full length OGG1 and NEIL1 proteins. These results establish that PAR binding may have important roles to play throughout the BER process in vivo. Finally, we have established that formaldehyde can be used to crosslink PAR to its binding peptides and proteins, and these results which may be important in future studies which probe for PAR-protein interactions in vivo.

## Materials and Methods

*Proteins and reagents* - Calf Thymus histone H1 was purchased from CalBioChem (San Diego, CA). Purified *Xenopus laevis* histone H3 was kindly provided by Dr. Lisa Gloss (WSU), and human NEIL1 and murine MYH were donated by Dr. Tapas Hazra (UTMB) and Dr. Shelia David (UC-Davis), respectively. High Specific Activity PARP-1 (EC2.4.2.30) was purchased from Trevigen (Gaithersburg, MD). [α-$^{32}$P]-NAD$^{+}$ was purchased from Perkin-Elmer (Waltham, MA; specific activity 800Ci / mmol). Glutathione-Agarose resin and Thrombin were purchased from Invitrogen (Carlsbad, CA), and the UnosphereS cation exchange resin was obtained from BioRad (Hercules, CA). 37% Formaldehyde was purchased from JTBaker (Phillipsburg, NJ).

*Synthesis and purification of poly(ADP-ribose)*- $^{32}$P-labeled poly(ADP-ribose) (PAR) was prepared using a modified published procedure.(66,67) Briefly, 2 units of high specific activity PARP-1, 1 mM NAD$^{+}$, 25 µg histone H1, 3 µL [α-$^{32}$P]-NAD$^{+}$, and 22.5 µg digested pET DNA were mixed in 100 mM Tris, 10 mM $MgCl_2$ pH 8.8 buffer, and the mixture was incubated at 37°C for 60 minutes. After TCA precipitation, the resulting protein-PAR pellet was washed twice with diethyl ether, and the heterogeneous PAR polymers were cleaved from the acceptor proteins by heating the resuspended pellet for 3 hours at 65°C in 1 M KOH with 50 mM EDTA. After dilution in AAEG9 buffer (6 M Guanidinium-HCl, 250 mM ammonium acetate, 10 mM EDTA, pH 9.0), the mixture was applied to a dihydroxy boronate BioRad (DHBB) column, prepared using the method of

Wielckens et al.(68) The column was washed with AAEG9 buffer followed by 1 M ammonium bicarbonate, 10 mM EDTA, pH 9.0 buffer. Finally, the PAR polymers were eluted in 10 mM HCl. After lyophilisation, the PAR polymers were resuspended in water and ethanol precipitated. The pellet was washed with 70% ethanol, resuspended in water, and stored at -20°C.

*Peptide Identification, Synthesis, and Purification* – The known PAR-binding sequence of Histone H3 (IRRYQ KSTEL LIRKL PFQRL VR; H3(52-73)) (69) and the full amino acid sequences of the human DNA glycosylases MPG, MYH, NEIL1, NEIL2, NTH1, hOGG1, SMUG1, TDG, and UDG were aligned using the LALIGN program (70) with the Blosum 62 scoring matrix. A DNA glycosylase was considered to have a putative PAR-binding sequence if the homology between H3(52-73) and any glycosylase amino acid sequence of greater than 15 amino acids was ≥20%. The glycosylases with sequences homologous to H3(52-73), and the amino acid compositions of their putative PAR-binding sequences, are listed in Figure 4.

The following peptides were obtained using FMOC-based, solid-phase peptide synthesis: H3(52-73) RYQKS TELLI RKLPF QR; OMP – GAATL ASTPA LWASI PCPRS EL; OGG1(14-30) RTLAS TPALW ASIPC PR, MPG(263-284) GEWAR KPLRF YVRGS PWVSV VD, NTH1 (39-60) LRREA AAEAR KSHSP VKRPR K; MYH(402-423) PSVTW EPSEQ LQRKA LLQEL QR; and NEIL1(321-342) PSRTR RARD LPKRT ATQRP EG. After synthesis, all peptides were purified by reverse phase HPLC (Alltech Macrosphere 300 C8 column) using a

Waters 2960 system coupled to a 996 Photodiode Array Detector. After lyophilisation, the peptides were resuspended in water (hOGG1(14-30) was resuspended in water and 0.1% β-mercaptoethanol) and stored at -20°C. Protein concentrations were determined using a Cary Bio100 UV-Vis spectrophotometer (Varian) by measuring $A_{210}$ (71), and when appropriate, $A_{280}$. The identity of each peptide was verified by MALDI mass spectrometry before use (data not shown).

*hOGG1 expression and purification* – GST-tagged OGG1 was expressed in E. coli BL21(DE3) cells following similar protocols to Audebert et al.(72) Briefly, transformed cells were grown at 37°C to $OD_{600}$ = 0.6 in LB media with 100 μg/mL ampicillin and 25 μg/mL chloramphenicol. Expression was induced by adding 1 mM IPTG and incubating the cells 25°C for 18 hours. The cells were harvested by centrifugation, washed once with PBS, and lysed by sonication. After centrifugation of the cell lysate, the soluble fraction was applied to Glutathione-Agarose resin. The resin was washed once with PBS, and the GST-OGG1 was eluted with 30 mM glutathione, 50 mM Tris, pH 8.0 buffer. After cleavage of the GST-OGG1 fusion protein by thrombin, OGG1 was purified using an NaCl gradient elution from a Unisphere S cation exchange column (BioRad). Fractions containing OGG1 were identified using $A_{280}$ and SDS-PAGE. Pooled fractions were concentrated using Amplicon Ultra-15 spin concentrators. The purity of OGG1 was assessed at >95% by SDS-PAGE. OGG1 activity was verified using $^{32}$P-labeled 8-oxoguanine-modified duplex DNA (data not shown).

*Slot-blot assays* – A modified literature procedure (73) was utilized in these experiments. Peptides (500 ng) or proteins (5 µg) were applied to a nitrocellulose membrane using a vacuum manifold, followed by drying at 80°C for 1 hour. Next, the membranes were immersed in TBST (10 mM Tris•HCl, 150 mM NaCl, 0.05% Tween 20, pH = 7.4) supplemented with 30,000 counts of $^{32}$P-PAR for 1 hour at room temperature. The membrane was subjected to a stringency wash using TBST containing 1 M NaCl.(69) The membrane was washed with TBST, dried, and subjected to autoradiography using a Molecular Dynamics Phosphorimager 445 SI system. After autoradiography, the membranes were stained with Amido Black to verify the presence of the spotted peptides / proteins. All blots were performed in triplicate.

*Phenol partitioning assays* – 500 ng of peptide or 5 µg of protein was mixed with 1,000 counts of $^{32}$P-PAR in 100 mM Tris (pH 8.4) and the solution was incubated at room temperature for 10 minutes. An equal volume of phenol/chloroform/isoamyl alcohol was added and the aqueous and organic layers separated by centrifugation. After extracting the organic layer with another aliquot of Tris buffer, the $^{32}$P counts in the organic and the combined aqueous layers were measured using a 1900CA TRI-CARB scintillation counter (Packard, Meriden, CT).

*Formaldehyde crosslinking* – PAR–peptide and PAR-protein crosslinking was performed using a slightly modified phenol partitioning procedure. After mixing the peptides / proteins with PAR, 0.01% formaldehyde was added and allowed to react for 10 minutes at room temperature. Then SDS (2% final concentration) was added to disrupt all non-covalent PAR-protein / peptide complexes, followed by phenol extraction and scintillation counting of the aqueous and organic layers.

## Results

*Identification of a PAR binding motif in several human BER glycosylases* – Traditionally, the PAR binding potential of nuclear proteins has been assessed using experimental protocols like membrane blotting.(50-52) As an alternative approach, Pleschke et al. (53) utilized bioinformatic tools to discern PAR binding sequences in nuclear proteins (Figure 4). Their identified motif has a characteristic N-terminal K/R rich region and a C-terminal alternating hydrophobic / basic amino acid pattern, as illustrated by the H3 sequence in Figure 4. Instead of using a pattern search algorithm to identify PAR-binding motifs in proteins, we hypothesized that a simpler procedure would suffice. In particular, we theorized that using a homology-based sequence alignment of a known PAR binding sequence to a test protein primary structure would suffice to identify PAR-binding candidates. Since all four histone proteins bind PAR (52) we chose H3(52-73) as our probe sequence. We used the LALIGN program (70) with the Blosum62 scoring matrix for homology scoring and set our selection criteria as (i) the amino acid sequence with homology to H3(52-73) must be of ≥

15 amino acids in length and (ii) the identity must be ≥ 20%. We first tested this alignment protocol by screening the full length sequences of the PAR-binding proteins identified by Pleschke et al.(53) This procedure identified the PAR-binding motif in >50% of these proteins (data not shown), indicating that while it is not 100% efficient; it is able to identify the PAR-motifs in diverse nuclear proteins. Furthermore, it shows that a significant subset of all known PAR-binding motifs in nuclear proteins can be identified simply by searching for homology to the sequence in histone H3.

The nine glycosylase proteins we chose to screen for PAR-binding sequences were MPG, MYH, NEIL1, NEIL2, NTH1, OGG1, SMUG1, TDG, and UDG. Of this group, OGG1, MPG, MYH, NEIL1, and NTH1 were all found to have a sequence that fit our selection criteria (Figure 4). After this initial screen, we then moved forward to test these sequences for PAR-binding ability using the experimental protocols described as follows.

*Analysis of PAR binding by peptides corresponding to the identified BER glycoslyase sequences* – The most common experimental technique used to demonstrate protein-PAR binding is membrane blotting. This approach uses either the electrophoretic transfer of proteins onto a nitrocellulose membrane(53), or the direct spotting of peptides and proteins on to a membrane,(74) followed by incubation of the membrane with $^{32}$P-labeled PAR to assay for PAR binding. We used the direct spotting method but added the use of a slot-blot vacuum manifold for better containment of the peptides on the membrane. The membrane was

spotted with 500 ng of each peptide, dried, and soaked in TBST containing $^{32}$P-PAR. After a stringency wash in TBST supplemented with 1 M NaCl, conditions which disrupt non-specific PAR-protein and PAR-nitrocellulose interactions,(75) the membrane was dried and visualized using autoradiography. A representative slot-blot experiment carried out on the glycosylase peptides is shown in Figure 5. Panel A shows the autoradiography of the membrane where binding of $^{32}$P-PAR to the peptides is detected. Panel B shows the same membrane after staining with Amido Black, a general protein stain which verifies that similar levels of peptide were present on the membrane. From Panel A in Figure 5, it is observed that the peptides NTH1(39-60), NEIL1(321-342), MPG(268-289), and OGG1(12-33) all bind to PAR. However, the MYH(402-423) did not display PAR binding. As controls, we spotted H3(52-73) and observed PAR binding, while slots without peptide showed the low background of randomly bound PAR on these membranes. To further verify PAR-binding by the glycosylases, we used a mutant peptide of the OGG1 sequence, named OMP (OGG1 mutant peptide; Figure 4), in which the two N-terminal basic residues of OGG1(12-33), His13 and Arg14, were mutated to alanine. PAR binding by OMP (Figure 5) is not observed, indicating that the N-terminal basic residues are essential for PAR-binding in OGG1(12-33). From these results, OMP will serve as a negative PAR-binding control peptide for our further experiments.

As a second method to test for specific PAR-peptide binding, we modified a phenol partitioning assay that was originally developed to verify histone-PAR binding.(75) These assays rely on the fact that the partitioning of $^{32}$P-PAR

between aqueous and organic layers is modulated by the presence of a bound peptide or protein, with protein binding leading to the loss of $^{32}$P-PAR from the aqueous layer. In the Panzeter method, this loss of counts in the aqueous layer was visualized by DNA sequencing gels. (75) We modified these assays to be more quantitative by using scintillation counting of the separated organic and aqueous layers of a phenol extraction in the presence and absence of peptides. We hypothesized that if a peptide bound PAR, we would observe an increase of $^{32}$P-PAR counts in the organic layer of the extraction. Our first control reaction was to measure the background of PAR carried over to the organic layer in the absence of peptide. The bar labeled "PAR" in Figure 6 shows this background, and the error bar is the standard error of the mean from triplicate, independent experiments. Next, we performed control reactions using H3(52-73) as a positive-binding control and OMP as a negative-binding control. The addition of H3 shows a sharp increase in $^{32}$P-PAR carried over to the organic layer, while the results from OMP are statistically identical to PAR. Having validated this methodology, we next tested the PAR-binding ability of the five PAR-binding peptides. Because of increased $^{32}$P-PAR counts in the organic phase, OGG1(12-33), MPG(268-289), NEIL1(321-342), and NTH1(39-60) are all deemed to bind PAR, while MYH(402-423) does not. We note that these results are consistent with those observed from slot blotting.

For statistical analysis of these findings, a one-way analysis of variation (ANOVA) was used because more than two independent groups were tested in triplicate. ANOVA analysis of the amount of PAR-binding confirms that NEIL1

and NTH1 peptides bind PAR at a significant level; however, OGG1 and MPG peptides do not bind PAR at levels that are statistically different from the negative controls. We believe that these latter sequences do bind PAR, as the diagnostics with formaldehyde crosslinking will show below. Finally, the MYH peptide did not bind to $^{32}$P-PAR as consistent with the results of slot blotting (Figure 5).

*Analysis of PAR binding by BER glycoslyase proteins* – We next set out to verify that full-length glycosylase proteins possessing a PAR-binding motif could non-covalently bind to PAR. The same methods used for testing the glycosylase peptides were utilized in these experiments. To establish a protein test set, we obtained the following purified, full-length proteins *Xenopus laevis* Histone H3 human NEIL1, and murine MYH (mMYH) from other laboratories and human OGG1 from our own efforts. The Xenopus laevis histone H3 (NP_001091119) that we used has an identical PAR-binding sequence as the human sequence that we used. The mouse form of MYH was used in these studies because of availability, but it was deemed suitable for our needs for the following reasons. First, the mMYH and human MYH proteins are 73% identical and are therefore very similar in overall sequence. Second, the putative PAR motif in human MYH and mMYH are 86% identical, with a Leu at position 406 and two His residues at positions 412 and 414 in the mMYH sequence. Since none of these amino acid changes effects the important residues in the Pleschke et al PAR-binding motif, we decided to use mMYH as a suitable surrogate for human MYH. (75)

Figure 7 shows the results of a representative slot-blot performed with the BER glycosylases. Panel A shows the autoradiography of the nitrocellulose membrane and Panel B shows the Amido Black staining of the proteins. This slot-blot was performed using the same protocol as for the glycosylase peptides, with the exception that 5 μg of protein were loaded to keep the total amount of PAR-binding sequence in each slot roughly the same. The H3 positive control shows PAR binding, while the BSA negative control does not. NEIL1 binds to $^{32}$P-PAR at a level similar to H3, while OGG1 and mMYH appear to have a lower, but detectable, amount of PAR bound to them. While PAR binding by NEIL1 and OGG1 was expected from the peptide analysis, the binding of mMYH was not.

The OGG1, mMYH, and NEIL1 proteins were next utilized in a phenol partitioning assay to assess the ability of these glycosylases to bind PAR in their native, folded state. These reactions were carried out in an identical fashion to those performed earlier on the peptides, except 5 μg of protein were used in each reaction. Figure 8 shows that the H3 positive control had an increased level of $^{32}$P-PAR in the organic layer, while the negative controls BSA (acidic protein) and lysozyme (basic protein) are statistically identical to the PAR-only control (data not shown). The glycosylases OGG1 and NEIL1 bound PAR at a level closer to H3, indicating that these three proteins bind to PAR with a slightly lower affinity in their native state. On the other hand, the folded mMYH protein is indistinguishable from the negative controls and therefore does not bind to PAR with high affinity. We note that in the cases of OGG1 and NEIL1, the two experimental assays led to similar conclusions about their ability to bind to PAR.

However, for mMYH the slot blot assay appeared to lead to an inconclusive positive binding since the phenol partitioning assay was negative for PAR binding.

*Formaldehyde crosslinking of PAR and native proteins* – Chemical crosslinking of nucleic acids and proteins is a standard technique and is used extensively in cell biology, e.g. Chromatin immunoprecipitation (ChIP) assays and earlier studies that identified DNA-histone contact points.(76) However, to our knowledge no one has attempted to use formaldehyde crosslinking to analyze PAR-protein complexes, nor was it clear going into this work that such complexes could be crosslinked due to a lack of structural characterization of PAR-protein complexes. Therefore, we performed formaldehyde in vitro crosslinking experiments on both PAR-peptide and PAR-protein complexes as a proof of principle. The crosslinking assays were carried out using modified phenol partitioning experiments in which 0.01% formaldehyde was added to the protein-PAR mixture before phenol extraction. Additionally, we added a 2% SDS step between the formaldehyde treatment and phenol extraction. Since it has been previously shown that 2% SDS will disrupt non-covalent PAR-protein interactions,(52) this treatment serves as a stringency control in our experiments to ensure that the proteins and PAR were physically crosslinked by the formaldehyde treatment. Figure 9 shows the results of the experiments on PAR-peptide mixtures. Panel A redisplays the results of the phenol partitioning in Figure 6 for reference. Panel B shows that all of the peptides do not bind to PAR at a significant level when

treated with 2% SDS compared to negative controls analyzed with ANOVA one-way analysis (data not shown). Panel C shows that OGG1(12-33), MPG(268-289), NEIL1(321-342), and NTH1(39-60) all bind to PAR and can be crosslinked using formaldehyde at a level similar to that observed for H3(52-73). The levels of OGG1(12-33) and MPG(268-289) crosslinking verifies that these two peptide sequences do in fact bind PAR, however with a seemingly lower affinity than either NEIL1(321-342) or NTH1(39-60).

Since the glycosylase peptides all are susceptible to formaldehyde crosslinking when bound to PAR, we next carried out similar experiments on full length proteins (Figure 10). In Panel A, the H3, BSA, and OGG1 phenol partitioning results from Figure 8 are redisplayed for reference. Panel B shows the disruption of protein-PAR binding when 2% SDS is added before phenol extraction. In Panel C, the crosslinking of PAR to histone H3 and OGG1 is evident after formaldehyde is added. As expected from previous studies(74,76) there was no formaldehyde crosslinking of the PAR nucleic acid to BSA in these experiments. ANOVA analysis of the level of PAR binding by OGG1 demonstrates that it is statistically similar to the positive control histone H3 (data not shown).

## Discussion

Using both in silico and in vitro experimental procedures, we have shown that several human BER glycosylases are capable of binding non-covalently to

Poly(ADP-ribose). To identify putative PAR-binding regions in these proteins, we tested the hypothesis that simple homology searching using a known PAR-binding sequence would suffice to identify similar sequences in other, non-related nuclear proteins. In particular, we have shown that a significant subset of the PAR-binding regions in nuclear proteins can be identified by their homology with H3(54-70), the PAR-binding region of histone H3. This approach is in contrast to other methodologies which rely on more complex sequence pattern analyses to identify potential PAR-binding motifs in nuclear proteins.(69,77,78) When we screened nine human glycosylases, we identified five which contained a contiguous sequence of ≥15 amino acids with ≥ 20% homology to H3(52-73). The glycosylases with putative PAR-binding regions were OGG1, MPG, MYH, NEIL1, and NTH1, while SMUG1, UNG1, NEIL2, and TDG did not harbor a sequence of high homology to H3(52-73). We note that the potential positive PAR-binders all fall into a group that repairs either oxidative (OGG1, MYH, NEIL-1, and NTH1) or alkylation damage (MPG) arising from cellular stress.(55) In contrast, the glycosylases without a putative binding sequence largely repair DNA damage which arises from the spontaneous deamination of pyrimidines—e.g. SMUG1 and UNG2 remove deoxyuracil residues,(79) while TDG is involved in the repair of G:T mismatches.(80) The differences in homology with H3(54-70) between NEIL1 and NEIL2 is not surprising since these two glycosylases share very little sequence homology with one another.(81)

After their identification, peptides corresponding to the putative PAR-binding regions of OGG1, NEIL1, MYH, NTH1, and MPG were tested for PAR-

binding using two separate experimental techniques. First, we used peptides spotted on nitrocellulose membranes to probe for PAR binding. Next, we used phenol partitioning experiments to interrogate PAR binding. Our motivation for using these two techniques in tandem is that while slot blotting is a quicker screen for PAR-binding, it does not probe for non-covalent PAR-peptide or PAR-protein interactions under non-denaturing conditions, such as those present during phenol partitioning. Using these assays in tandem, we discovered that the tested peptides bind to PAR in the following approximate order of affinity; H3 ~ NEIL1 ~ NTH1 > OGG1 ~ MPG >> MYH.

To better understand these results, Figure 4 shows the peptide sequences aligned to the PAR-binding motif of Pleschke et al.,(69) with the amino acids which fit their pattern indicated in bold. The first observation from this alignment is that the three peptides with the highest affinity for PAR have the highest number of amino acids which match the PAR motif (11 each). In contrast, MPG only has 7, MYH has 5, and OGG1 has 4. Perhaps more important than the number of amino acids which fit the PAR-binding motif is their distribution within the peptide sequence. For instance, both of the low affinity, positive binding peptides (OGG1(12-33) and MPG(268-289)) have basic residues in the N-terminal K/R rich region, whereas MYH does not. To see if this observation can explain the lack of PAR-binding in MYH, we generated the OMP peptide (Figure 4). OMP is identical to OGG1(12-33) except His13 and Arg14 are mutated to alanine to knock out all basic residues in the N-terminal region. As Figures 5 and 6 show, OMP behaves identical to MYH and does not bind to PAR. This result

indicates that a future refinement of our homology search methodology to identify putative PAR-binding motifs would be to ensure that candidate sequences possess lysine or arginine residues in their N-terminal region.

Surprisingly, our analysis of the PAR-binding motif in OGG1 leads to results which are exactly the opposite reported by Pleschke et al.(53) These authors took the PAR-binding sequence from the MARCKS effector peptide M and selectively substituted different amino acids with alanine. They observed by membrane blotting that replacing all of the upstream K/R residues did not result in the loss of PAR-binding. However, when they replaced all of the hydrophobic residues with alanine they did observe a loss of PAR-binding. In OMP we have left the hydrophobic residues alone, but mutated the N-terminal basic residues to alanine and observed a loss of PAR binding. We have no explanation for this discrepancy, but we suspect that it indicates that there is much more to be learned about the roles of the individual amino acids in PAR-binding, as well as the molecular interactions important for the stabilization of PAR-protein complexes.

Once these peptide sequences were identified, we next wanted to verify that the PAR-binding motif in the full length glycosylase proteins was active. While we were not able to study all of the proteins corresponding to the peptides in Figure 4, we were able to obtain OGG1, NEIL1, Histone H3, and mMYH as an initial test set. We used mMYH because of availability, and also included BSA and lysozyme as acidic and basic protein controls, respectively. The slot blotting experiments showed that the H3 positive control, NEIL1, mMYH, and OGG1

bound to PAR, although weakly in the case of the latter two proteins. The observation that mMYH bound to PAR in this assay was a surprise, given that the MYH peptide had shown negative PAR-binding and MYH(402-423) was the only sequence in human MYH which fit our homology criteria with H3(52-73). However, when all of these proteins were tested in their native, folded conformation in the phenol partitioning experiments, we observed that H3, OGG1, and NEIL1 did bind to PAR, while mMYH did not. We suspect that the discrepancy we observe between the slot blotting and phenol partitioning experiments on mMYH indicates that probing membrane-bound proteins with $^{32}$P-PAR to look for PAR binding is a method which potentially generates false positives. Therefore, while slot blots can be used as a quick screen for PAR-binding, they should always be validated through a second methodology, such as phenol partitioning, that interrogates PAR-binding by proteins in their native conformation.

Mapping the location of the PAR-binding motifs onto the known molecular structures of H3, NEIL1, and OGG1 can tell us something about the potential for PAR-protein binding in other glycosylases and nuclear proteins. Under the conditions we have studied, H3 is largely unstructured(82) and PAR binding is facile. However, the H3(54-70) sequence is found in the central $\alpha$-helix of the histone fold, which makes intermolecular contacts with Histone H4 in the central tetramer of the nucleosome core particle(83). Since the difference in thermodynamic stability between H3 in the nucleosome and H3 bound to PAR is currently unknown, it is not clear if H3-PAR binding would be prevalent in vivo.

However, the previous observation that PAR is capable of disrupting chromatin structure could indicate that PAR-histone binding is thermodynamically feasible and it can compete with DNA-histone and histone-histone binding.(84) In the crystal structure of OGG1, the PAR binding motif is near the N-terminus of the protein, on a solvent exposed exterior face of the folded core and away from the active site or DNA-binding surface. Therefore, PAR-binding by OGG1 is not expected to lead to large changes in the structure of the protein. Another interesting observation about OGG1 comes from looking at the sequence of this enzyme in yeast, a eukaryote which only recently was found to have a PARP-like protein as part of their proteome. (85) From the sequence alignment of the only major difference between human OGG1 and yeast OGG1 is truncation of the N-terminus of the latter which results in the lack of a PAR motif in yeast OGG1. This result indicates that PARsylation reactions and PAR-motifs in some nuclear proteins, such as OGG1, may have an evolutionary connection. Finally, the PAR-binding region of NEIL1 is outside of the folded core of the protein observed in crystal structures. Therefore, the PAR-binding sequence in this glycosylase is most likely located in a non-structured, disordered region that could explain the efficient binding of PAR and NEIL1.

We can extend these structural observations to make predictions about the other glycosylases used in this paper. First, the putative PAR-binding sequence in NTH1 is located near the N-terminal region of the protein, far removed from the C-terminal catalytic domain. Therefore, it is reasonable to expect that the PAR-binding region in NTH1 is in a disordered region like NEIL1,

and therefore we predict that folded NTH1 will bind to PAR. MPG is also known as 3-aminoadenine glycosylase, and its crystal structure was reported by Lau et al.(85) The PAR motif in MPG is located in the folded domain of this glycosylase, but it is far removed from the active site and DNA binding sites in a region of mixed β-strand and α-helical structure. In fact, the folded structure of the putative PAR-binding sequence in MPG looks very similar to that observed for OGG1, and therefore we predict that MPG can bind to PAR in its native, folded state. Finally, we turn our attention to MYH. The PAR-binding sequence in MYH lies within the folded, catalytic domain of MYH so it is not surprising that there was no binding of the folded protein to PAR. We note that the positive PAR-binding signal for mMYH in the slot blot could be due to denaturation of the protein, leading to increased accessibility to the PAR-binding region. Also, the putative PAR-binding region of mMYH is different from human MYH by three amino acids, including two histidine residues in the C-terminal region. If these resides were protonated on the membrane then this change could be responsible for the weak PAR-binding in the slot blot analysis.

Another outcome of these studies is the novel observation that chemical cross-linking agents like formaldehyde can be used to covalently trap PAR-protein complexes. This result immediately leads us to speculate that in the near future in vivo PAR-protein complexes may be purified from cells using techniques akin to ChIP assays. Also, the observation of formaldehyde cross-linking provides us some clues about the currently unknown molecular details of protein PAR interactions. In other nucleic acids formaldehyde reacts with the highest

frequency at the exocyclic amines of the nucleobases.(46,47) This makes the amino groups at the 6 position of the adenines in PAR excellent candidates for reaction with formaldehyde. In proteins, there are several amino acids which will react with formaldehyde, but the basic residues Lys and Arg appear to be more reactive than most others.(48) In the peptide sequences of Figure 4, we note that there is an abundance of Arg residues; in fact every positive PAR-binding sequence has at least one Arg in both the N-terminal and C-terminal regions. Therefore, we speculate that formaldehyde is creating cross-links between the Arg residues in the peptides or proteins and the 6-amino group of adenine residues in PAR. This contention is further supported by Seeman's work(86) which showed that a favorable interaction between nucleobases and amino acids is hydrogen bond formation between the guanidinium side chain of Arg and the N7 and 6-$NH_2$ groups of adenine. Furthermore, this specific type of molecular interaction would help explain another experimental observation—even though PAR-binding peptide sequences are rich in basic residues, PAR-peptide complexes are not stabilized solely by electrostatic forces, and other intermolecular forces must be contributing. Obviously, an NMR or X-ray crystal structure is needed to further characterize the molecular contacts responsible for the binding of select amino acid sequences to poly(ADP-ribose).

This work has added new members to the growing list of in vivo PAR-binding protein candidates. In particular, these glycosylases join other proteins involved in BER on the list of PAR-binding proteins, including XRCC-1 and DNA Ligase III.(53) These latter proteins are involved in the late stages of BER, while

the glycosylases studied here are involved in the initial damage recognition and base excision steps. Therefore, PARP-1, which has been shown to associate with the BER machinery, and its product PAR may regulate the dynamics of BER in the nucleus. As a first model for the role of PAR in BER, the ability of BER enzymes to bind to PAR may play a role in localizing the repair machinery to sites of DNA damage. The envisioned steps in this process would include: 1) DNA damage initiation and the related dramatic increase in PARP-1 enzymatic activity (40), 2) binding of BER enzymes to this newly synthesized PAR via their PAR-binding motifs, thus localizing the repair machinery near sites of genomic damage, and 3) release of the BER enzymes by the degradation of PAR by PARG. (17,87) As a second model for the role of glycosylase-PAR binding in BER regulation, we hypothesize that these interactions may help protect the genome by limiting the number of single strand breaks which occur after oxidative stress by stalling the glycosylases from acting on damaged DNA substrates. The combined activity of these glycosylases and APE1 is to generate single strand breaks, the preferred binding substrate of PARP-1 and the type of DNA lesion which leads to highest PARP-1 activity in vitro. (88) If the genome incurs massive single strand breakage due to stress, the PAR generated by PARP-1 could provide a scaffolding to temporarily hold the glycosylase proteins and allow the latter stages of BER to progress so that the number of single stand breaks can be limited. Clearly, there is much still to be learned about the roles of PARsylation in cellular processes, especially DNA repair. The

research presented in this paper, again, has hopefully laid the groundwork for the cellular investigation to be built from.

## CHAPTER THREE

## CONCLUSIONS AND FUTURE DIRECTIONS

*Follow up to the glycosylase study of Chapter 2-* Since this study only tested the PAR binding ability of 3 out of the 5 glycosylases with a PAR-binding motif, it would be of immediate interest to perform assays on the two remaining glycosylases, MPG and NTH1, to see if the native proteins are able to bind to PAR. A second set of experiments could focus on testing the 4 glycosylases not found to have PAR binding motifs according to our search criteria, namely NEIL2, SMUG1, TDG, and UDG. While these glycosylases did not possess a sequence homologous to H3(52-73), they may still bind to PAR. If they do possess a PAR motif, our homology search may have missed them, and a protein pattern search, such as the one developed by Pleschke et al.(53) may be more successful.

Once the entire BER glycosylase family has been tested, the next step should be to verify that the PAR binding peptide regions we have identified are truly responsible for PAR-glycosylase binding in the full length proteins. This could be accomplished in a couple of different ways. The most traditional method would be to mutate residues in the PAR binding motif of each protein and then evaluate the potential for PAR binding, as was done with alanine scanning in the case of the MARCKS peptides.(51) These experiments are feasible for virtually all human glycosylases since they are routinely overexpressed in *E. coli*. Furthermore, it is feasible to generate catalytically inactive mutants of these proteins using techniques such as Quikchange Site Directed Mutagenesis (Stratagene), so generation of PAR binding sequence mutants should be

possible. We would initiate sit-specific mutagenesis of glycosylases with PAR-binding domains is in a disordered region, such as NEIL1 and NTH1, since these mutations would not expected to interfere with protein folding or stability.

A second, complementary approach would be to use formaldehyde crosslinking of the PAR – glycosylase complex to identify the protein region in contact with PAR. The following protocol could be developed to accomplish this goal. First, PAR-glycosylase complexes would be crosslinked by formaldehyde treatment. After the quenching of formaldehyde, protease digestion could be carried out to generate peptides bound to PAR, and other protein fragments free in solution. At this point, the peptides could be analyzed through two methodologies. 1) SDS-PAGE analysis would be performed on PAR-bound proteins digested by proteases; the peptides derived from the protease digestion would be compared with digested glycosylases not bound to PAR. The unique peptides could be analyzed through amino-terminal sequencing or tandem MS-MS to identify the specific sequences. 2) After proteolysis, the protease digestion mixture could be coupled to a boronate chromatography step to isolate only the peptides that are crosslinked to PAR. After elution from the column the formaldehyde crosslinks could be reversed by hydrolysis at 65°C, followed by peptide sequenced using the MS techniques described above.

*Methods for elucidating PAR-Protein association in cells –* Even though PAR binding by nuclear proteins has been established in vitro, to date there is no evidence for these interactions in the cell. A major reason for this arises from a

lack of experimental methods which would allow researchers to selectively isolate protein-PAR complexes away from the higher abundance DNA-protein and even RNA-protein complexes. Our discovery that non-covalent PAR-protein complexes can be chemically crosslinked using formaldehyde is envisioned as a first step in overcoming these limitations. Formaldehyde crosslinking is established in probing the cellular context for protein-protein interactions as well as protein-DNA binding. (89,90) As a zero-order crosslinking agent, formaldehyde will only bind two compounds in direct contact with each other as shown through studies that showed that no crossliking will occur between two non-binding compounds BSA and the lac operon even if the concentration of one is greatly increased. (74)

Even though formaldehyde crosslinking of any PAR-protein complexes in vivo is feasible, a methodology which would allow selective isolation of these complexes away from other nucleic acid-protein complexes will be needed. As an initial method, boronate chromatography would be optimal in our opinion. First, this technique has been used to isolate specific nucleic acids and carbohydrates (91) since the boronate moiety selectively binds to sugars with cis-diol groups. The two cis-diol groups of each ADP-ribose monomer in PAR makes this nucleic acid bind strongly to this chromatographic substrate. In fact, this chromatography step has been used to isolate PAR polymers from both in vitro synthesis reactions and from cellular lysates.(21,68)

Coupling both formaldehyde crosslinking and boronate chromatography should allow proteins that are crosslinked to PAR in cells to be isolated from the

rest of the cellular milieu. Once the PAR polymers are removed from the proteins in the boronate elution, via alkaline lysis, the proteins can be identified by gel electrophoresis coupled with either Western blotting or Mass Spectrometry techniques to specifically identify the proteins.

*Does BER glycosylase-PAR binding lead to the formation of a repairosome? -*

This work has shown that BER glycosylases can bind to PAR through two different in vitro analyses. The functional application of this work is not clear. However, two potential models for how these findings fit into the cellular context have been proposed at the end of the last chapter. The elucidation of this functionality will come from future work using the tools developed here employing formaldehyde crosslinking of PAR-binding proteins and PAR coupled to the specific targeting of PAR polymers by boronate chromatography. Without the cell-based results, speculation about PAR and glycoslyase protein binding can be made using previous results of protein-protein interactions in the establishment of a BER repairosome.

Previous work with XRCC1 has shown that it interacts with several BER proteins through its BRCT domain. (48) Since XRCC1 does not have enzymatic activity, its interactions with DNA polymerase β, DNA ligaseIII, apurinic/apyrimidinic endonulcease 1 (APE1), and PARP-1 is thought to function as a scaffolding to perhaps assemble these players into a repairosome. (92-95) Support for this claim comes from looking at the binding to PAR and XRCC1 as a sequential process.

PARP-1 has been shown to immediately localize near areas of DNA damage caused by focused ionizing radiation. (96,97) Once PARP-1 is turned on at this area and PAR is created, XRCC1 can bind along with other BER proteins. (53) The PAR polymer will be degraded rapidly (87,98), and then the proteins are localized facilitating the repairosome formation through interactions with XRCC1. Along with interactions between XRCC1 and BER proteins, other BER proteins have been shown to have protein-protein contacts. NEIL1 and DNA polβ have been found to have direct protein-protein contact. (99) Future work to establish how many of these protein-protein contacts can be made simultaneously will help further elucidate if a repairosome is in fact formed near areas of DNA damage.

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**Figure 1.** The structure of PARP-1.

This schematic shows the modular structure of PARP-1. The amino-terminus is the DNA binding domain (DBD) containing two zinc-fingers (F1 and F2) and the nuclear localization sequence (NLS). The recently found third zinc finger (F3) is shown just after the the NLS. The central region contains the BRCT domain (breast cancer C-terminal) involved in protein-protein interactions. The automodification domain (AMD) is found downstream of the BRCT domain. The carboxy-terminus of the protein contains the catalytic domain.

**Figure 1**

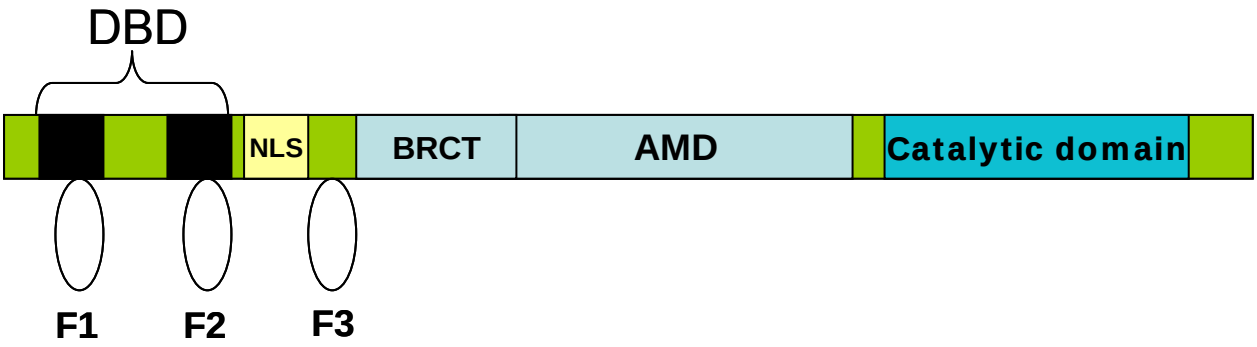


**Figure 2.** Three steps in the poly(ADP-ribose) formation.

This diagram shows the PAR polymer by displaying the three different actions of the PARP-1 protein. The initial step in the process is the attachment of the first ADP-ribose monomer on to an accepting protein (1) with the concomitant release of a nicotinamide molecule. The next step is the elongation of the PAR polymer (2). Finally, the polymer can be branched at various points (3). See text for more detailed explanation of PARP-1's activity. Image borrowed from http://www.biochemj.org/bj/388/0493/bj3880493f01.gif.

**Figure 2**

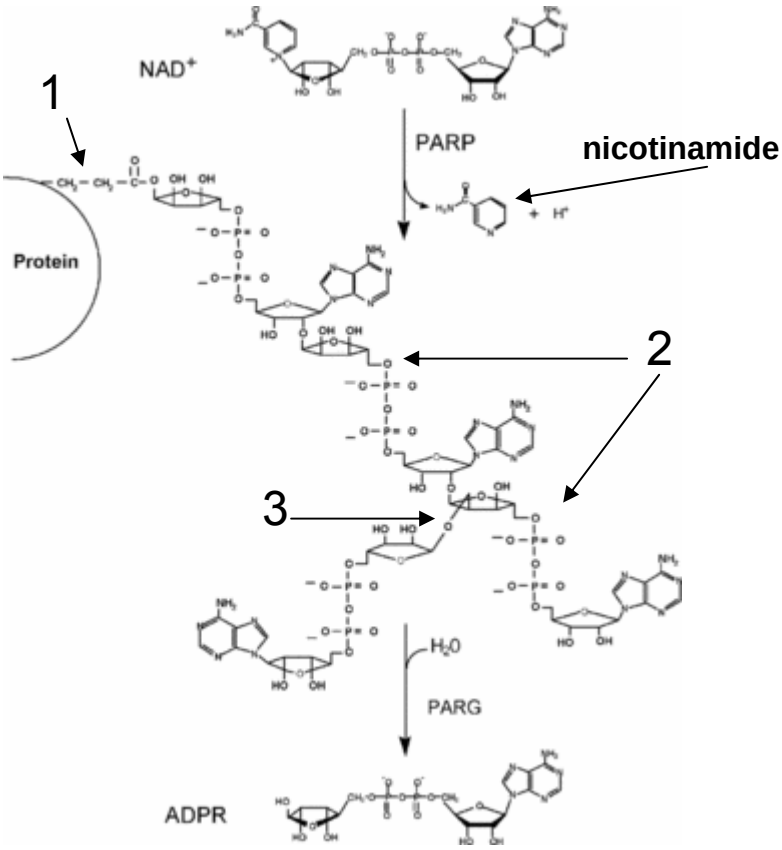


**Figure 3**. The histone shuttling mechanism.

This diagram shows the steps proposed in the histone shuttling mechanism. First, DNA strand breakage occurs either by a DNA damaging agent or by enzymatic activity followed by PARP-1's binding at the strand break via its zinc-finger motif(1). Next, the PARP-1 dimerizes and its enzymatic activity is turned on (2). The PAR polymer is synthesized and attached to histones in the nucleosome core particle (NCP - blue cylinders) (3). Once the PAR polymer is synthesized, the histones of the NCP lose their affinity for DNA and the nucleosome dissociates from the DNA (4). PARG breaks down the PAR polymer into monomers of ADP-ribose (5). Once the PAR is digested, the NCP can reassemble and return to its position in the chromatin fiber (6).

**Figure 3**

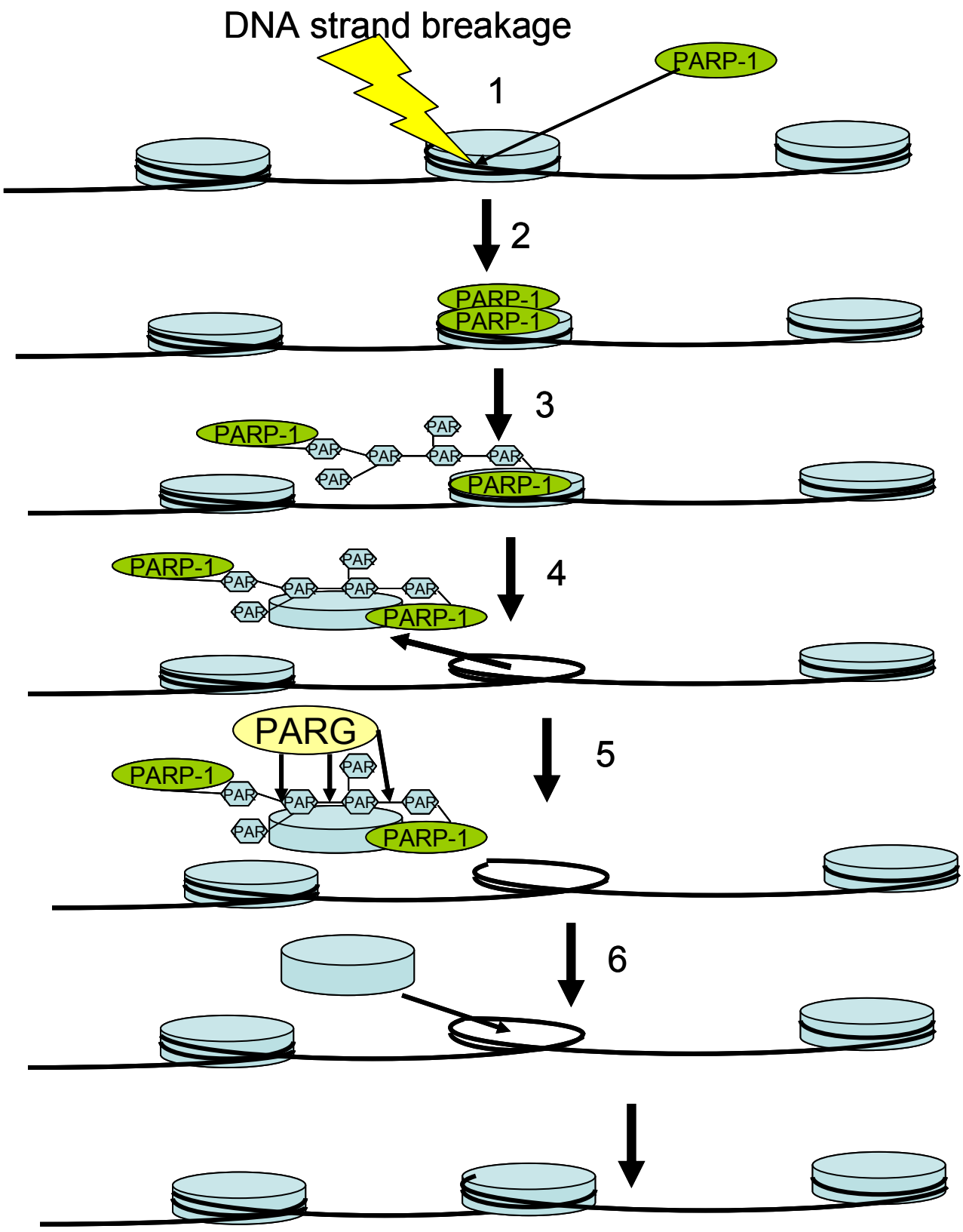

DNA strand breakage
PARP-1
1
2
PARP-1
PARP-1
3
PARP-1
PAR
PARP-1
4
PARP-1
PARP-1
PARG
5
PARP-1
PARP-1
6

**Figure 4.** Alignment of the primary sequences of BER glycosylases with histone H3.

The H3 PAR binding motif was aligned with the primary sequences of the BER glycosylases OGG1, MPG, NTH, MYH, and NEIL1 using LALIGN and the Blosum62 matrix. Residues in bold fit the PAR binding motif delineated by Pleschke et al (2000). The OMP peptide was generated by replacing His 13 and Arg 14 of the OGG1 sequence and was used as a negative control as described in the text.

## Figure 4

| PAR binding motif | [ R / K ] | | | | | | | | | h | | b | | h | h | b | b | h | h | b |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| H3(52-73) | I | **R** | **R** | Y | Q | **K** | S | T | E | L | L | **I** | R | **K** | L | **P** | **F** | Q | **R** | **L** | **V** | **R** |
| OMP | G | A | A | T | L | A | S | T | P | A | L | **W** | A | S | I | **P** | C | P | **R** | S | E | L |
| OGG1(12-33) | G | H | **R** | T | L | A | S | T | P | A | L | **W** | A | S | I | **P** | C | P | **R** | S | E | L |
| MPG(268-289) | G | E | W | A | **R** | **K** | P | L | **R** | F | Y | V | R | G | S | **P** | **W** | V | S | **V** | **V** | D |
| NTH1(39-60) | L | **R** | **R** | **R** | E | A | A | A | E | A | **R** | K | S | **H** | S | **P** | **V** | **K** | **R** | **P** | R | **K** |
| MYH(402-423) | P | S | V | T | W | E | P | S | E | Q | L | Q | R | **K** | A | **L** | **L** | Q | E | **L** | Q | **R** |
| NEIL1(321–342) | P | S | **R** | T | **R** | **R** | A | **K** | **R** | D | L | **P** | K | **R** | T | **A** | **T** | Q | **R** | **P** | E | G |

**Figure 5.** Slot Blot analysis using glycosylase peptides.

Each position contains 500 ng of peptide. Positions: 1 = NTH1(39-60); 2 = NEIL1(321-342); 3 = MYH(402-423); 4 = MPG(268-289); 5 = OGG1(12-33); 6 = OMP (negative control peptide); 7 = H3(52-73) (positive control peptide). Panel A shows the autoradiography of the slot blot. Following incubation with 30,000 counts of $^{32}$P-PAR and a 1 M salt stringency wash. Panel B shows the same membrane in Panel A after staining with Amido Black to verify the presence of the spotted peptides.

**Figure 5**

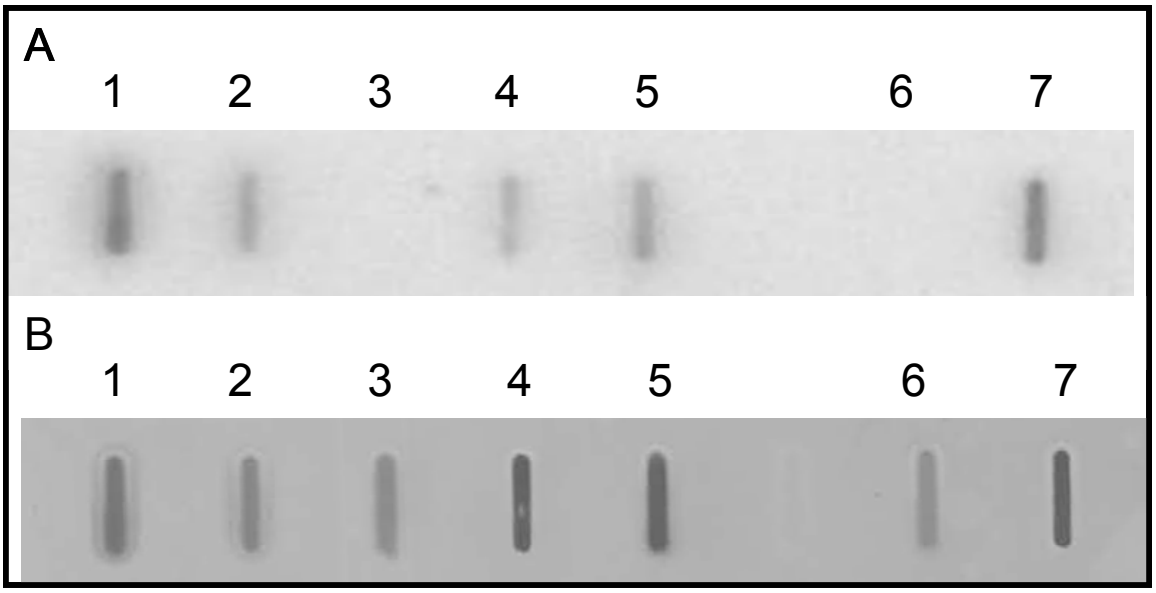


**Figure. 6**. Results of phenol partitioning assays using glycosylase peptides.

The y-axis indicates the amount of $^{32}$P-PAR carried over into the organic phase of a phenol-chloroform extraction of $^{32}$P-PAR – peptide mixtures. The reactions were carried out using 500 ng of each peptide mixed with 1,000 counts of $^{32}$P-PAR. The background signal from the $^{32}$P-PAR without added peptide is shown as the bar labeled “PAR”. H3(52-73) (positive control), OGG1(12-33), MPG(268-289), NEIL1(321-342), and NTH1(39-60) all carry PAR over to the organic phase, whereas the OMP negative control and MYH(402-423) are indistinguishable from background (PAR). Error bars represent the standard deviation as calculated from 3 independent experiments (*, ** represent levels that are statistically similar in value from one-way ANOVA analysis).

**Figure 6**

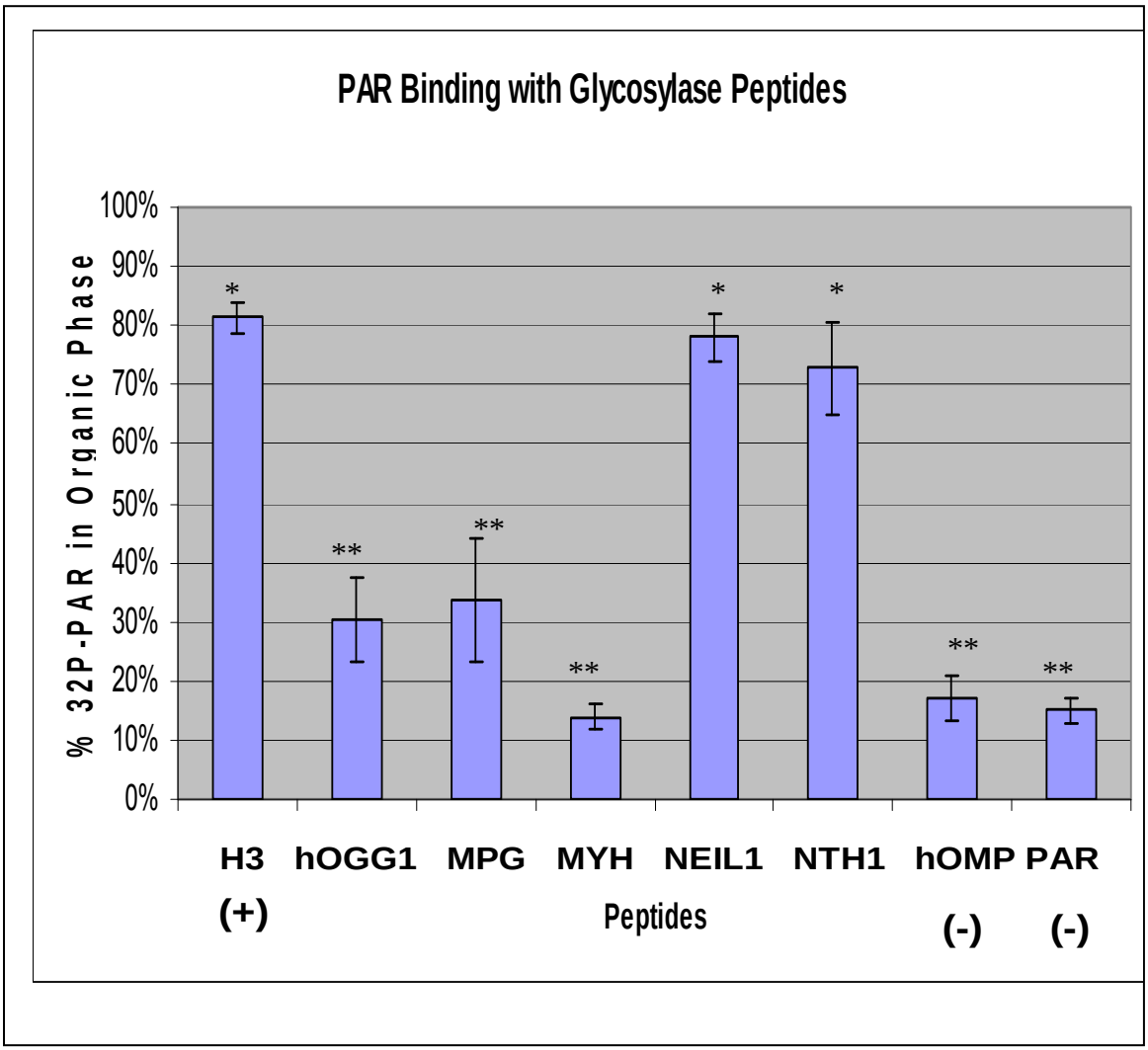

PAR Binding with Glycosylase Peptides
% 32P-PAR in Organic Phase
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
*
**
**
**
*
*
**
**
H3 (+)
hOGG1
MPG
MYH
NEIL1
NTH1
hOMP (-)
PAR (-)
Peptides

**Figure 7.** Slot Blot analysis performed on full length proteins.

Postions: 1 = histone H3 (positive control); 2 = BSA (negative control); 3 = OGG1; 4 = NEIL1; 5 = mMYH. Panel A is the autoradiography showing binding of 32P-PAR to H3, OGG1, NEIL1, and mMYH. 5 µg of each protein was spotted in each lane and the membrane was incubated with 30,000 counts of $^{32}$P-PAR. Panel B shows detection of all proteins on the membrane using Amido Black staining.

**Figure 7**

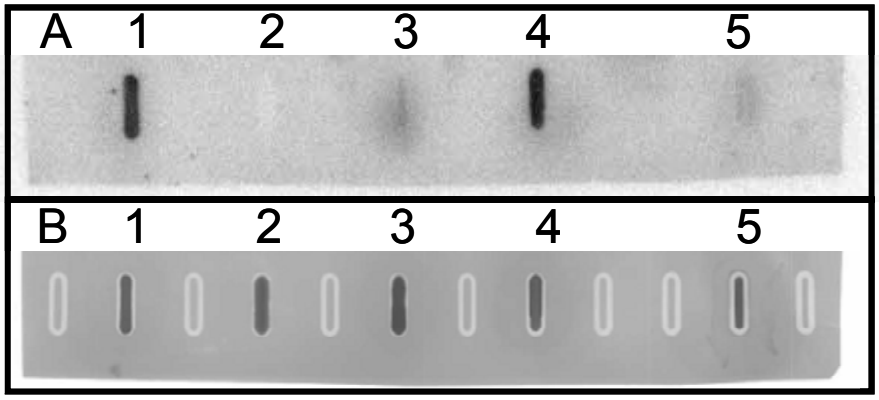


**Figure 8.** Results of phenol partitioning experiments performed with full-length proteins.

The y-axis shows the amount of $^{32}$P-PAR bound by the glycosylase and control proteins after phenol extraction of a reaction mixture containing 5 µg of protein and 1,000 counts of $^{32}$P-PAR. Both OGG1 and NEIL1 bind PAR with similar affinity as Histone H3 (positive control). Conversely, mMYH is similar to both BSA (acidic negative control) and Lysozyme (basic negative control). Error bars represent the standard deviation from three independent experiments (*, ** represent levels that are statistically similar in value from one-way ANOVA analysis)..

**Figure 8**

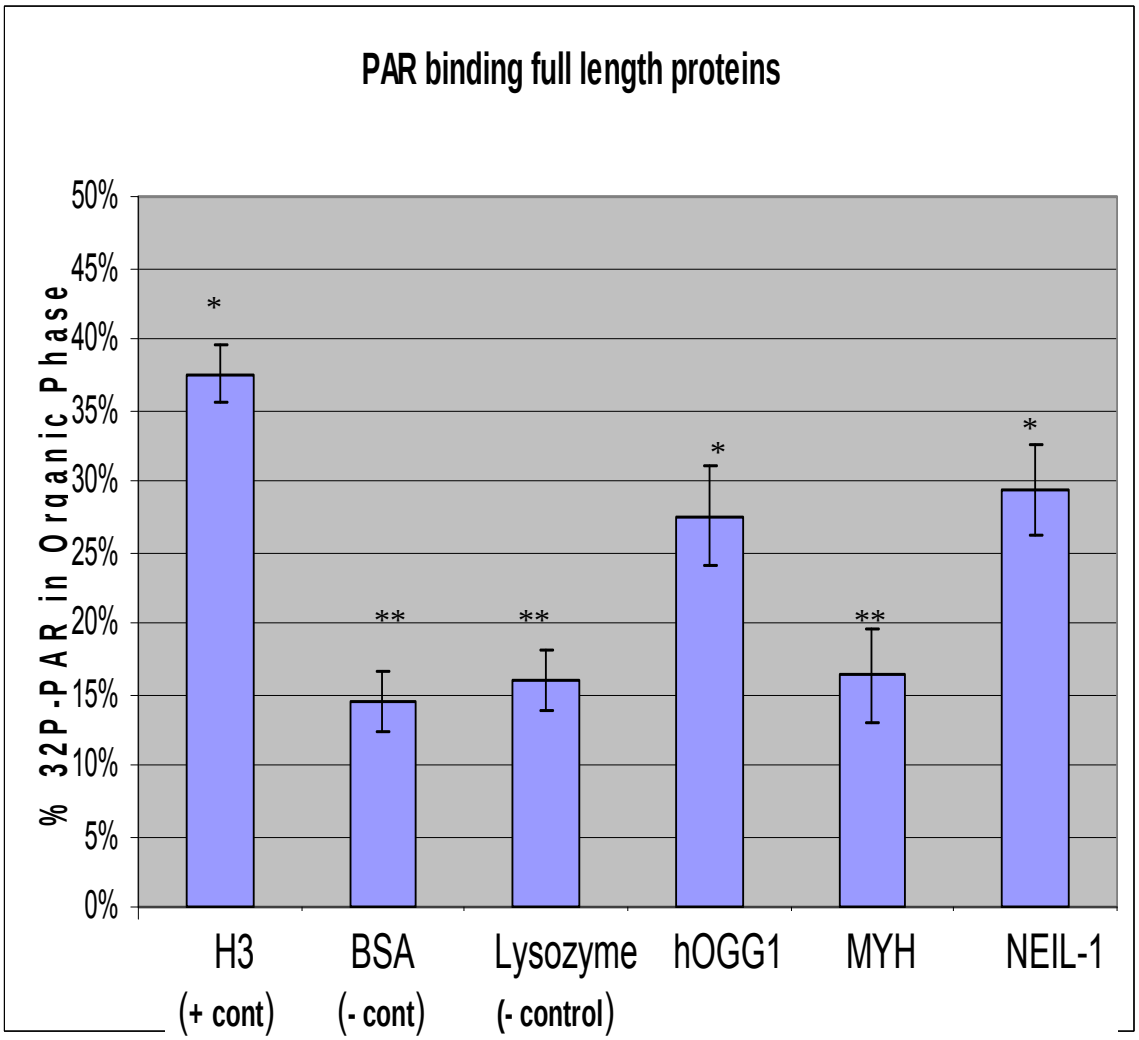

PAR binding full length proteins
% 32P-PAR in Organic Phase
50%
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
*
**
**
*
**
*
H3
(+ cont)
BSA
(- cont)
Lysozyme
(- control)
hOGG1
MYH
NEIL-1

**Figure. 9.** Formaldehyde can be used to crosslink 32P-PAR to peptides.

Positions: 1 = H3 (52-73); 2 = OGG1(12-33); 3 = MPG(268-289); 4 = MYH(402-423); 5 = NEIL1(321-342); 6 = NTH1(39-60; 7 = OMP; 8 = PAR. Panel A is the phenol partitioning of all peptides, reproduced from Figure 1. Each reaction was carried out with 500 ng of peptide and 1,000 counts of $^{32}$P-PAR. Panel B demonstrates that the addition of 2% SDS nearly disrupts all PAR-peptide binding. Panel C displays the results when PAR-peptide complexes are first treated with 0.01% formaldehyde, followed by 2% SDS. Error bars represent the standard deviation of three independent experiments(*, **, a, b, c represent levels that are statistically similar in value from one-way ANOVA analysis)..

**Figure 9**

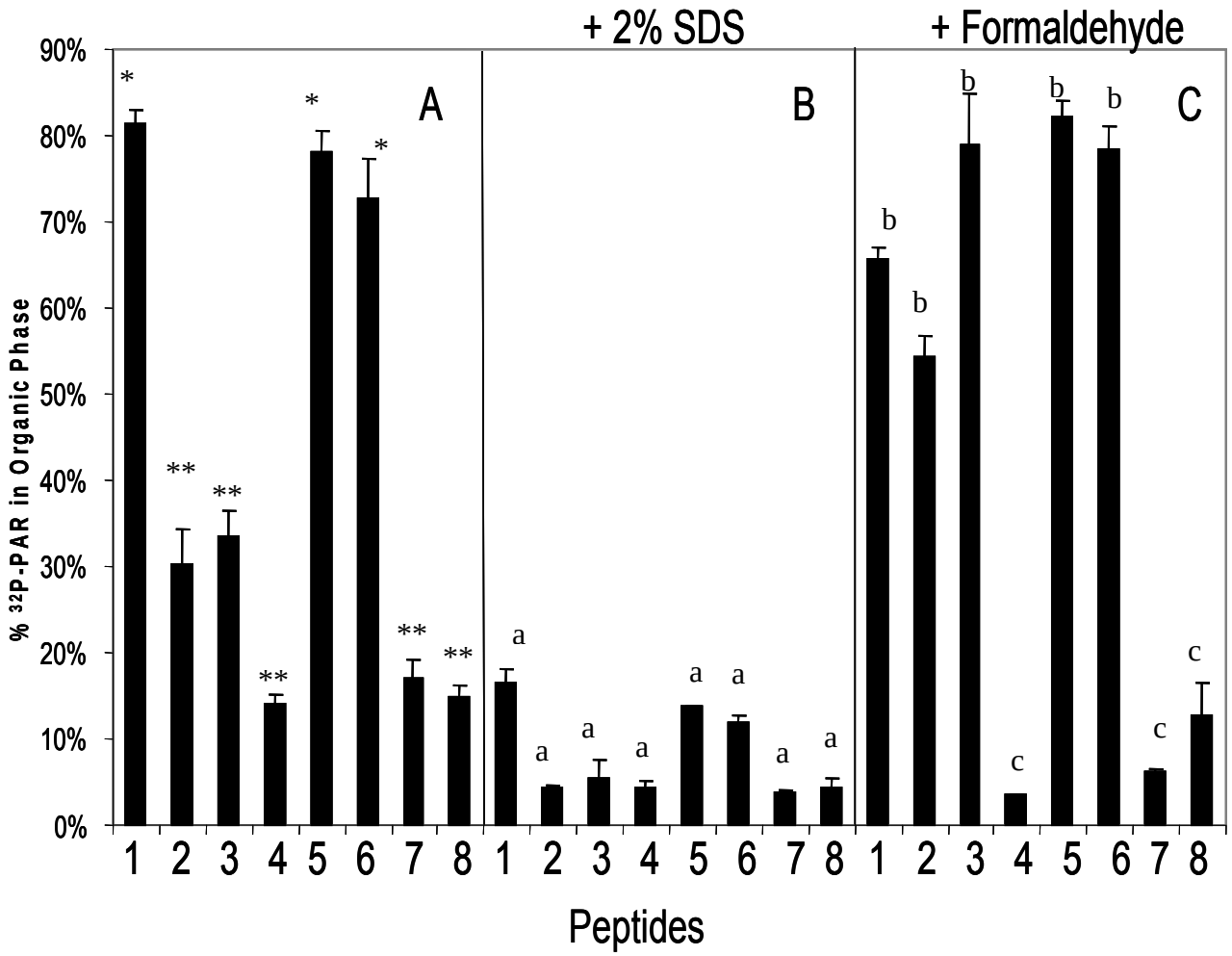
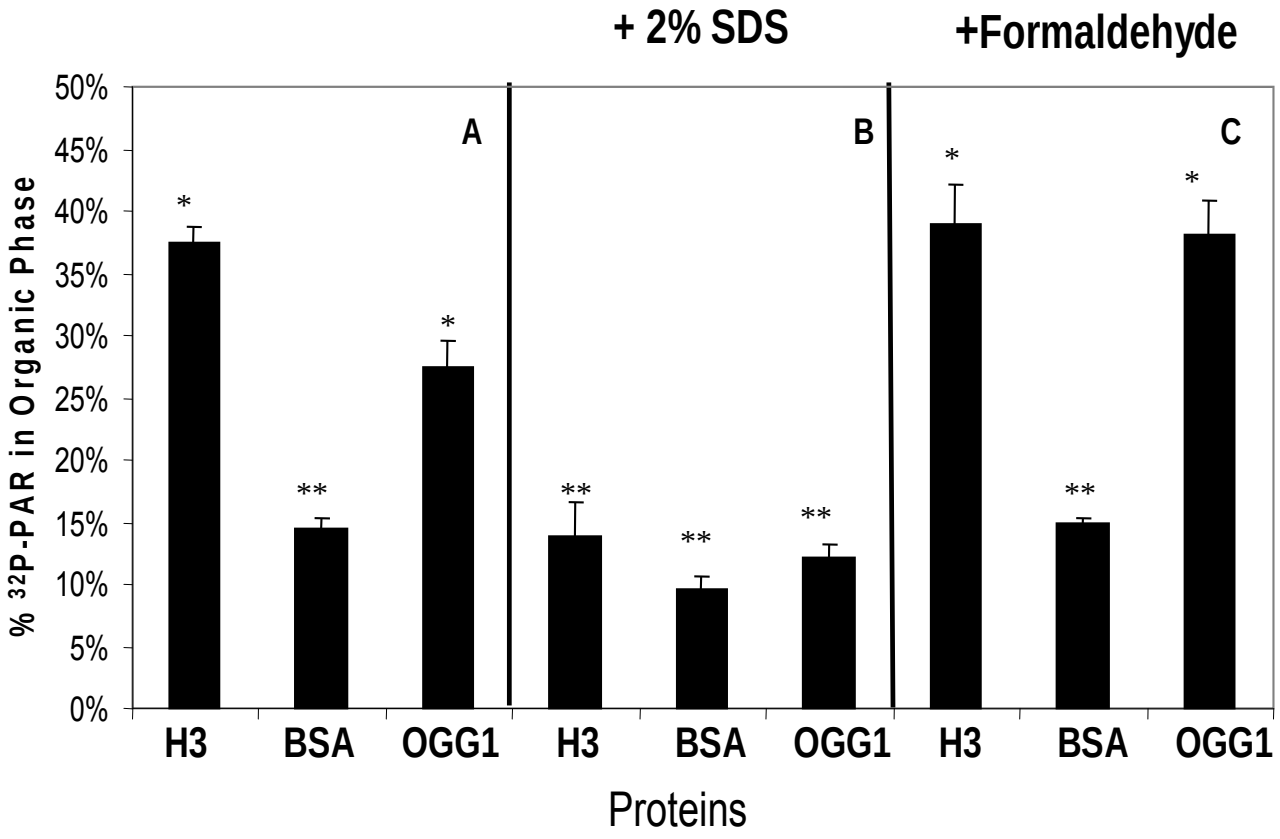

+ 2% SDS
+ Formaldehyde
A
B
C
% 32P-PAR in Organic Phase
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8
Peptides

**Figure. 10.** Formaldehyde crosslinking of $^{32}$P-PAR to histone H3 and OGG1.

The y-axis indicates the amount of $^{32}$P-PAR carried over into the organic phase of a phenol-chloroform extraction of $^{32}$P-PAR (1,000 counts) – protein (5 µg) mixtures. Panel A shows the phenol partitioning of histone H3, BSA, and OGG1. Panel B shows that the PAR binding of both histone H3 and OGG1 can be disrupted by treating with 2% SDS before extraction. Panel C shows that H3 and OGG1 can be crosslinked to PAR by formaldehyde, while mixtures containing the non-PAR binding protein BSA are not affected. Error bars represent the standard deviation from three independent reactions(*, ** represent levels that are statistically similar in value from one-way ANOVA analysis)..

**Figure 10**


+ 2% SDS
+Formaldehyde
% 32P-PAR in Organic Phase
50%
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
A
B
C
*
**
H3
BSA
OGG1
Proteins

## LIST OF ABBREVIATIONS

PTM – post-translational modification

PARP – poly(Adenosine Diphosphate Ribose) polymerase

PAR – poly(Adenosine Diphosphate Ribose)

BER – Base Excision Repair

TBST – Tris Buffered Saline with Tween 20

AAEG9 – Acetic Acid, EDTA, Guanidinium- HCL, pH 9.0

OGG1 - human 8-oxo-guanine glycosylase 1

NTH - human endonuclease III

NEIL1 - human endonuclease1

MPG - human methylpurine glycosylase

MYH - human mutY homolog

SMUG1 - single-strand selective monofunctional uracil DNA glycosylase

UDG – uracil DNA glycoslase

TDG – thymine DNA glycosylase

APE-1 - apurinic/apyrimidinic endonulcease 1

OMP – 8-oxoguanine glycosylase mutant peptide

$NAD^+$ - nicotinamide adenine dinucleotide

NLS – nuclear localization signal

DBD – DNA binding domain

BRCT – breast cancer c-terminal associated

AMD – automodification domain

PARG – poly(ADP-ribose) glycohydrolase

MNNG - N-methyl-N'-nitro-N-nitrosoguanidine

ATP – adenosine triphosphate

XRCC1 – x-ray cross complementation protein 1

SDS – sodium dodecyl sulfate

MARCKS - myristoylated alanine-rich C kinase substrate

APLF - aprataxin PNK-like factor

CHFR - checkpoint protein with FHA and RING domains

Pol β – DNA polymerase β

Pol ε – DNA polymerase ε

EDTA - ethylene diamine tetraacetic acid

DHBB – dihydroxy boronate BioRad

ANOVA – analysis of variation

MS – mass spectrometry