Study on interacting proteins with human NTH1 involved in oxidative DNA damage repair

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学位論文要旨

ABSTRACT

Thymine glycol (Tg) is one of the predominant oxidative DNA lesions caused by ionizing radiation and other oxidative stresses such as H_2O_2. Tg efficiently blocks DNA replication and is thought to mainly cause cell death rather than mutation. NTH1 is a major DNA glycosylase that removes Tg in the first step of base excision repair (BER). Several cellular factors, YB-1, APE1 and XPG, have been reported to stimulate the NTH1 activity probably through their interactions. We have searched for the other factors interacting with NTH1 by using a pull-down assay with human cell-free extracts or purified recombinant proteins. Here we show that NTH1 physically interacts with p53 and PCNA as well as XPG. Furthermore, p53 stimulated the Tg DNA glycosylase/AP lyase activity of NTH1, while PCNA did not show any stimulation. We also confirmed the previous observations that XPG stimulates the NTH1 activity. These results provide an insight into the positive regulation of BER reaction and suggest a possible linkage between BER of Tg and other cellular mechanisms, DNA replication, transcription and damage signaling pathways.

Reactive oxygen species (ROS) is spontaneously generated as by-products by oxidative phospholylation system. ROS is so highly reactive to oxidize lipids, proteins and nucleic acids. Especially, oxidative DNA damage is thought to cause mutation and genome instability, possibly leading to cancer and aging. Thymine glycol (Tg) is the most predominant form of oxidative pyrimidine lesions produced by ROS and ionizing radiation. Tg blocks DNA replication and is thought to mainly cause cell death rather than mutation.

Base excision repair (BER) is the major repair pathway, which excises modified DNA base damage including Tg. BER is initiated by the action of DNA glycosylase and

NTH1 is a bifunctional DNA glycosylase for Tg, which has two enzymatic activity DNA glycosylase and AP lyase activity. Recently, several cellular factors including YB-1, APE1 and XPG have been reported to stimulate the NTH1 activity probably through their interactions. In this study, in order to understand the molecular mechanisms of the cellular responses to the deleterious Tg lesions, I have prepared active recombinant NTH1 proteins using an *E. coli* expression system and searched for the factors that interact with NTH1 and modulate its activity.

Recombinant GST-NTH1 protein was overproduced in *E. coli* and purified by using a glutathione-sepharose affinity column. The purified GST-NTH1 was tested for DNA glycosylase/AP lyase activity by incubating with ^{32}P-laveled DNA substrate containing a single Tg. Incision products increased depending on the reaction time and the amount of GST-NTH1, indicating that the recombinant GST-NTH1 is functionally active as a Tg DNA glycosylase/AP lyase.

In literature, there are two reports showing that one of nucleotide excision repair factors, XPG stimulates NTH1 activity by enhancing the NTH1 binding to Tg-containing DNA. However, the data on the physical interaction between the two proteins has been controversial. To test whether NTH1 interacts with XPG, GST-NTH1 was incubated with cell-free extracts (CFEs) from human cell lines and pulled down with glutathione-sepharose 4B beads. Immunoblot analysis with anti-XPG antibody revealed that XPG was coprecipitated with GST-NTH1 beads, but not GST beads, suggesting the association between NTH1 and XPG. To determine whether the association of the two proteins is direct or indirect, I overproduced recombinant FLAG-tagged XPG protein in the baculovirus/insect cell system and purified it to near homogeneity. The purified FLAG-XPG was specifically detected in the bound fractions to GST-NTH1, indicating that NTH1 directly interacts with XPG. Furthermore, I confirmed that XPG stimulates the Tg DNA glycosylase/AP lyase activity of GST-NTH1 in a dose dependent manner. These results suggest that XPG positively affects the NTH1 activity through the protein-protein interaction.

The association between BER and DNA replication has been suggested from several groups. Human UNG2, nuclear form of uracil DNA glycosylase, and MYH, DNA glycosylase against adenine misincorporated to template 8-oxoG, have been shown to interact with PCNA and RPA, and to colocalize with PCNA in replication foci, suggesting the presence of replication-coupled repair. I wished to test the possibility that NTH1 also interacts with PCNA and/or RPA, since Tg efficiently blocks replication. After the pull down experiments with GST-NTH1 and human CFEs, the precipitates were analyzed by western blotting with anti-PCNA and anti-RPA(p34) antibodies. PCNA was specifically detected in the bound fractions to GST-NTH1, while RPA was not detected. To test the physical

interaction between NTH1 and PCNA, human recombinant PCNA was employed for the pull down experiments and found to bind to GST-NTH1 with a high specificity. These results indicate that NTH1 physically interacts with PCNA.

p53 has been also shown to associate with BER. p53 is well known as a tumor suppressor and the gatekeeper for maintaining the genomic integrity. Various stress signals including oxidative DNA damage induce the posttranslational accumulation of p53 and enhanced its transactivation activity, leading to the up-regulation of down-stream genes involved in cell cycle arrest, DNA repair and apoptosis. Interestingly, it has been reported that p53 directly interacts with DNA polymerase β and APE1, and stimulates BER reaction for uracil and natural AP site. These observations prompted me to examine whether NTH1 interacts with p53. I performed similar pull down experiments with human CFEs and found that native p53 specifically binds to GST-NTH1. Furthermore, with the CFEs from human cells pretreated with H_2O_2, larger amounts of p53 were coprecipitated, corresponding to the elevated p53 levels in the cells. I next asked whether NTH1 directly interacts with p53. Human recombinant $(His)_6$-tagged p53 was prepared from the baculovirus/insect cell system, and found to directly interact with GST-NTH1. Moreover, in the reverse pull down experiments, GST-p53 fusion protein was also shown to bind to NTH1 prepared after cleavage of GST-NTH1 by thrombin. Taken together, I concluded that NTH1 directly interacts with p53.

To examine whether the physical interaction between NTH1 and PCNA or p53 affects the NTH1 activity, I performed a Tg DNA incision assay in the presence or absence of recombinant PCNA or His-p53. The addition of PCNA did not enhance the NTH1 activity, even when 4-fold excess molar PCNA was used. On the contrary, His-p53 stimulates the incision rates of GST-NTH1 in a dose dependent manner.

In this study, I have found that human NTH1 physically interacts with PCNA and p53, and p53 but not PCNA stimulates the Tg DNA glycosylase/AP lyase activity of NTH1. I have also answered the controversial question whether NTH1 interacts with XPG or not. The findings would provide a clue to uncover not only the mechanism of efficient BER but also the association of BER with other cellular responses such as replication, transcription and p53 signaling pathway.

学位論文審査結果の要旨

尾山将樹氏から提出された学位論文について，上記５名の審査委員による査読の後に平成 16 年２月３日に口頭発表会が行われた。同日に最終の審査委員会が開かれ，審議の結果，以下のとおり判定した。

チミングリコールは，DNA 複製や転写反応を阻害することが知られている DNA 酸化型損傷の一つであり，ヒト細胞では塩基除去修復機構によって修復される。本論文は，塩基除去修復の第 1 段階で働く DNA グリコシラーゼ NTH1 に関する解析であり，NTH1 と相互作用する新しい因子の同定を行ったものである。まず，細胞粗抽出液を用いたプルダウン法で検索したところ，これまでに相互作用が示唆されていたヌクレオチド除去修復因子 XPG に加えて，DNA 複製因子である PCNA，およびストレス応答に関与するがん抑制遺伝子産物 p53 との相互作用が新しく見出された。そこで，各因子のリコンビナントタンパク質を調製し、これらの相互作用が直接的なものか否かを検討した結果，いずれの因子も他の因子を介さず直接 NTH1 と相互作用することがわかった。さらに，NTH1 の活性に対する影響を調べ，XPG と p53 は NTH1 の活性を増強させ，PCNA は活性に影響を及ぼさないことを明らかにした。以上の結果より，塩基除去修復以外の機構で働いている因子が，相互作用を介して NTH1 の活性調節をしている可能性が示唆された。

本研究は，NTH1 の新規相互作用因子を同定し，この修復酵素の新たな活性調節機構を示唆したものと評価され，博士（薬学）の学位に値すると判定した。