Chapter 2

Mono-Ubiquitination of Nuclear Annexin A1 and Mutagenesis

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1. Introduction

Annexin A1, a protein previously termed as lipomodulin and lipocortin, is a member of the protein family that binds to phospholipids in a Ca^{2+} dependent manner (Hirata, 1998; Gerke & Moss, 2002; Lim & Pervaiz, 2007). This protein was first discovered as a phospholipase A_2 inhibitory protein, and from its chemical nature was thought to be closely associated with membrane functions such as membrane organization, trafficking and metabolism (Hirata, 1998; Gerke & Moss, 2002; Lim & Pervaiz, 2007). On the other hand, annexin A1 is a major substrate of oncogenic kinases such as c-met and c-src, and is thus, proposed to be involved in signal transduction of growth factors and mitogens (Hirata et al., 1984; Skouteris & Schröder, 1996). Therefore, this protein is thought to have some regulatory roles in cancer development. Indeed, certain types of cancers such as hepatoma and pancreas cancers have higher levels of annexin A1 (Lim & Pervaiz, 2007). However, transfection of cDNA encoding annexin A1 often results in apoptosis of cells or interference of cell proliferation, consistent with tumor suppressing functions (Debret et al., 2003; Hsiang et al., 2006). In keeping with this interpretation, some types of cancers such as esophageal carcinoma and prostate cancer have decreased levels of annexin A1 (Lim & Pervaiz, 2007). However, recent pathohistochemical evidence with esophageal carcinoma and neck squamous carcinoma suggests that such down-regulation of annexin A1 is partially attributed to nuclear translocation, and the nuclear translocation of annexin A1 is facilitated by tyrosine and/or serine phosphorylation and Ca^{2+} signals as well as by oxidative stress (Rhee et al., 2000; Kim et al., 2003; Liu et al., 2003; Cui et al., 2007; Lin et al., 2008). The presence of annexin A1 in nuclei is now proposed to be a poor prognostic marker of squamous cancer or to be associated with malignancy of gastric carcinoma, while changes in cellular expression of annexin A1 may not be involved in tumorigenesis (Lin et al., 2008; Zhu et al., 2010). Therefore, nuclear annexin A1 is thought to play an important role in cell proliferation and/or cell transformation. Since this protein is reported to reside on DNA synthesomes

within nuclei (Lin et al., 1997), it is likely that nuclear annexin A1 is involved in DNA replication, especially DNA damage induced gene mutation, since DNA damage induced mutagenesis plays an important role in tumorigenesis.

Mutagenesis is largely the outcome of insults to DNA by environmental agents including alkylating agents and by endogenous metabolic oxidative metabolites such as reactive oxygen, and plays an important role in initiation, progression, and ultimately formation of cancer (Wang, 2001). Heavy metals such as As^{3+} are known to promote the mutagenic action of another DNA damaging agent including reactive oxygen, while they alone are weakly- or non-mutagenic (Sekowski et al., 1997; Maier et al., 2002). Such promotion by heavy metals is attributed not only to inhibition of DNA repair systems such as mismatch repair but also to relaxation of the semi-conservative replication machinery for translesion DNA synthesis that bypasses sites of damage (Calsou et al., 1996; Jin et al., 2003). Translesion DNA synthesis is catalyzed by error-prone DNA polymerases, and exchange of DNA polymerases is promoted by ubiquitination of nuclear proteins such as proliferating cell nuclear antigen (PCNA) (Ulrich, 2005). Translesion DNA synthesis is thought to be the major cause of mutagenesis rather than incorrect repair of damaged DNA (Kunz et al., 2000). Accordingly, we have investigated how annexin A1 in nuclei stimulates DNA damage-induced mutagenesis.

2. Modifications of annexin A1 in nuclei with ubiquitin and ubiquitin-like proteins

Ubiquitin and ubiquitin-like modification systems are related pathways that covalently attach a protein modifier to a lysine residue of a target protein. Ubiquitin classically marks proteins for proteosomal destruction typically when polymeric chains (longer than four ubiquitin subunits) assemble *via* ubiquitin-ubiquitin isopeptide-linkages (Gill, 2004; Chen & Sun, 2009). However, other functions of ubiquitin have been recently discovered that do not involve the proteosome (Hicke, 2001; Chen & Sun, 2009). The small ubiquitin-related modifier, SUMO, post-translationally modifies many proteins with diverse functions including regulation of transcription, chromatin structure and DNA repair, and facilitates their nuclear translocation (Gill, 2004). In addition, repair of and tolerance to DNA damage are regulated by modifications with ubiquitin and SUMO (Ulrich, 2005), and their modifications are shown to have antagonistic effects on functions of the target proteins (Hilgarth et al., 2004; Huang & D'Andrea, 2006).

Since annexin A1 contains the consensus sequence, ψKxE/D, for SUMOylation, purified bovine annexin A1 was incubated with human recombinant Ubc9 (E2) and SAE I/SAE II (E1) in the presence of SUMO 1, 2 or 3 to test whether annexin A1 can be SUMOylated. When the reaction mixtures were analyzed by Western blots, monospecific anti-SUMO antibodies stained two proteins with apparent molecular weights around 38,000 and 34,000 Da (F. Hirata et al., 2010). Anti-annexin A1 antibody detected a broad single protein band with an apparent molecular weight around 37,000 Da but not a protein band with a molecular weight of 34,000 Da. The protein band with a molecular weight of 34,000 was identified,

using anti-Ubc9 antibody as SUMOylated Ubc9 (F. Hirata et al., 2010). With SUMO 2 or 3 as a substrate, SUMOylation of annexin A1 was apparently facilitated. Rates of annexin A1 SUMOylation with SUMO 1, 2 or 3 were approximately 1:3:5, providing that the amounts of SUMOylated Ubc9 formed with SUMO 1, 2, or 3 were essentially the same. Ca^{2+} was required for the maximal modification, and increased SUMOylation by 2.6 fold *vs*. without Ca^{2+}. Therefore, we concluded that annexin A1 was conjugated with SUMOs under these conditions. While the SUMOylation barely altered the molecular weight of bovine annexin A1 (37,000 Da) as detected by anti-annexin A1 antibody, the protein band with an apparent molecular weight around 38,000 Da could not be detected by anti-SUMO antibody in the absence of E1 or E2 for SUMOylation or in the absence of annexin A1. To further confirm that annexin A1 is covalently modified with SUMO, the reaction mixtures in the absence and presence of annexin A1 for SUMO 3 modification were scaled up by 5 fold, and the incubation was continued overnight. Then, the reaction mixtures were analyzed using FLPC (Amersham Biosciences) with a MiniQ column. Native and SUMOylated annexin A1 were separately eluted as measured by conductance, suggesting that charges in native and SUMOylated annexin A1 are distinct. To establish this contention, 2D electrophoresis was performed. Native annexin A1 was detected at pI 6.4. The reaction mixture containing SUMOylated annexin A1 showed a new annexin A1 location in the pI 6.1 area as detected by anti-annexin A1 antibody, while its mobility on SDS gel electrophoresis was barely shifted. This new protein with pI 6.1 was stained with anti-SUMO antibody as well as with anti-annexin antibody, and its density increased, when the incubation was prolonged. Omission of SUMO or ATP from the reaction mixture resulted in no protein around pI 6.1. These observations supported the conclusion that annexin A1 was covalently modified with SUMO, even though no significant mobility shift on SDS electrophoresis was detected after the modification of annexin A1 with SUMOs. Since ^{160}LKRD in the annexin repeat domain II is conserved among mammalian annexin A1 proteins (Gerke & Moss, 2002), it is likely that SUMOylation takes place in the core domain II. Mutation experiments by mutating K to R will be essential for determination of the site of SUMOylation.

On the other hand, the molecular weight of annexin A1 shifted from 37,000 Da to approximately 45,000 Da after ubiquitination. The ubiquitination of annexin A1 required UbcH2A (Rad6 homologue) together with HeLa S100 lysate that contained E3 ubiquitin ligases. Since HeLa S100 lysate pretreated with anti-Rad 18 antibody did not catalyze ubiquitination of annexin A1, it is most likely that Rad18 is an E3 ligase for ubiquitination of annexin A1. Ca^{2+} was required for the maximal ubiquitination, but its stimulation was not as much as seen with SUMOylation, when amounts of ubiquitinated annexin A1 were adjusted with the total amounts of annexin A1 (free and ubiquitinated annexin A1). These observations suggest that the modification site with ubiquitin is distinct from that with SUMO. HeLa S100 lysate also contained annexin A1 as detected by anti-annexin A1 antibody, but under the present experimental conditions, no significant ubiquitination of endogenous annexin A1 was detected in the presence of ubiquitin and an ATP generating system without UbcH2A. UbcH2A could be equally replaced by its related enzyme, UbcH2B but not by E2-25K. These observations suggest but do not necessarily prove that annexin A1

is ubiquitinated by the Rad6–Rad18 system which is closely associated with response to DNA damage (Kunz et al., 2000; Ulrich, 2005). The difference in stimulation of SUMO and ubiquitin conjugation by Ca^{2+} is apparently attributed to sites of modification and Ca^{2+} induced conformational changes, in which the N-terminal domain is exposed and flexibility of the core domain residues are increased by Ca^{2+} (Shesham et al., 2008). Therefore, we suggest that SUMOylation takes place in the core domain regions, while ubiquitination takes place outside the core domain regions.

3. DNA damage and modification of annexin A1

Among post-translational modification systems, ubiquitin and ubiquitin-like molecules including SUMO are a unique family of protein modifiers that play pivotal roles in regulation of protein stability and function (Hicke, 2001; Gill, 2004; Hilgarth et al., 2004; Huang & D'Andrea, 2006; Chen & Sun, 2009). SUMOylation is involved in protein stabilization, nucleo-cytoplasmic trafficking, cell cycle regulation, maintenance of genome integrity and transcription. Indeed, annexin A1 present in nuclei is mostly modified with SUMO or ubiquitin (F. Hirata et al., 2010). SUMO 1-modified annexin A1 resulted in enhanced helicase activity, while SUMO2/3 were the better substrates for *in vitro* annexin A1 conjugation, suggesting that SUMOylated annexin A1 might be involved in cell proliferation and differentiation (Yang & Paschen, 2009). On the other hand, mono-ubiquitination is thought to enable the modified proteins to interact and form complexes with other proteins via ubiquitin binding proteins and ubiquitin receptors. Mono-ubiquitination changes subcellular localization and alters certain structural and targeting properties, while polyubiquitination targets proteins for the degradation pathway (Huang & D'Andrea, 2006). Mono-ubiquitination by the Rad6-Rad18 system is proposed to play an important role in DNA damage response (Ulrich, 2005).

To ask whether mono-ubiquitination of annexin A1 is, indeed, involved in DNA damage response, we investigated if nuclear annexin A1 is modified with SUMO or ubiquitin in mouse L5178Y *tk*(+/-) lymphoma cells treated with DNA damaging agents, 15 μM MMS or 3 μM $AsCl_3$ for 3 or 6 hr. Under these conditions, the mutation rate of the thymidine kinase gene increased from 23 x 10^{-6} (vehicle control) to 67 x 10^{-6} and 104 x 10^{-6} with $AsCl_3$ or MMS, respectively, as measured by the number of colonies with trifluorothymidine resistance according to the method described by Honma et al. (1999). These observations suggest that DNA damage was induced under these conditions. After 3 hr treatments with 15 μM MMS or 3 μM As^{3+}, nuclear and cytoplasmic extracts were isolated and were analyzed by Western blots with anti-annexin A1 antibody. Nuclear annexin A1 was increased by the treatments, while cytoplasmic annexin A1 was decreased. These observations suggest nuclear translocation of annexin A1 following DNA damage signaling. Nuclear annexin A1 exhibited apparent molecular weights around 38,000 and 45,000 Da, whereas the molecular weights of cytoplasmic annexin A1 were 37,000, 30,000 and 27,000 Da (F. Hirata et al., 2010). Annexin A1 with the molecular weights of 30,000 and 27,000 Da are reported as products of N-terminal cleavage (Kim et al., 2003; Sakaguchi et al., 2007). Such cleavage was also

detected in nuclear annexin A1. Polyubiquitination of annexin A1 has recently been shown to be catalyzed by E6AP in the presence of Ca^{2+}, and polyubiquitinated annexin A1 is degraded by proteosomes (Shimoji et al., 2009). Although poly-ubiquitination of proteins is proposed to be a major pathway of protein degradation (Hilgarth et al., 2004; Huang & D'Andrea, 2006; Wilkinson et al., 2008), poly-ubiquitinated annexin A1 was not detected in either cytosol or nuclei. The direct cleavage of annexin A1 was thought to be its major degradation pathway under our experimental conditions. The Western analysis of the extracts with anti-SUMO 1 and anti-ubiquitin antibodies suggested that majority of nuclear annexin A1 might be modified, while cytoplasmic annexin A1 was not. MMS increased ubiquitination of annexin A1 in nuclei by 2 fold, whereas these treatments decreased its SUMOylation by 70%. $AsCl_3$ alone was less effective but was more than additive, when MMS was present. Cytosolic annexin A1 was not stained with anti-SUMO 1 or anti-ubiquitin antibodies (data not shown). Accordingly, we concluded that the modifications of annexin A1 with SUMO or ubiquitin facilitate its nuclear translocation and that ubiquitinated annexin A1 is involved in DNA damage response, in which the Rad6-Rad18 system plays an important role (Ulrich, 2005).

4. DNA helicase activity of nuclear annexin A1

Annexins have a common internal structure comprising 4 or 8 repeats of a conserved 70 amino acid domain, and differ primarily in the length and composition of the amino-terminal domains (F. Hirata, 1998; Gerke and Moss, 2002; Lim and Pervaiz, 2007). Since this amino-terminal domain contains the sites for phosphorylation and glycosylation, it is considered a regulatory domain. A defining feature of annexins is their ability to bind, in a Ca^{2+}-dependent manner, to negatively charged phospholipids such as phosphatidylserine (PtdSer). This functional feature is attributed to the conserved C-terminal domain, and is essential for biological functions of the annexins. The cell-cycle dependent existence of annexin A1 and A2 in nuclei suggests a close association with nuclear functions, while they are major substrates of the oncogenic tyrosine kinases, met and src (Katoh et al., 1995; Rydal et al., 1992). Thus, annexin A1 and A2 are proposed to be a biological marker of proliferating cells (cancer cells) (Masaki et al., 1994). In accord with this notion, the treatments of A347 and HeLa cells with antisense annexin A1 and A2 oligonucleotides reduce the synthesis and subsequent phosphorylation at tyrosine of the annexins, thereby inhibiting cell proliferation (Kumble et al., 1992; Skouteris & Schröder, 1996). Translocation of annexins from cytosol to nuclei apparently requires their phosphorylation at tyrosine and Ca^{2+} signaling (Mohiti et al., 1997). Purified annexin A1 and A2 can stimulate DNA synthesis in cell free systems of HeLa cells, Xenopus oocytes and rat hepatocytes (Vishwanatha & Kumble, 1992; Vishwanatha et al., 1992; Tavokoli-Nezhad et al., 1998). In addition, annexin A1 is present in DNA synthesomes and annexin A2 is located in nuclear matrix (Kumble et al, 1992; Lin et al., 1997). These observations strongly suggest that nuclear annexins regulate DNA replication.

Annexin A2 functions in DNA replication as a primer recognition factor for Pol α (Vishwanatha & Kumble, 1993), while this protein is also reported to be an RNA binding

protein, interacting with c-myc (Fillipenko et al., 2004). The binding of annexin A2 to RNA and DNA requires Ca^{2+}. Accordingly, it was thought that DNA and RNA bind to acidic phospholipid binding sites via ionic interaction. Our laboratory has investigated details of annexin A1 binding to RNA and DNA (Hirata & Hirata, 1999; 2002). Annexin A1 purified from rat liver nuclei binds to purine clusters in RNA, while it preferentially binds to pyrimidine clusters in DNA. The size of maximal recognition for binding was around 20-25 nt. Since phospholipids, especially acidic phospholipids such a phosphatidylserine, enhanced DNA and RNA binding, the binding of annexin A1 to RNA and DNA was not due to simple ionic interactions, and the sites for binding to phospholipids and DNA/RNA were distinct. Indeed, the RNA binding site of annexin A2 was reported to be C-D helices of Domain IV (Aukrust et al., 2007), while the consensus sequence for phosphatidylserine binding is proposed to be (R/K)XXXK-(B-C helices)-(R/K)XXXXDXXS(D/E) in Domain I and II (Montaville et al., 2002).

Annexins are also reported to interact with ATP and GTP (Bandorowicsz-Pikula & Pikula, 1998), although they do not have consensus sequences for typical ATP binding sites such as Walker A. As seen with annexin A7 that forms ion channels in lipid bilayers, GTP and other nucleotides are thought to regulate Ca^{2+} gating, and/or Ca^{2+} dependent membrane trafficking such as exocytosis (Caohuy et al., 1996). Cotton fiber annexin and N-terminal deleted annexin A1 can hydrolyze GTP in the presence of Mg^{2+}, and Ca^{2+} is not required for this hydrolysis (Hyun et al., 2000; Shin & Brown, 1999). Since annexin A1 can bind not only ssDNA but also dsDNA in the presence of both Mg^{2+} and $Ca^{2+,}$ we examined effects of various DNAs on ATP hydrolysis by annexin A1 (Hirata & Hirata, 2002). dsDNA such as calf thymus DNA and annealed M13mp18 but not ssDNA stimulated ATP hydrolysis by annexin A1. When DNA was analyzed, dsDNA was unwound to form ssDNA, suggesting that annexin A1 has DNA helicase activity (Hirata & Hirata, 2002). Interestingly, its annealing reaction did not require Mg^{2+} nor ATP, but Ca^{2+} was necessary. Therefore, binding of dsDNA requires Mg^{2+}, and that of ssDNA takes place in the presence of Ca^{2+} (Fig. 1).

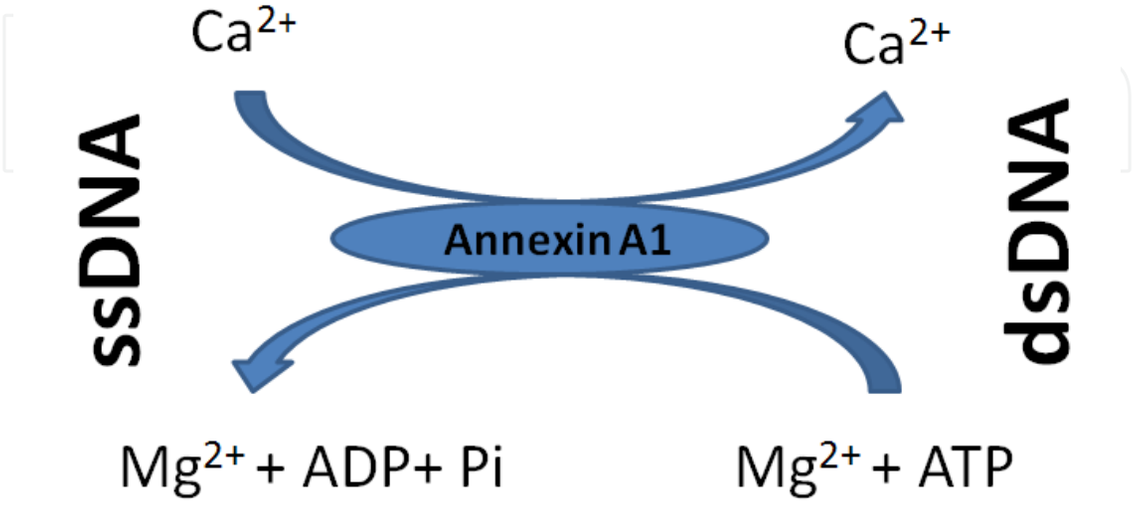

Figure 1. Helicase activity of annexin A1

5. Modification with SUMOylation and ubiquitination and annexin helicase activity

Purified rat nuclear annexin A1 had an apparent molecular weight of approximately 92,000 ± 2,000 Da, and was ubiquitinated as detected by anti-ubiquitin antibody (A. Hirata et al., 2010). Under reducing conditions, its molecular weight was approximately 45,000 ± 1,000 Da on SDS-PAGE, suggesting that high molecular weight annexin A1 is a homodimer of mono-ubiquitinated annexin A1 rather than a heterodimer complex with S100 as previously thought. Since this homodimer with an apparent molecular weight of 92,000 Da exhibited a Ca^{2+}- and Mg^{2+}-regulated helicase activity, we performed helicase assays of the reaction mixtures for ubiquitination and SUMOylation of annexin A1. The reaction mixtures containing annexin A1 modified with SUMO1 and native annexin A1 showed helicase activity as measured by unwinding of dsDNA . While purified native annexin A1 also exhibited low helicase activity, SUMOylated annexin A1 exhibited much higher activity. Since under the present conditions, approximately 10% and 20% of annexin A1 were modified in the absence and presence of Ca^{2+} with SUMO1, we calculated that SUMOylation stimulated the helicase activity of annexin A1 by approximately 3.5 fold (F. Hirata et al., 2010).

HeLa S100 lysate that was required for ubiquitination reactions contained other types of helicases beside annexin A1, and thus increased control activity. Despite this challenge, the reaction mixtures containing mono-ubiquitinated annexin A1 demonstrated clearly enhanced helicase activity (F. Hirata et al., 2010). Assuming that annexin A1 in HeLa S100 exhibits helicase activity equal to purified bovine annexin A1, mono-ubiquitination of annexin A1 results in an approximately 6-fold activation as compared with native annexin A1. Since purified rat nuclear annexin A1 shows 9 fold higher activity than native rat annexin A1 (A. Hirata et al., 2010), these observations suggest that the conjugation of annexin A1 with ubiquitin or SUMO enhances its helicase activity.

6. Heavy metals and annexin A1

Annexin A1 has 3 different types of Ca^{2+} binding sites, type II, type III and type III (AB) (Weng et al., 1993). Type II calcium binding sites have the highest affinity for Ca^{2+}, and are found only at AB loops. The coordination of the type II sites is octahedral. It consists of three peptide oxygens from the AB loops with the (K, R)-(G, R)-X-G-T sequence and bidentate ligands from the acidic groups of either an aspartate or a glutamate residue downstream in the sequence. The remaining two calcium coordinating sites show electro-density for water molecules. The calcium ions at the type III sites coordinate to two backbone carbonyl oxygens and one nearby acidic side chain. Water molecules have been found at most of the remaining three coordinating sites to complete the six-ligand octahedral coordination. The type III sites correspond to the two minor calcium sites labeled by lanthanum in annexin A5.

As seen with EF band calcium binding proteins that have type I Ca^{2+} binding sites, phospholipid aggregation experiments suggest that other divalent metals such as Pb^{2+} and Zn^{2+} can replace Ca^{2+} in annexin A1 (Mel'gunov et al., 2000). Since type III Ca^{2+} binding sites

can be labeled with La^{3+}, we tested not only Pb^{2+} and Cd^{2+} but also As^{3+} and Cr^{6+} for DNA helicase assays (A. Hirata et al., 2010). Pb^{2+} alone stimulated the DNA binding activity of purified mono-ubiquitinated nuclear annexin A1 in the absence of Mg^{2+} and Ca^{2+}. Heat denatured nuclear annexin A1 did not exert DNA binding even with these metals. Therefore, we thought that Pb^{2+} and Ca^{2+} were acting in essentially similar manners on mono-ubiquitinated annexin A1. Similar results were observed with Cd^{2+}, suggesting that these divalent metals were able to replace Ca^{2+} for DNA binding activity of annexin A1 as previously shown for its phospholipid binding activity (Mel'gunov et al., 2000). To clarify whether carcinogenic heavy metals such as As^{3+} and Cr^{6+} can promote or block the DNA binding activity of nuclear annexin A1, we tested the effects of As^{3+} on binding of nuclear annexin A1 to P0G, a 80mer polynucleotide that is complimentary to the *ori* of M13mp18 (see below). As^{3+} was synergistic with Ca^{2+} and with Mg^{2+}, yet As^{3+} alone apparently promoted the binding of annexin A1 to ssDNA. With As^{3+}, formation of the nuclear annexin A1-P0G complex increased in a concentration-dependent manner. The concentration of As^{3+} for half-maximal activation was 2.2 μM in the absence of phospholipids. Similar concentration-dependent activation was observed with Pb^{2+}, Cd^{2+}, and Cr^{6+}. Half-maximal binding in the absence of phospholipids was observed at 30, 0.1, and 2 μM for Pb^{2+}, Cd^{2+}, and Cr^{6+}, respectively. Phospholipids increased complex affinity for heavy metals by approximately 10 fold, as seen previously with Ca^{2+} for phospholipid binding (Gerke & Moss, 2002). These observations suggest that carcinogenic divalent and some multivalent heavy metal cations are able to replace Ca^{2+} in the DNA binding activity of mono-ubiquitinated annexin A1. It is noted that Cd^{2+} at concentrations higher than 5 μM caused potent inhibition, possibly due to thiol oxidation of the annexin A1 molecule.

Ca^{2+} facilitates the annealing of C_{20}-P0G to M13mp18 by nuclear annexin A1 (Hirata & Hirata, 2002). Although poly(dC)$_{20}$ was added at the 3'- or at the 5'-end of P0G as a binding site for annexin A1, which demonstrates a higher affinity for poly(dC)$_{20}$ (Hirata & Hirata, 2002), nuclear annexin A1 bound to P0G without a poly(dC)$_{20}$ tail. To test the interpretation that As^{3+} or Cr^{6+} can replace Ca^{2+}, the annealing activity of nuclear annexin A1 was measured in the presence of As^{3+} or Cr^{6+} and the absence of Ca^{2+} or Mg^{2+}. As expected, As^{3+} promoted the annealing of P0G to M13m18 by nuclear annexin A1 in a concentration-dependent manner. Half-maximal stimulation was observed at 1.4 μM $AsCl_3$. This concentration was consistent with that required for half maximal binding of ssDNA. Similar results with DNA annealing were obtained with CrO_3, $PbCl_2$ and $CdCl_2$, with concentrations for half-maximal annealing approximately 3,30 and 0.1 μM, respectively. Since heat denatured nuclear annexin A1 did not promote DNA annealing even with heavy metals under the present experimental conditions (data not shown), it was concluded that carcinogenic heavy metals As^{3+} and Cr^{6+} and divalent metals Pb^{2+} and Cd^{2+} can replace Ca^{2+} for the ssDNA binding and DNA annealing activities of nuclear annexin A1 (A. Hirata et al., 2010).

7. DNA damage and mono-ubiquitinated annexin A1

Mono-ubiquitination of nuclear proteins is mainly involved in tolerance of DNA damage, while SUMOylated nuclear proteins generally function in repair of damaged DNA (Ulrich,

2005). Therefore, we tested nuclear annexin A1 for binding to damaged DNA. We synthesized the 80mer, 5′-GTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGC-GAAAAACCGTCTATCAGGGCGATGGCCCACTACGTGAACCA-3′ (P0G), and 3 additional 80mers, each with a selected single G in position 14, 30 or 37 replaced by 8-oxo-guanosine (8-oxo-G) to model DNA damaged at a specific site by oxidation (A. Hirata et al., 2011). These damaged DNAs were designated as P14G, P30G, and P37G. We previously demonstrated that nuclear annexin A1 binds to ssDNA in a Ca^{2+}-dependent manner, and binding to dsDNA or aggregated DNA occurs in a Mg^{2+}-dependent manner (Hirata & Hirata, 1999; 2002). In the presence of 50 μM Ca^{2+}, mono-ubiquitinated annexin A1 purified from rat liver exhibited higher affinity for damaged DNA, while SUMOylated annexin A1 did not show much of difference in preference. In the presence of 50 μM Ca^{2+}, *Km* values for P30G, P37G and P14G were 0.20, 0.28, and 0.44 nM. All showed significantly higher affinity than P0G which had a *Km* value of 0.62 nM. Guanosine damaged by oxidation in the middle of the polynucleotide rather than at its ends appears to be readily tolerated by mono-ubiquitinated annexin A1.

Ca^{2+} induces annexin A1 conformational changes (Shesham et al. 2008). Since As^{3+} and Cr^{6+} also bind to the Ca^{2+} binding sites of annexin A1, we tested As^{3+} and Cr^{6+} for damaged DNA binding of mono-ubiquitinated annexin A1. The carcinogenic heavy metals, As^{3+} and Cr^{6+}, increased the affinity of nuclear annexin A1 for the oxidatively damaged DNA, P30G, but not for undamaged P0G. The *Km* value in the presence of 50 μM Ca^{2+} for G30P (0.20 nM) was significantly changed to 0.12 and 0.10 nM in the presence of 10 μM Cr^{6+} and 30 μM As^{3+}, respectively. However, maximal binding did not appear to be significantly altered (0.42 and 0.39 mol ssDNA/mol protein for G0P and G30P, respectively). Pb^{2+} and Cd^{2+} also increased affinity, but effects were much smaller. Ca^{2+} and heavy metals promote annealing of the damaged DNAs to M13mp18 by nuclear annexin A1 (Hirata & Hirata, 2002; A. Hirata et al., 2010). K_m values for the annealing reaction were essentially the same as values for the binding reaction. The affinity of nuclear annexin A1 for oxidatively damaged DNA was much higher than that for undamaged P0G and amounts of the DNA annealed with oxidatively damaged P14G were higher than with undamaged P0G. The specificity of nuclear annexin A1 for various oxidatively damaged DNAs was not substantially altered in the presence of heavy metals.

The damaged oligonucleotide-M13mp18 duplexes were also unwound in the presence of Mg^{2+} and ATP by mono-ubiquitinated annexin A1. ATP was hydrolyzed under these conditions. Unwinding velocities appeared similar for undamaged and damaged DNA. The unwinding of damaged polynucleotide-M13mp18 duplexes was inhibited by Ca^{2+} and heavy metals as reported previously (Hirata & Hirata, 2002). *Ki* values for heavy metal inhibition of the unwinding reaction were essentially the same with the *Ka* values for binding and annealing reactions. These heavy metals did not inhibit but rather stimulated dsDNA-dependent ATPase activity. Therefore, the apparent inhibition of unwinding by heavy metals most likely resulted from metal-induced increases in the annealing reaction, which in turn supplied the substrate (A. Hirata et al., 2010).

8. Translesion DNA synthesis by mono-ubiquitinated annexin A1

Helicases and DNA binding proteins are among the first proteins to encounter sites of DNA damage during transcription and DNA replication. The Werner syndrome protein enhances DNA synthesis during strand replacement of damaged DNA through its helicase activity (Harrigan et al., 2003). To test if mono-ubiquitinated annexin A1 stimulates translesion DNA synthesis, Pol β was used as an error-prone DNA polymerase that bypasses 8-oxo-guanine during DNA replication (Avkin & Livneh, 2002). DNA synthesis was measured by extension of the primer, 5′-TGGTTCACGTAG-3′ annealed to P0G or oxidatively damaged DNA oligonucleotide (P30G) templates. Mono-ubiquitinated annexin A1 and Ca^{2+} increased DNA replication by approximately 2.6 fold as measured by the synthesis of 80mer, a full size of the template, G30G. When DNA synthesis was terminated at the damaged site, the size of DNA newly synthesized should be around 50 mer. Because ATP is not required for the maximal activation, it was conceivable that mono-ubiquitinated annexin A1 promoted annealing of the primer or stabilized the ssDNA template by binding rather than promoting unwinding. Translesion DNA synthesis was greatly enhanced by mono-ubiquitinated annexin A1, when primer was added separately at low concentrations. The amount of primer required for half maximal DNA synthesis in the presence of nuclear annexin A1 and Ca^{2+} decreased significantly to 1 pmol from 5 pmol in the absence of annexin A1. These observations suggest that mono-ubiquitinated annexin A1 promoted annealing of primer to template, while this primer was not necessarily the best substrate for nuclear annexin A1 with regards to length and base composition (Hirata & Hirata, 1999). It is noteworthy that even when the primer was annealed to the template prior to the experiments, annexin A1 was able to enhance translesion DNA synthesis by Pol β, suggesting that annexin A1 stabilizes the ssDNA region of the template. Taken together, these observations suggest that mono-ubiquitinated annexin A1 acts as a primer-recognizing protein that anneals primers to templates, a ssDNA binding protein, or both (Jindal & Vishwanatha, 1990). Notably, the amount of mono-ubiquitinated annexin A1 required for maximal DNA synthesis was not a function of Pol β, indicating that annexin A1 did not directly interact with this polymerase.

Given that binding of annexin A1 to damaged DNA was dependent on heavy metals, the effects of heavy metals on translesion DNA synthesis by Pol β was examined directly. The concentrations of metals required for half-maximal activation of DNA synthesis equaled those for half-maximal ssDNA binding. With P30G, Ka values for Ca^{2+}, Pb^{2+}, Cd^{2+}, As^{3+} and Cr^{6+} were 12.5, 30, 0.1, 1.4, and 3 μM, respectively, without phosphatidylserine. These values are essentially the same as those observed with these heavy metals for helicase activity.

9. Annexin A1-dependent promotion of translesion DNA synthesis by L lymphoma nuclear extracts

L lymphoma, L5178Y *tk*(+/-) cells contain annexin A1 with an apparent molecular weight 45,000 Da in nuclei (F. Hirata et al., 2010). The majority of nuclear annexin A1 was present in DNA synthesomes (Lin et al., 1997). Heavy metals inhibited DNA unwinding and

stimulated DNA annealing by the nuclear extracts, and these metal effects were blocked by anti-annexin A1 antibody but not by anti-IgG (A. Hirata et al., 2010). To establish if nuclear annexin A1 plays a role in translesion DNA synthesis, we tested nuclear extracts for DNA synthesis using the P30G oligonucleotide as a template. Synthesis of 80mer DNA was considered as full translesion synthesis. Translesion DNA synthesis from damaged DNA templates was stimulated only in the presence of Ca^{2+} or heavy metals. Heavy metal-stimulated translesion DNA synthesis was partially blocked by monospecific anti-annexin A1 antibody but not by anti-IgGs, indicating that translesion DNA synthesis was dependent on annexin A1. However, it is noted that some degree of replication by nuclear extracts with damaged DNA templates took place in the absence of heavy metals or Ca^{2+}. Therefore, it cannot be ruled out that nuclear annexin A1 can function in recruiting error-prone DNA polymerases to the site of DNA synthesis. A recent study by O'Brien and colleagues (2009) demonstrates that Pol τ plays an important role in Cr^{6+}-induced mutation. It is also possible that the type of error prone polymerase that is recruited depends on the specific type of DNA damage.

When L5178Y *tk*(+/-) lymphoma cells were exposed to 20 μM MMS and 3 μM $AsCl_3$, mutation of the *tk* gene was induced as measured by the number of clones resistant to 5-fluoro-thymidine. Such mutation was suppressed by pretreatment of L5178Y *tk*(+/-) cells with an annexin A1 anti-sense oligonucleotide (unpublished data by F. Hirata). These observations suggest that DNA damage induced mutagenesis is mediated through annexin A1.

10. Summary

Cellular contents of annexin A1 increase in a variety of cancer cells including pancreatic cancer, glioma and hepatoma (Lim & Pervaiz, 2007). Not only precancerous hepatocytes but also proliferative hepatocytes following liver damage express annexin A1, while normal hepatocytes do not. Therefore, annexin A1 is proposed to be a biomarker of cell transformation and/or hyperproliferative state (Masaki et al., 1994). In contrast, some cancer cells such as prostate, breast and esophageal cancers demonstrate downregulation of annexin A1 expression (Lim & Pervaiz, 2007). Transfection of cDNA encoding annexin A1 into some cells alters the MAP kinase pathway *via* interaction of annexin A1 with Grb2, thereby delaying the cell cycle (Lim & Pervaiz, 2007). These observations lead to the proposal that annexin A1 is a suppressor gene that promotes cellular differentiation rather than an oncogene that promotes cancer. However, recent studies have shown that the down-regulation of annexins in certain types of malignant cancers is mainly attributable to epigenetic regulation by DNA methylation and histone acetylation, and to deletion of the annexin genes due to chromosomal instability following malignant transformation (Lim & Pervaiz, 2007). In esophageal epithelial carcinoma cells, nuclear annexin A1 is increased by its translocation from the cytosol, and cytosolic annexin A1 is consequently decreased (Liu et al., 2008). Therefore, it is proposed that nuclear annexin A1 rather than cytosolic annexin A1 is more closely associated with cell transformation, namely, cancer initiation¸ while changes of annexin A1 expression may not be involved in tumorigenesis.

Annexin A1 contains sequence and structural motifs for binding of nucleotides (non-Walker A type), binding of Ca^{2+} and heavy metals (non-EF hand type II and III) and DNA and/or RNA (helix-loop or turn-helix). Further, this protein is present in DNA synthesomes, suggesting some roles in the replication machinery. Nuclear annexin A1 is modified with ubiquitin and ubiquitin like molecules, and mono-ubiquitination of annexin A1 increases in response to DNA damage. Mono-ubiquitinated annexin A1 exhibits a helicase activity which has higher affinity for damaged DNA. Since helicases are among the first proteins that encounter damaged sites of DNA, this protein may regulate assembly of proteins required for repair of and tolerance to DNA damage. Mono-ubiquitinated but not SUMOylated nor native annexin A1 stimulates error-prone DNA polymerases such as Pol β with damaged DNA as a template *via* its helicase activity. In keeping with this interpretation that annexin A1 plays an important role in error prone lesion bypassing DNA synthesis in response to DNA damage, nuclear extracts of L5178Y *tk*(+/-) lymphoma cells promote translesion DNA synthesis in an annexin A1 dependent manner. Carcinogenic heavy metals such as As^{3+} and Cr^{6+} bind to the Ca^{2+} binding sites of annexin A1, and promote translesion DNA synthesis via annexin A1. DNA damage induced mutagenesis was found to be annexin A1 dependent both *in vivo* and *in vitro* (Fig. 2).

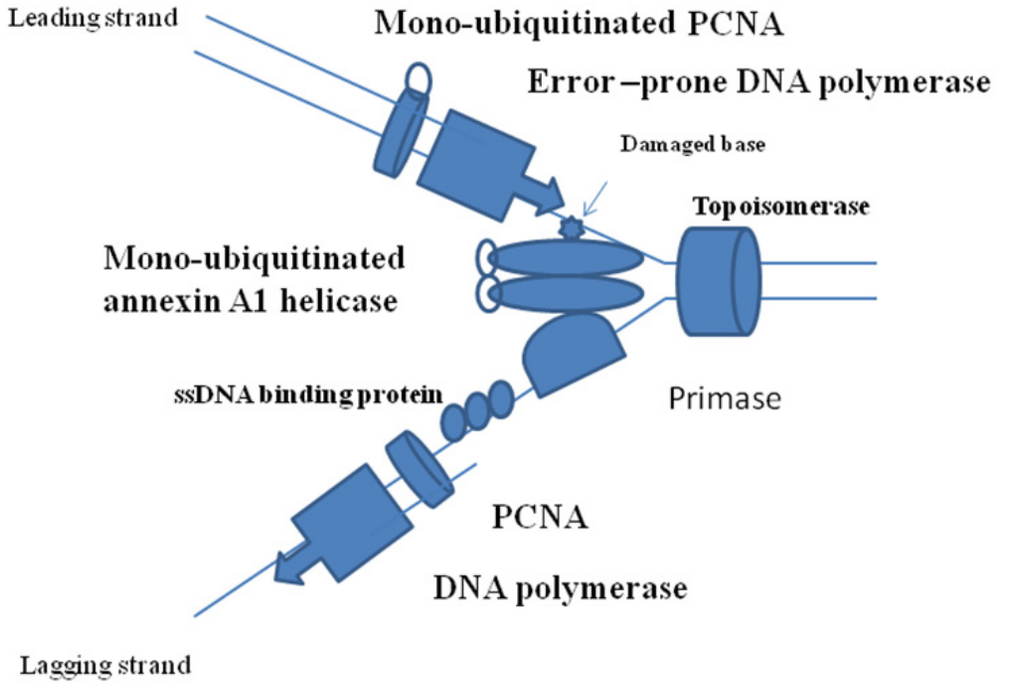

Figure 2. Annexin A1 mediated translesion DNA synthesis

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