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Substrate specific stimulation of NEIL1 by WRN but not the other human RecQ helicases

Venkateswarlu Popuri, Deborah L. Croteau, and Vilhelm A. Bohr*

Laboratory of Molecular Gerontology, National Institute on Aging, NIH, 251 Bayview Blvd, Suite 100, Baltimore, MD 21224, USA

Abstract

NEIL1, the mammalian homolog of *Escherichia coli* endonuclease VIII, is a DNA glycosylase that repairs ring-fragmented purines, saturated pyrimidines and several oxidative lesions like 5-hydroxyuracil, 5-hydroxycytosine etc. Previous studies from our laboratory have shown that Werner Syndrome protein (WRN), one of the five human RecQ helicases, stimulates NEIL1 DNA glycosylase activity on oxidative DNA lesions. The goal of this study was to extend this observation and analyze the interaction between NEIL1 and all five human RecQ helicases. The DNA substrate specificity of the interaction between WRN and NEIL1 was also analyzed. The results indicate that WRN is the only human RecQ helicase that stimulates NEIL1 DNA glycosylase activity, and that this stimulation requires a double-stranded DNA substrate.

Keywords

DNA repair; base excision repair; NEIL1; NEIL2; RecQ helicase; WRN

1. Introduction

Most oxidative DNA lesions are repaired by the base excision repair (BER) pathway. BER is initiated by a DNA lesion specific N-glycosylase, which hydrolyzes the N-glycosidic bond between the modified base and the sugar moiety to release the base and generate an abasic site. 8-oxoguanine DNA glycosylase, OGG1, and NTH1 were the first mammalian DNA glycosylases to be identified and characterized, and the Fpg/Nei-related family of DNA glycosylases was identified more recently [1]. Three mammalian members of the Fpg/Nei protein family have been identified to date: NEIL1, NEIL2 and NEIL3 [1-3,7]. Human NEIL1 is the best characterized member of this protein family and is structurally similar to *Escherichia coli* Nei and Fpg [4]. Like *E. coli* Fpg and Nei, NEIL1 is a bifunctional glycosylase/lyase that catalyzes β - and δ -elimination at DNA lesions leading to strand scission [5,6]. NEIL1 recognizes DNA lesions in both ssDNA, dsDNA, as well as bubble substrates, whereas the 8-oxoguanine glycosylase (OGG1) is active only on dsDNA lesions [7]. Lesions recognized by NEIL1 include 5-hydroxyuracil (5-OHU), 5-hydroxycytosine (5-OHC), dihydrothymine, FapyA, FapyG, thymine glycol (Tg), hydantoin lesions and its derivatives like guanidinohydantoin and spiroiminodihydantoins [8]. Recent studies also

*Corresponding author: Vilhelm A. Bohr, Laboratory of Molecular Gerontology, National Institute on Aging, NIH, 251 Bayview Blvd, Suite 100, Baltimore, MD 21224, USA. Tel: 410 558 8162 Fax: 410 558 8157 vbohr@nih.gov.

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implicate NEIL1 in repair of bulky DNA lesions including psoralen-induced thymine monoadducts and interstrand DNA crosslinks [9,10]. Given its preference for partial ssDNA substrates and the fact that the level of NEIL1 is higher during S phase of the cell cycle, it has been proposed that NEIL1 may play a role in replication-associated and/or transcription-coupled DNA repair [2,12]. Consistent with this idea, NEIL1 interacts with and is stimulated by replication proteins including PCNA [12], FEN1 [13] Pol β & Lig III [14] and CSB [15]. Thus, human NEIL1 likely plays a critical role in processing oxidative DNA damage in human cells [2,5,7,11].

RecQ helicases are highly conserved from bacteria to man and play very important roles in the maintenance of genomic stability. *E. coli*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* express a single RecQ homolog, while in higher eukaryotic cells multiple RecQ homologs have been identified. Human cells express five RecQ helicases, including RECQ1, WRN, BLM, RECQ4 and RECQ5. Mutations in BLM, WRN, and RECQ4 are associated with the human genetic disorders Bloom, Werner, and Rothmund–Thomson syndromes, respectively. Mutations in the *RECQ4* gene are also found in patients with RAPADILINO and Baller-Gerold syndromes. A wide range of proteins involved in DNA replication, DNA repair, and telomeric maintenance have been found to interact with RecQ helicases [16,17].

The RecQ helicases are called the guardians of the genome, and are generally involved in many DNA metabolic processes including DNA repair, DNA replication and transcription. It is of great interest and a considerable challenge to understand why there are five RecQ helicases in humans while only there is only one member in yeast and other lower eukaryotes. Are they collaborating or is it redundancy? This question has not been answered. It is therefore very important to compare the role of these helicases in specific processes, something that has not been done because all five RecQ helicases have not been available for comparative studies. Clinical, genetic and biochemical studies of the human RecQ helicases indicate that they play complementary, non-redundant biological roles and may not fully substitute for each other in vivo [18,19].

WRN has been implicated in oxidative DNA damage and BER. Previous studies showed that WRN has functional interactions with the BER proteins APE1, POL β and FEN1, suggesting that WRN may play a role in BER [20,21]. Extracts from WRN knockdown cells have a reduced capacity for short patch and long patch BER [21] and WRN knockdown cells are hypersensitive to oxidizing and alkylating agents and accumulate oxidative base lesions [22]. WRN also interacts physically with NEIL1 *in vivo* and *in vitro* and stimulates the DNA glycosylase activity of NEIL1 *in vitro* [23], but the substrate specificity requirements for the WRN stimulation of NEIL1 has not been characterized.

The goal of this study was two-fold: to examine whether the other human RecQ helicases interact with NEIL1 during repair of oxidative DNA damage, and to analyze the substrate specificity of the WRN/NEIL1 stimulatory interaction. The results show that WRN is unique in its ability to stimulate NEIL1, while other human RecQ helicases lack this ability under the conditions tested here. Furthermore, WRN and other RecQ helicases fail to stimulate NEIL2, and WRN only stimulates NEIL1 activity only on dsDNA substrates. These results demonstrate a fairly high degree of specificity in the WRN/NEIL1 interaction, which is consistent with the proposal that this interaction plays an important biological role in human cells.

2. Results

WRN, but not other RecQ helicases, specifically stimulate NEIL1 on duplex substrates

A goal of this study was to characterize the interaction between all five human RecQ helicases and NEIL1. For this purpose, DNA incision assays were performed with NEIL1 in the presence of variable concentrations of WRN, RECQ1, BLM, RECQ4 or RECQ5. The DNA incision assays were carried out with ssDNA, a bubble DNA substrate and a dsDNA substrate carrying a unique centrally-located 5-OHU or FapyA residue, respectively. Additionally, the ability of the human RecQ helicases to stimulate NEIL1 and NEIL2 was compared on the 5-OHU bubble DNA substrate.

2.1. WRN stimulates NEIL1 incision of 5-OHU bubble substrates

Previous studies showed that NEIL1 has higher affinity for “bubble” substrates than for duplex substrates or ssDNA [2,7]. Das *et al.* also showed that WRN stimulates NEIL1 cleavage of 5-OHU, FapyG, and 8-oxoG in bubble substrates in the absence of WRN helicase activity [23]. In this study, DNA incision assays were conducted using NEIL1 and WRN under conditions similar to those used by Das *et al.*, and the results are shown in Figure 1A & 1B. In these experiments, the NEIL1-catalyzed incision of a 5-OHU-containing bubble DNA substrate was stimulated up to 3-fold by increasing concentrations of WRN (up to 5-fold WRN: NEIL1 molar excess). In the presence of equimolar WRN, NEIL1 incision activity increased 1.5-fold, while in the presence of a 2-fold excess of WRN, NEIL1 incision activity increased 2-fold (Figure 1A, compare lanes 3 & 4). In contrast, RECQ1, BLM, RECQ4 and RECQ5 did not stimulate NEIL1-catalyzed incision of the same DNA substrate under the same conditions at the same (Fig. 1C-F) or higher molar ratio (i.e., 10:1 RecQ helicase: NEIL1) (data not shown). Control experiments showed that all RecQ helicases were active DNA helicases (data not shown). Interestingly, the stimulation of NEIL1 studied here is independent of active helicase catalytic activity, thus the reactions were performed in the absence of ATP.

2.2. WRN stimulates NEIL1 incision of FapyA dsDNA substrates

Although NEIL1 can act on a broad range of substrates, FapyA and FapyG are its preferred substrates. Therefore, the ability of WRN to stimulate NEIL1 cleavage of FapyA in a dsDNA substrate was determined. Figure 2 shows that WRN stimulated NEIL1 incision of FapyA in dsDNA 5-fold, when present at a 5:1 molar ratio to NEIL1. Quantification of these data (Figure 2A & B), revealed that WRN stimulated NEIL1 activity 2-fold at a 1:1 molar ratio and 4-fold at a 2-fold molar excess (i.e. 2:1 WRN: NEIL1). In contrast, the other human RecQ helicases, including RECQ1, BLM, RECQ4 and RECQ5 did not stimulate NEIL1 incision of FapyA under the same conditions and the same (Fig. 2C-F) or higher molar ratio (10:1) (data not shown). Combined, figures 1 and 2 show that the WRN stimulation of NEIL1 is specific and not a general feature of the RecQ family of helicases.

2.3. WRN does not stimulate NEIL1 incision of 5-OHU or FapyA on ssDNA substrates

The above results show that WRN stimulates NEIL1 incision of 5-OHU in a bubble DNA substrate and FapyA on a dsDNA duplex substrate. The ability of WRN to stimulate NEIL1 incision activity of 5-OHU or FapyA was also examined on a ssDNA substrate. However, using the ssDNA substrate, no significant stimulation of NEIL1 incision was observed with up to 5-fold molar excess of WRN to NEIL1 (Fig. 3A& 3B). In contrast, FEN1 stimulated NEIL1-catalyzed incision of 5-OHU on the ssDNA substrate (Figure 3C), as previously reported by Hegde *et al.* [19]. Thus, the ability of WRN to stimulate NEIL1-catalyzed DNA incision appears to depend on the structure of the DNA substrate. This study indicates that WRN stimulates NEIL1 incision only on dsDNA substrates.

2.4. NEIL1 specifically interacts with WRN but not with RECQ4 and RECQ5

Co-immuno precipitation assays (Co-IP) were performed to analyze the association of NEIL1 with the RecQ helicases. Our results show that NEIL1 specifically interacts with WRN (Fig. 4 A). Previous results from Das et al, indicated the association of ectopically expressed Flag-tagged NEIL1 with WRN [23]. Our results, for the first time, show that endogenously WRN and NEIL1 interact (Fig. 4 A). RECQ4 and RECQ5 have been shown to interact with and modulate BER proteins in our laboratory [25,34], therefore we sought to extend our analysis to these two RecQ helicases. Our results also show that the interaction is specific with WRN and NEIL1 does not interact with exogenously expressed RECQ4 or RECQ5 (Fig. 4 C). Further the association of endogenous RECQ1 was also tested with the same negative result (Fig. 4 B).

2.5. RecQ helicases do not stimulate the incision activity of NEIL2

The ability of WRN and other RecQ helicases to stimulate NEIL2-catalyzed incision of a 5-OHU-containing bubble DNA substrate was also examined. Before assaying for stimulation of NEIL2 by all five human RecQ helicases, NEIL2 was titrated into the DNA incision assay, and the linear range of the assay was determined (Fig. 5A). At a 1 nM concentration of NEIL2, 5 % of the bubble substrate was incised. Subsequently, incision assays were performed in the presence of increasing concentrations of WRN, BLM, RECQ1, RECQ4 or RECQ5. These assays were performed with up to 10-fold molar excess of the RECQ enzyme to NEIL2. The results showed that WRN and the other RecQ helicases do not stimulate NEIL2 (Fig. 5B-D).

3. Discussion

The main conclusion of this study is that WRN but not the other RecQ helicases specifically stimulate NEIL1 DNA glycosylase and that this stimulation requires a dsDNA substrate because no stimulation was seen when lesions were positioned within single stranded DNA (Fig. 1-3). NEIL1 stimulation with the dsDNA substrates only, might suggest that the co-operative binding of WRN along with NEIL1, on to the duplex DNA might be responsible for the stimulation. This substrate specific stimulation of NEIL1 by WRN could be implied as a co-operation of both proteins in the repair of oxidative lesions during replication and /or repair of stalled / damaged replication forks (refer 3.1).

None of the five RecQ helicases stimulate NEIL2, further supporting the specific association of NEIL1 and WRN (Fig. 5). WRN stimulation of NEIL1 could promote the removal of oxidative lesions during replication fork progression and thus play an important role in BER. Additionally, the loss of this activity could contribute to the higher oxidative DNA damage load observed in cells without WRN [22,24]. The specificity of this interaction, both at the level of protein-protein and protein-DNA recognition/interaction indicates that it is likely to have biological significance.

Co-IP of endogenous NEIL1 and WRN confirms our previous findings that these proteins interact (Fig. 4). We sought to extend the protein: protein interaction study to other RecQ helicases, as previous studies from our lab have been shown that both RECQ4 and RECQ5 regulate other BER proteins like APE1, FEN1 etc. [25,34]. Our co-IP experiments here, however, show that NEIL1 does not interact with RECQ1, RECQ4 or RECQ5, further supporting our proposal that the NEIL1: WRN is a specific interaction.

Our results support a model in which WRN and NEIL1 collaborate on the repair of oxidative DNA lesions. However, a number of questions remain to be answered about the interaction between WRN and NEIL1, such as which biological pathways this physical and functional interaction has implications for. Furthermore, the molecular mechanism by which WRN

stimulates NEIL1 remains undetermined at present. Additionally, it may be interesting to determine which other proteins are present in the complex with WRN and NEIL1. While these issues are beyond the scope of this paper, it should be noted that this is the first study where biochemical interactions studies have been done with all the five human RecQ helicases.

3.1. WRN and NEIL1 might collaborate functionally during replication and/or DNA repair

Previous studies showed that the level of NEIL1 increases during S phase of the cell cycle and that NEIL1 and WRN both interact with PCNA [2,12,26]. For example, PCNA stimulates NEIL1 glycosylase activity on forked duplexes and enhances loading of NEIL1 onto the DNA substrate. Dou *et al.* proposed that PCNA-bound NEIL1 may initiate repair by recognizing the site of the oxidative lesion in either the leading or lagging strand template ahead of the polymerase [12]. Subsequent stalling of the replication fork can lead to replication fork collapse (*i.e.*, formation of a “chicken foot” structure). WRN is also known to be recruited to stalled replication forks, where it promotes fork regression, resolution of the collapsed replication fork and replication restart [27,28]. These results suggest that the DNA repair functions of WRN and NEIL1 may be targeted to nascent DNA during DNA replication, and that they could interact functionally in the vicinity of stalled DNA replication forks.

3.2. Distinct biological roles of human RecQ helicases

Previous studies established that WRN interacts with BER proteins including FEN1, POL β , and APE1 and plays a functional role in BER in human cells [20,21,29]. This study demonstrates a functional interaction between WRN and NEIL1 that is unique among the RecQ helicases. The present study provides *in vitro* evidence that WRN exclusively stimulates NEIL1 glycosylase, while the other RecQ helicases do not under similar reaction conditions. This observation is consistent with the notion that the human RecQ helicases have complementary functions and therefore are not redundant in human cells. It is of great interest to further explore whether human RecQ helicases are complementary in other important DNA metabolic reactions. Such studies are in their infancy because most studies are confined to one RecQ helicase and do not include a comparative study of all RecQ helicases. Additional studies are underway to better define the roles of the multiple human RecQ helicases. This study underscores the possible importance of WRN in the repair of oxidative DNA damage via BER.

4. Materials and Methods

4.1. Recombinant proteins

The antibodies for RECQ1 and NEIL1 were commercially bought from Santa Cruz and Calbiochem respectively. Recombinant WRN was expressed and purified from baculovirus / SF9 insect cells as previously described in Orren et al [30]. BLM and RECQ1 were expressed and purified from yeast and baculovirus, respectively. RECQ4 and RECQ5 were expressed and purified from *E.coli* as previously described [31,32]. The recombinant protein NEIL1 was a kind gift from Dr. Sankar Mitra, professor, Dept. of Biochemistry and Molecular biology, university of Texas Medical Branch, Galveston, Texas. Recombinant NEIL2 was kindly provided by Dr. Magnar Bjorås, Oslo University. FEN1 was generously provided by David Wilson III, NIA. All proteins were judged to be greater than 95% pure by PAGE gel analysis and coomassie staining (data not shown).

4.2. DNA substrates

All DNA substrates were synthesized and PAGE purified by Midland Certified Reagent Company (Midland, Tx) with the exception of the FapyA containing oligonucleotide (Table I). The FapyA oligonucleotide was kindly provided by Dr. Marc Greenberg from Johns Hopkins University. The 5-OHU bubble substrate was made from the 51-mer oligonucleotides, BSX1 and BSX2. BSX1 contains the 5-OHU lesion at residue 26. The FapyA duplex substrate was made from 30-mer oligonucleotides FpA1 and FpA2, with the oligonucleotide FpA1 containing the FapyA lesion at residue 16. The DNA strand containing the adduct was ^{32}P end labeled with T4 polynucleotide kinase according to the manufacturers recommendations. Free ^{32}P was removed by size exclusion chromatography using the G25 spin columns (GE Biosciences).

Oligonucleotides that required duplex formation were annealed to their complementary partners at a 1:2 molar ratio in the presence of 50 mM KCl.

4.3. Incision assay

NEIL1 AP lyase activity was assayed by incubation of γ P^{32} - labeled substrate with indicated amounts of NEIL1 alone or together with increasing concentrations of RecQ helicase, as indicated, in a buffer containing 40 mM Tris pH 8.0, 4 mM MgCl_2 , 5 mM DTT, 0.1 $\mu\text{g}/\mu\text{l}$ BSA at 37 °C for 15 min. After stopping the reaction with 70% formamide/30 mM NaOH, the products were analyzed by 12 % denaturing gel electrophoresis. Incision assays containing NEIL2 or FEN1 were conducted in the same buffer as that for NEIL1 incision assays.

4.4: Co-immuno precipitation assays

Co-IP assays were performed in order to further evaluate the potential protein interactions between NEIL1 and the RecQ helicases WRN, RECQ4 and RECQ5. The association of NEIL1 and WRN was used as a positive control. Nuclear extracts from HeLa cells (1 mg) were incubated over night at 4°C, with the Invitrogen protein AG agarose beads (and pre-cleared with 5% BSA) and the anti-rabbit NEIL1 antibody (at a dilution 1-200) as per the manufacturer's instructions (Cat # DR1038). The beads were then washed three times with 50 nM Tris HCl (pH 7.5) and 150 mM NaCl. The bound proteins were separated by SDS PAGE, transferred to PVDF and the blots were probed for WRN using a mouse monoclonal WRN antibody (clone 2D06, [33]). The association of RECQ1 with NEIL1 was also tested similarly to that of WRN as described above, except that the blot was probed with the polyclonal anti-goat RECQL1 antibody from the Santa Cruz Biotechnology (SC-16154). Due to poor commercial antibodies against RECQ4 and RECQ5, C-terminal 3× Flag tagged RECQ4 or RECQ5 (with N-terminal 3×Flag and C-terminal Myc tag, proteins were ectopically expressed in HeLa cells and pulled down with the Sigma mouse Flag M2 beads. The beads were processed as described above for the WRN IP. The upper portion of the blot was probed with Anti Flag antibody produced in rabbit from the Sigma Aldrich (F7425), and the lower portion of the blot was probed with the Anti- rabbit NEIL1 antibody from Calbiochem.

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Abbreviations

5-OHU	5-hydroxy uracil
8-oxo-Gua	7,8-dihydro-8-oxo Guanine

FapyA	4,6-diamino-5-formamidopyrimidine
OGG1	8-oxoguanine DNA glycosylase
BER	base excision repair

Fig. 1. WRN specifically stimulates NEIL1 incision activity on bubble substrate with 5-OHU lesions, but not by other RecQ helicases

0.5 nM substrate DNA (51-mer bubble 5-OHU) was incubated at 37 °C for 20 min, with 1 nM of NEIL1 and increasing concentrations (1, 2, 4, 5 and 10 nM) of WRN (A) or the other RecQ helicases (C-F, 1-10 nM), respectively. The concentration of the RecQ helicase without NEIL1 was 10 nM. The plot is the average of three independent experiments with standard deviation indicated by error bars (B).

Fig. 2. Stimulation of NEIL1 incision activity on duplex substrate with FapyA lesions specifically by WRN, but not by other RecQ helicases

0.5 nM substrate DNA (30-mer duplex FapyA) was incubated at 37 °C for 20 min, with 2 nM NEIL1 and increasing concentrations (2, 4, 8 and 10 nM) of WRN (A) or the other RecQ helicases, (C-F, 2-10 nM) respectively. The plot is the average of three independent experiments with standard deviation indicated by error bars (B).

Fig. 3. WRN does not stimulate NEIL1 on single stranded DNA

0.5 nM single stranded DNA, with FapyA (A) or 5-OHU (Fig. B, C), were incubated at 37 °C for 20 min, with 2.5 nM NEIL1 and increasing concentrations (2.5, 5, 10 and 12.5 nM) of WRN (A & B) or FEN1 (B & C, 2.5 – 12.5 nM FEN1). FEN1 alone was tested at 12.5 nM. The plot (D) comparing the stimulation of FEN1 with WRN on the ss-DNA with the 5-OHU is the average of three independent experiments, with standard deviation indicated by error bars

Fig. 4. Interaction of NEIL1 with WRN but not with other RecQ helicases

Fig. 4A, NEIL1 was pulled down from HeLa nuclear extracts with anti-rabbit NEIL1 antibody, and the blot was probed with anti-mouse WRN antibody. Fig. 4A lane 2 shows the specific endogenous association of NEIL1 with WRN, and lanes 1 and 3 are input and IgG control pull down, respectively. Fig. 4B shows the NEIL1 IP performed as described above, and probed with the polyclonal anti-goat anti RECQ1 antibody (Santa Cruz). Lane 2 shows the negative IP of NEIL1 when probed with anti RECQ1 antibody, while lanes 1 and 3 are input and IgG control pull downs, respectively. Fig. 4C, Flag tagged RECQ4 and RECQ5 proteins were ectopically expressed in HeLa cells and pulled down with the Sigma Flag M2 beads. The expression of Flag RECQ4 and RECQ5 were shown in the upper panel

of Fig. 4C, and NEIL1 is shown in the lower panel. The left panel of the figure shows the supernatants' of the Empty Flag vector (3×) transfection, Control mouse IgG pull down, Flag RECQ4 and RECQ5 transfected pull downs respectively, while the right panel of the figure shows the IP's of the Empty flag vector, IgG pull down, Flag RECQ5 and RECQ4 transfected pull downs respectively. The control IgG pull down has the Flag RECQ4 transfected in it. Fig. 4C lower panel shows the blot probed for NEIL1. Neither RecQ4 nor RecQ5 interact with NEIL1 (right lower panel, lanes 3 and 4), while the left lower panel shows the NEIL1 in the inputs.

Fig. 5. Neither WRN nor other RecQ helicases stimulate NEIL2

Fig. 5A, Increasing concentrations of NEIL2 (0.5 nM – 20 nM) was incubated with 51 mer bubble 5-OHU (0.5 nM) at 37 °C for 20 min. Fig. 5B-D, The 51 mer bubble 5-OHU substrate (0.5 nM), 1 nM of NEIL2 and increasing concentrations (1, 2, 4, 5 and 10 nM) of WRN or the other RecQ helicases, respectively, were incubated at 37 °C for 20 min. The concentration of the RECQ helicase without NEIL2 was 10 nM. The plot is the average of three independent experiments with standard deviation indicated by error bars (E).

TABLE I
The oligonucleotides used in the study are shown in Table I

The oligonucleotide BSX1 contains the 5-OHU lesion at residue 26, denoted with X. The bold sequence represents the 11 nucleotide bubble portion of the substrate. The FapyA duplex substrate was made from 30 mer oligonucleotides FpA1 and FpA2, with the oligonucleotide FpA1 containing the FapyA lesion at residue 16, indicated by Y in italics.

BSX1	5'- GCTTAGCTTGGAAATCGTATCAT GTAXACTCGTGTGCCGTGTAGACCGTGCC -3'
BSX2	5'- GGCACGGTCTACACGGCACAA ACAGCCCACGG ATACGATTCCAAGCTAAGC -3'
FpA1	5'- CGTTCAACGTGCACTYACAGCACGTCCCAT -3'
FpA2	5'- GCAAGTTGCACGTGATTGTCGTGCAGGGTA -3'