RNase H 2010

Organized by

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NIH Office of Rare Disease Research

Monday September 20, 2010

Session I: 9:00 AM

9:00-9:30

An uORF and context of the two AUG codons affect the abundance of mitochondrial and nuclear RNase H1.
Yutaka Suzuki, J. Bradley Holmes, Susana M. Cerritelli, Kiran Sakhuja, Michal Minczuk, Ian J. Holt and Robert J. Crouch

9:35-10:05

Possible interplay between RNase HI and RNases involved in RNA decay and processing in *E. coli*.
Valentine Usongo, Jill Egbe Bessong and Marc Drolet

10:10-10:40

The N-terminal hybrid binding domain of RNase HI from *Thermotoga maritima* is important for substrate binding and Mg2+-dependent activity
Nujarin Jongruja, Dong-Ju You, Eiko Kanaya, Yuichi Koga, Kazufumi Takano, and Shigenori Kanaya

Coffee Break 10:45-11:00

11:10-11:40

Identification of the genes encoding novel type 1 RNases H from compost metagenome
Nhan Tri Nguyen, Eiko Kanaya, Clement Angkawidjaja, Yuichi Koga, Kazufumi Takano, and Shigenori Kanaya

11:45-12:15

Utility of boranophosphate nucleic acids in supporting RNase H-type cleavage via antisense agents and in siRNA gene silencing
Barbara Ramsay Shaw, Xin Wang, Zinaida Sergeeva, Allison Hall, Kenneth Alexander, Jing Wan

12:20-12:35

Structure-function correlates of 2'-Fluoroarabinose (2'FANA) oligonucleotides in RNase H1-dependent oligo•RNA duplex cleavage
Nageswara R. Alla, Alan M. Gewirtz, Masad J. Damha, and Allen W. Nicholson

Lunch 12:45-2:15

Monday September 21, 2010

Session II:

2:15-2:30 Susana M. Cerritelli Cellular RNases H1 and H2

2:30-3:00

What RNases H substrates reveal about their functions
Susana M. Cerritelli, Hyongi Chon, and Robert J. Crouch

3:00-3:30

Chromatin loops emanating from R-loops
György Fenyőfalvi, Éva Hegedüs, Lóránt Székvölgyi, Zsolt Bacsó and Gábor Szabó

3:35-4:05

The consequences of incorporating ribonucleotides into DNA
Stephanie A. Nick McElhinny , Dinesh Kumar, Alan B. Clark, Danielle L. Watt, Jessica S. Williams, Brian E. Watts, Else-Britt Lundström, Erik Johansson, Andrei Chabes , Thomas A. Kunkel

Coffee Break 4:10-4:25

4:25-4:55

Structural studies of RNases H2
Marcin Nowotny, Monika Rychlik, Hyongi Chon, Susana M. Cerritelli,
Robert J. Crouch

5:00-5:30

Reconstitution of the yeast RNase H2 complex to establish structure-function relationship among their subunits
Tuan Anh Nguyen, Chul-Hwan Lee, Young-Hoon Kang, Il-Taeg Cho, Min-Jung Kang, and Yeon-Soo Seo

Reception (Time to be announced)

Tuesday September 21, 2010

Session III:

8:30-9:00

The structure of the mammalian RNase H2 complex provides insight into RNA-DNA hybrid processing to prevent immune dysfunction
Nadine M. Shaban, Scott Harvey, Fred W. Perrino, and Thomas Hollis

9:05-9:25

Localisation of the known AGS mutations on the human RNase H2 structure
Martin A.M. Reijns, Doryen Bubeck, Lucien Gibson, Stephen C. Graham, George Baillie, E.Yvonne Jones, and Andrew P. Jackson

9:25-9:50

Localization of the known AGS mutations on the human RNase H2 structure
Marcin Nowotny, Małgorzata Figiel, Hyongi Chon, Susana M. Cerritelli, Robert J. Crouch

Coffee Break 10:00-10:15

10:15-10:30

AGS an innate immune disease
Andrew P. Jackson Min Ae Lee-Kirsch

10:30-11:00

RNASEH2B is required for the nuclear localisation of human RNase H2 and its presence at replication foci
Katy R. Astell, Martin A. M. Reijns and Andrew P. Jackson

11:10-11:40

The phenotypic spectrum of systemic autoimmunity in Aicardi-Goutières syndrome suggests a role of RNASEH2 as modulator of the innate immune response
Sarah Koss, Franziska Schmidt, Manuela Dobrick, Sophia Blum, Hedwig Guttmann, Mike Lorenz, Rayk Behrendt, Georgia Ramantani, Min Ae Lee-Kirsch

11:50-12:20

Catalytic properties of the RNase H2 subunit A mutations in Aicardi-Goutières Syndrome
Stephanie R. Coffin, Gillian Rice, Yanick J. Crow, Thomas Hollis, and Fred W. Perrino

12:25-12:55

PCNA directs Type 2 RNase H activity in DNA replication and repair
Martin A.M. Reijns, Doryen Bubeck, Stephen C. Graham, Katy R. Astell,
E. Yvonne Jones, and Andrew P. Jackson

Session IV:

2:30-2:45 Stuart Le Grice – Retroviral DNA Replication

2:45-3:15

Determining the trajectory of nucleic acids bound to HIV-1 reverse transcriptase by site-specific chemical footprinting
Greg L. Beilhartz, Egor P. Tchesnokov, Stuart Le Grice and Matthias Götte

3:20-3:50

The position of HIV-1 reverse transcriptase on the template-primer is a determinant of primer mispair extension efficiency
Scott J. Garforth and Vinayaka R. Prasad

3:55-4:25

Effects of RNase H-inactivating mutations on the fidelity of HIV-1 group O reverse transcriptase
Mar Álvarez, Raquel N. Afonso and Luis Menéndez-Arias

Coffee Break 4:30-4:45

4:45-5:15

N348I in HIV-1 Reverse Transcriptase Can Counteract the Nevirapine-mediated Bias toward RNase H Cleavage during Plus-Strand Initiation
Mia J. Biondi, Greg L. Beilhartz, Suzanne McCormick, Matthias Götte

5:15-5:45

The N348I mutation of HIV-1 RT causes nevirapine resistance by an RNase H-independent mechanism that is based on decreased inhibitor binding (cancelled)
Matthew M. Schuckmann, Bruno Marchand, Atsuko Hachiya, Eiichi N. Kodama,

Karen A. Kirby, Kamalendra Singh, and Stefan G. Sarafianos

(substitute)
A small step for RT, a giant step for HIV: conformational dynamics of tRNA-primed initiation of reverse transcription

Stuart F. J. Le Grice et al.

5:50-6:20

Alizarine derivatives as new dual inhibitors of the HIV-1 reverse transcriptase(RT)-associated DNA polymerase and Ribonuclease H (RNase H) activities effective also on the RNase H activity of non-nucleoside resistant RTs
Francesca Esposito, Tatyana Kharlamova, Simona Distinto, Luca Zinzula1, Yung-Chi Cheng, Ginger Dutschman, Giovanni Floris, Patrick Markt and Enzo Tramontano

Dinner together –time to be announced

Wednesday September 22, 2010

Session V:

8:30-9:00

N-Hydroxynaphthyridone inhibitors of HIV Ribonuclease H
Jay A. Grobler, Peter D. Williams, Hua Poo Su, Daria J. Hazuda, Michael D. Miller

9:10-9:40

In Vitro Combination Studies between Nonnucleoside Reverse Transcriptase Inhibitors and Ribonuclease H Inhibitors against HIV-1 Reverse Transcriptase
Sanjeewa Dharmasena and Nicolas Sluis-Cremer

9:50-10:20

Small molecule inhibitors of the RNase H function of HIV-1 reverse transcriptase.
Thorsten A. Kirschberg, Mini Balakrishnan, Neil H. Squires, Tiffany Barnes, Katherine M. Brendza, Eugene J. Eisenberg, Weili Jin, Nilima Kutty, Stephanie Leavitt, Albert Liclican, Qi Liu, Xiaohong Liu, John Mak, Jason Perry, Michael Wang, William J. Watkins, Eric B. Lansdon

Coffee Break 10:25-10:45

10:45-11:15

A 2.7 Å Resolution X-ray Crystal Structure for the Inhibitor Manicol Bound at the RNase H Active Site of HIV-1 Reverse Transcriptase
Daniel M. Himmel, Krzysztof Wojtak, William Ho, Joseph D. Bauman, Arthur D. Clark, Jr., Chhaya Dharia, Kalyan Das, John Beutler, Stuart F. J. Le Grice, and Eddy Arnold

11:10-11:40

The 3.0 Å resolution crystal structure of BTDBA, a diketo acid RNase H inhibitor, in complex with full length HIV-1 reverse transcriptase
William C. Ho, Joseph D. Bauman, Daniel M. Himmel, Chhaya Dharia, Arthur D. Clark, Jr.[1], Eddy Arnold

11:50-12:20

Sensitivity of Xenotropic Murine Leukemia Virus-Related Retrovirus Reverse Transcriptase to α-Hydroxytropolone-Derived Ribonuclease H Inhibitors
Suhman Chung, Daniel Himmel, Jian-Kang Jiang, Brian Scarth, Yi Wang, Jason W. Rausch, Edward Arnold, Matthias Götte, Craig R. Thomas, and Stuart F.J. Le Grice

Summary

An uORF and context of the two AUG codons affect the abundance of mitochondrial and nuclear RNase H1.

Yutaka Suzuki[1] † , J. Bradley Holmes[1,2], Susana M. Cerritelli[1], Kiran Sakhuja[1], Michal Minczuk[2], Ian J. Holt[2] and Robert J. Crouch[1]

[1]Program in Genomics of Differentiation, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892. [2]Medical Research Council Mitochondrial Biology Unit, Wellcome Trust/Medical Research Council Building, Cambridge CB2 OXY, England, UK. †Present address: Cell Biology, University of Kaiserslautern, Germany.

This and all work in subsequent abstracts with Robert J. Crouch and Susana M. Cerritelli was supported in part by the NIH Intramural Program of Eunice Kennedy Shriver National Institute of Child Health and Human Development

RNase H1 in mammalian cells is present in nuclei and mitochondria. Its absence in mitochondria results in embryonic lethality due to the failure to amplify mtDNA. Dual-localization to mitochondria and nuclei results from differential translation initiation at two in-frame AUGs (M1 and M27) of a single mRNA. Here we show that expression levels of the two isoforms depend on the efficiency of translation initiation at each AUG codon and on the presence of a short upstream ORF (uORF) resulting in the mitochondrial isoform being about 10% as abundant as the nuclear form. Translation initiation at M1-AUG is restricted by the uORF while expression of the nuclear isoform requires re-initiation of ribosomes at M27-AUG after termination of uORF translation or new initiation by ribosomes skipping the uORF and M1-AUG. Such translational organization of RNase H1 allows tight control of expression of RNase H1 in mitochondria, where its excess or absence can lead to cell death, without affecting the expression of the nuclear RNase H1.

Possible interplay between RNase HI and RNases involved in RNA decay and processing in *E. coli*.

Valentine Usongo, Jill Egbe Bessong and Marc Drolet.

Département de microbiologie et immunologie, Université de Montréal, Montréal, P.Québec, Canada

Well known phenotypes of *Escherichia coli* cells lacking RNase HI (*rnh*A mutants) include synthetic lethality with *rec*B mutations and the ability to replicate the whole chromosome from R-loops in the absence of the normally used *ori*C/DnaA system (cSDR: constitutive stable DNA replication). More recently we have described a new phenotype of *rnh*A mutants involving the inhibition of gyrase, the enzyme that introduces negative supercoiling in the chromosome of bacteria. Here we show that only the ATP-dependent supercoiling activity of gyrase is inhibited in *rnh*A mutants, since the enzyme is still able to relax DNA in the absence of ATP. In order to better characterize the mechanism at play, we have used transposon mutagenesis to isolate suppressors of the growth defect of a *rnh*A mutant in which over-replication (in part involving cSDR) together with gyrase supercoiling inhibition caused chromosome segregation defects. The suppressors in the first category, that likely acted by reducing replication, were found to map in genes involved in nucleotide metabolism and in genes more directly implicated in replication. Interestingly, the most frequent suppressors, in the second category, mapped in the *rne* gene that encodes RNase E, the main endoribonuclease involved in RNA decay and processing in *E. coli*. In this category, we also isolated suppressors that caused the rnr gene to be overexpressed. This gene encodes RNase R, an exoribonuclease that was recently shown to possess a helicase activity allowing the enzyme to degrade structured RNAs. The third category includes suppressors that mapped in genes of unknown function. Genes(s) encoding a gyrase inhibitor might be found among these suppressors. We will also present a model for the regulation of R-loop formation that includes RNase E and R activity.

The N-terminal hybrid binding domain of RNase HI from *Thermotoga maritima* is important for substrate binding and Mg^{2+}-dependent activity

Nujarin Jongruja,[1] Dong-Ju You,[1] Eiko Kanaya,[1] Yuichi Koga,[1] Kazufumi Takano,[1,2] and Shigenori Kanaya[1]

[1]Department of Material and Life Science, Graduate School of Engineering, Osaka University
[2]CREST, JST

Thermotoga maritima RNase HI (Tma-RNase HI) contains a hybrid binding domain (HBD) at the N-terminal region. This domain consists of approximately 40 amino acid residues and is commonly present at the N-terminal regions of eukaryotic RNases H1 (1), which have been reported to be important for substrate binding (2), processivity (3), and positional preference (4). To examine whether bacterial HBD has a similar role as those of eukaryotic RNases H1, Tma-RNase HI, Tma-W22A with the single mutation at the HBD, the C-terminal RNase H domain (Tma-CD), and the N-terminal domain containing the HBD (Tma-ND) were overproduced in E. coli, purified, and biochemically characterized. Tma-RNase HI prefers Mg^{2+} to Mn^{2+} for activity, and specifically loses most of the Mg^{2+}-dependent activity by removal of the HBD and 87% of it by the mutation at the HBD. Tma-CD lost the ability to suppress the RNase H deficiency of an *E. coli rnh*A mutant, indicating that the HBD is responsible for in vivo RNase H activity. The cleavage-site specificities of Tma-RNase HI are not seriously changed by removal of the HBD regardless of the metal cofactor. Binding analyses of the proteins indicate that the binding affinity of Tma-RNase HI is reduced by removal of the HBD, regardless of the metal cofactor. Tma-CD was as stable as Tma-RNase HI, indicating that the HBD is not important for stability. The HBD of Tma-RNase HI is important not only for substrate binding but also for Mg^{2+}-dependent activity, probably because the HBD affects the interaction between the substrate and enzyme at the active site, such that the scissile phosphate group of the substrate and the Mg^{2+} ion are arranged ideally.

1) Cerritelli and Crouch (2009) FEBS J. 276, 1494-1505.
2) Nowotny et al. (2008) EMBO J. 27, 1172-1181.
3) Gaidamakov et al. (2005) Nucleic Acids Res. 33, 2166-2175.
4) Wu et al. (2001) J Biol Chem. 276, 23547-23553.

Identification of the genes encoding novel type 1 RNases H from compost metagenome

Nhan Tri Nguyen[1], Eiko Kanaya[1], Clement Angkawidjaja[1], Yuichi Koga[1], Kazufumi Takano[1,2], and Shigenori Kanaya[1]
[1] Department of Material and Life Science, Graduate School of Engineering, Osaka University
[2] CREST, JST

A metagenomic DNA library was prepared from leaf-and-branch compost and used for functional screening of the RNase H genes. Twelve different genes encoding type 1 RNases H were identified1). Two of them, the LC9-RNase HI and LC11-RNase HI genes, were overexpressed in *E. coli* for further characterization of the proteins. LC9-RNase HI, which lacks a typical active-site motif, exhibited enzymatic activity both in vivo and in vitro. Biochemical properties of this enzyme were not greatly different from those of other type 1 RNases H. Another new type 1 RNase H, LC11-RNase HI, which is a *Sulfolobus tokodaii* RNase HI (Sto-RNase HI) homologue, was also purified and characterized. LC11-RNase HI lacks the C-terminal anchoring region, which is important for hyperstability of Sto-RNase HI2). As expected, LC11-RNase HI was less stable than Sto-RNase HI by 37oC in Tm. LC11-RNase HI was slightly more active than Sto-RNase HI, but could not cleave the double-stranded RNA (dsRNA), which can be cleaved by Sto-RNase HI. The crystal structure of LC11-RNase HI was determined at 1.4 Å resolution. The main-chain fold and steric configurations of the four acidic active-site residues of LC11-RNase HI are very similar to those of Sto-RNase HI, without a C-terminal anchoring tail. However, Glu93 and Pro94 of LC11-RNase HI are located at the position in which Ala92 and Lys93 of Sto-RNase HI are located. These sites are expected to contact the DNA strand of the RNA/DNA hybrid, and may account for the capability of Sto-RNase HI to cleave dsRNA. To elucidate the mechanism by which Sto-RNase HI can recognize both RNA/DNA hybrid and dsRNA as a substrate, further mutational studies of Sto-RNase HI and LC11-RNase HI are now underway.

1) Kanaya et al. (2010) J. Appl. Microbiol. 109, 974–983
2) You et al. (2007) Biochemistry 46, 11494-11503

Utility of boranophosphate nucleic acids in supporting RNase H-type cleavage via antisense agents and in siRNA gene silencing

Barbara Ramsay Shaw*[1], Xin Wang[1], Zinaida Sergeeva[1], Allison Hall[2], Kenneth Alexander[2], Jing Wan[1],
Departments of Chemistry[1] and Pediatrics[2], Duke University, Durham NC 27708-0346.

The unique chemistry of *P*-boranophosphates (BPs) provides a new modality for designing effective siRNAs for RNAi-mediated gene-silencing and DNA oligomers for activation of RNAse H by antisense mechanisms. Being less electronegative than O, S, and P, the substitution of boron for non-bridging oxygen in the phosphate ester linkage results in useful changes in electronic effects: polarity, bond order, and lipophilicity. Interactions with nucleic acid metabolizing enzymes are functionally unique. BPs display unparalleled versatility in acting as surrogate NTP and dNTP substrates for RNA and DNA polymerases, including reverse transcriptase. Further, BPs function as good templates for replication, transcription, reverse transcription, and translation. Yet they are good inhibitors of many deoxy and ribo exonucleases. Previously, we described the utility of boranophosphate oligoribonucleotides in activating RNase H in hybrid BP-DNA:RNA duplexes. Here, we investigated the utility of boranophosphates as agents for silencing RNA. Natural small interfering RNAs (siRNAs) are potent tools for gene down-regulation, but are minimally stable when introduced as exogenous agents into cells. To improve their efficacy, we replaced non-bridging oxygens in the phosphodiester linkages of natural siRNAs with BH_3 groups

Anti-EGFP siRNAs containing BP, PO (natural), and PS (phosphorothioate) modifications were prepared by in vitro transcription with T7 RNA polymerase from the corresponding 5'-(alpha-*P*-modified) NTPs. After confirming the presence of the borane modifications with MALDI-MS, several properties of the double stranded siRNAs were investigated: (1) BPs maintained the A-form conformation of siRNA according to CD spectra; (2) BH_3 groups increased the *Tm* by 0.5 to 0.8 °C per modification; and (3) siRNAs with BH_3-modifications were at least 10-fold more resistant to RNase A digestion than natural ones. The resulting *P*-boranophosphates (BPs) have unique properties, including enhanced lipophilicity and nuclease resistance, altered H-bonding of the phosphate, and less interaction with metal ions.

When these modified siRNAs were used to down-regulate EGFP expression in HeLa cell cultures: (1) BP-siRNAs were consistently more effective than siRNAs with PS modifications; (2) BP-siRNAs were more effective than normal siRNAs provided that the center of the antisense strand was not heavily modified; and (3) BP-siRNAs were more potent at lower concentrations than normal or PS siRNAs.

Finally, with BP single-stranded (ss-siRNA) the silencing activity was comparable to that of unmodified ds-siRNA. The BP ss-RNA had excellent maximum silencing activity, was durable up to one week after transfection, and was highly effective at low concentrations. BP is thus a potential new class of gene modulating agents. *P*-Boranophosphates (BPs) are the most versatile and near perfect mimics of natural nucleic acids in permitting reading and writing of genetic information with high yield and accuracy, yet they are more resistant to nucleases and facilitate gene silencing.

Xin Wang, Mikhail Dobrikov, Dmitri Sergueev, & Barbara Ramsay Shaw, "RNase H Activation by Stereo-regular Boranophosphate Oligonucleotide." Nucleos., Nucleot. & Nucleic Acids, 22(5-8) 1151-53 (2003)

Allison H. S. Hall, Jing Wan, Erin E. Shaughnessy, Barbara Ramsay Shaw and Kenneth A. Alexander, "RNA Interference Using Boranophosphate siRNAs: Structure Activity Relationships". Nucleic Acids Res. 32, 5991-6000 (2004)Allison H. S. Hall, Jing Wan, April Spesock, Barbara Ramsay Shaw and Kenneth A. Alexander, "High Potency Silencing by Single-Stranded Boranophosphate siRNA" Nucleic Acids Res. 34, 2773-2781 (2006)

Structure-function correlates of 2'-Fluoroarabinose (2'FANA) oligonucleotides in RNase H1-dependent oligo•RNA duplex cleavage

Nageswara R. Alla[1], Alan M. Gewirtz[2], Masad J. Damha[3], and Allen W. Nicholson[4]

[1]Department of Chemistry, Temple University, Philadelphia, PA, USA, [2]Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA, USA, [3]Department of Chemistry, McGill University, Montreal, Quebec, Canada, and 4 Departments of Chemistry and Biology, Temple University, Philadelphia, PA, USA

Incorporation of 2'-deoxy-2'-fluoroarabinose (2'FANA) sugars into normal DNA backbones significantly increases antisense oligonucleotide (AS ON) mediated gene-silencing potency and duration of action. This is because 2'FANA-substituted AS ONs exhibit enhanced intracellular stabilities and higher affinities for their complementary RNA targets, compared to the corresponding unmodified, DNA-based AS ONs. Furthermore, 2'FANA-AS ON•RNA hybrids are substrates for RNase H1, the mammalian nuclear enzyme responsible for AS ON mediated RNA degradation and gene silencing. However, 2'FANA-AS ON•RNA hybrids are less reactive towards RNase H1 than DNA-AS ON. In order to capture the best gene silencing attributes of each chemistry, we have synthesized mixed 2'FANA/2'-deoxyribose AS ON. To optimize functionality we are determining in detail how 2'FANA substitution affects RNase H1 action. To this end, RNA cleavage assays were performed that employed recombinant human RNase H1, a 21nt RNA target sequence, and complementary 21nt phosphorothioate (PS) AS ONs with varying 2'FANA (F) substitutions, either at the "wings" of the molecule ("gapmer"; each wing=7 nts: FFFFFFF-DDDDDDD-FFFFFFF), or alternating with 3 nt DNA (D) segments ("altimer": FFF-DDD-FFF-DDD-FFF-DDD-FFF). The initial rates of cleavage of hybrids containing the gapmer or altimer AS ON were compared to that of the all-2'-deoxyribose AS ON hybrid. The hybrid containing the fully 2'FANA modified AS ON was the least reactive, and exhibited a relative initial cleavage rate of 0.02 (2%). In contrast, hybrids containing the gapmer or altimer exhibited relative initial rates of 0.26 and 0.88, respectively. Moreover, the processivity of RNase H1, as measured by the amount of shorter RNA products relative to longer RNA products, increased as a function of 2'-deoxyribose substitution. The enhanced reaction rate and processivity of the altimer correlates with its previously reported superior gene silencing activity in vivo. Ongoing experiments are assessing the effect of 2'FANA substitution on hybrid recognition and cleavage by the RNase H1 hybrid-binding and catalytic domains, respectively.

What RNases H substrates reveal about their functions

Susana M. Cerritelli, Hyongi Chon, and Robert J. Crouch

Program in Genomics of Differentiation. Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892. USA

Eukaryotic RNases H are more complex enzymes that their prokaryotic homologues. Eukaryotic RNases H1 have acquired a Hybrid Binding Domain (HBD), in one or two copies at their N-terminus, which confer processivity to the protein. Eukaryotic RNases H2 comprise a catalytic subunit, similar to the monomeric prokaryotic enzymes, and two additional subunits that are required for activity and that assist the catalytic component by adding processivity and interacting surfaces for other cellular complexes. Although type 1 and type 2 RNases H share little sequence similarity, they have the same fold of the catalytic core, called the RNase H fold, and together with other nucleic acid processing enzymes belong to the retroviral integrase superfamily (RISF). They share a common catalytic mechanism but differ in substrate specificity. Unique to RNase H2 enzymes is the ability to hydrolyze a single ribonucleotide embedded in a DNA sequence. This activity, sometimes called junction ribonuclease activity, is essential for removal of misincorporated ribonucleotides during DNA synthesis. Bacterial RNases H2 have a strong preference for cleavage on the 5' side of RNA in the RNA-DNA/DNA substrates while eukaryotic enzymes hidrolyze as well RNA/DNA substrates, which do not contain an RNA-DNA transition. Conversely, all RNases H1 require at least four ribonucleotides embedded in a DNA sequence for cleavage to occur. We are studying the specificity and substrate preference of RNases H from bacteria and eukaryotes in an attempt to determine what type of substrates each enzyme process in vivo. Although there are hybrids both enzymes can use as substrates, the location and/or temporal expression might also be a selecting factor in determining RNase H specificity. We are also performing in vivo studies using yeast that are deleted for one or both RNases H, and in combination with mutations in other genes to establish whether RNase H1 and RNase H2 have overlapping substrates and can functionally replace each other.

Chromatin loops emanating from R-loops

György Fenyőfalvi, Éva Hegedüs, Lóránt Székvölgyi, Zsolt Bacsó and Gábor Szabó

Department of Biophysics and Cell Biology, University of Debrecen, 4032 Debrecen, Egyetem ter 1., Hungary

We have shown (Szekvolgyi et al., PNAS, 104/38:14964–14969, 2007) that the genomic DNA of resting and proliferating mammalian cells and yeast spheroplasts harbor ss breaks, probably nicks, positioned at loop-size intervals that could be efficiently labelled in situ by DNA polymerase I holoenzyme, but not by Klenow fragment and terminal transferase unless after ribonucleolytic treatments. The RNA molecules involved appear to be R-loops as they are detected by the RNA/DNA hybrid specific S9.6 monoclonal antibody. Quantitative microscopic (CLSM, LSC) analysis of nicks and R-loops over a population of cell nuclei demonstrates that the R-loops are juxtaposed with the nicks. Data obtained by immuno-FISH were in line with this result: the breakpoint cluster region (bcr) of the Mixed Lineage Leukemia (MLL) gene previously shown to be a nick-prone region, colocalized with the nicks. R–loops and biotin-dUTP-tagged ss breaks also appear to be enriched within the MLL bcr in ChIP samples obtained with either the anti-R-loop or anti-biotin antibodies. On the other hand, topo II/α, although also associated with the MLL bcr as shown by ChIP, exhibits no genomewide colocalization with either the nicks or the R-loops. The R-loops, as detected by the S9.6 antibody, are localized to the matrix region in nuclear halo experiments. The halo size is sensitive to RNase H treatment throughout the cell cycle. The expression of the RNA/DNA-hybrids in the nucleus can be suppressed by treatment with actinomycin D. Mapping of R-loops and nick in the rDNA cluster of *S. cerevisiae* is in progress. The data obtained so far allow envision a model with ≥50 kb superhelical chromatin loops emanating from their anchorage sites that comprise RNA/DNA-hybrids and are also distinguished by nick-accumulation in their vicinity.

The consequences of incorporating ribonucleotides into DNA

Stephanie A. Nick McElhinny [1], Dinesh Kumar [2,3], Alan B. Clark [1], Danielle L. Watt [1], Jessica S. Williams[1], Brian E. Watts[1], Else-Britt Lundström[2], Erik Johansson[2], Andrei Chabes[2,3], Thomas A. Kunkel[1].

[1]Lab Molec. Genet. and Lab Struct. Biol., NIEHS, NIH, RTP, NC 27709, [2]Depart. Medical Biochem. & Biophys., Umeå University, SE-901 87, Umeå, Sweden, and [3]Lab for Molec. Infection Medicine Sweden (MIMS), Umeå University, SE-901 87, Umeå, Sweden

In reactions containing dNTP and rNTP concentrations present in vivo, *S. cerevisiae* DNA polymerases alpha, delta and epsilon incorporate rNMPs into DNA during DNA synthesis in vitro (PNAS 107:4949). Quantification of the amount of rNMPs incorporated, and extrapolations based on the roles of these three polymerases in replication, suggests that rNMPs could be among the most common non-canonical nucleotides introduced into the nuclear genome. To determine if ribonucleotides are incorporated during DNA replication in vivo, we substituted Leu or Gly for Met644 in the active site of Pol epsilon. Compared to wild type Pol epsilon, rNMP incorporation in vitro is 3-fold lower for M644L Pol epsilon and 11-fold higher for M644G Pol epsilon. This order is re-capitulated in vivo in yeast strains lacking RNase H2. Compared to control strains, a pol2-M644G rnh201Δ mutant strain progresses more slowly through S-phase, has elevated dNTP pools and generates 2-5 base pair deletions in repetitive sequences at a high rate. The data indicate that rNMPs are incorporated during replication in vivo, that they are removed by RNase H2-dependent repair, and that defective repair results in replicative stress and genome instability via strand misalignment.

STRUCTURAL STUDIES OF RNases H2

Marcin Nowotny[1], Monika Rychlik[1], Hyongi Chon[2], Susana M. Cerritelli[2], Robert J. Crouch[2]

[1]International Institute of Molecular and Cell Biology, Warsaw, Poland

[2]Program in Genomics of Differentiation, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, USA

Type 2 RNases H or RNases H2 cleave preferentially on the 5' side of 5'-RNA-DNA-3' junctions and can also cleave single ribonucleotides embedded in the DNA. It was recently shown that single ribonucleotides result from misincorporation by DNA polymerases and occur very frequently in the DNA in eukaryotic cells. They can lead to genomic instability and have to be removed by the joint action of RNase H2 and flap endonuclease. While bacterial and archaeal RNases H2 are monomeric, all eukaryotic RNases H2 comprise three subunits – the catalytic one and two auxiliary ones. Mutations of the human enzyme lead to a severe autoimmune disorder called Aicardi-Goutières syndrome (AGS).

In order to elucidate the mechanism of action of RNases H2, we have determined structures of a bacterial enzyme in complex with nucleic acid substrate. They revealed the mechanism by which the enzyme specifically recognizes the 5'-RNA-DNA-3' junction. The structures showed that two key metal ions are involved in the hydrolysis reaction. A conserved tyrosine residue distorts the nucleic acid at the junction. The deformation allows the phosphate group of the junction to coordinate one of the catalytic metal ions. The structure, together with biochemical experiments explains the differences in biochemical properties and substrate preference between type 1 and type 2 RNases H.

Reconstitution of the yeast RNase H2 complex to establish structure-function relationship among their subunits

Tuan Anh Nguyen, Chul-Hwan Lee, Young-Hoon Kang, Il-Taeg Cho, Min-Jung Kang, and Yeon-Soo Seo*

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RNase H2 has been implicated in Okazaki fragment processing by virtue of its intrinsic ability to remove RNA hybridized to DNA and ribonucleotides erroneously incorporated by DNA polymerases. The *Saccharomyces cerevisiae* RNase H2 complex is a heterotrimeric enzyme that consists of Rnh201, Rnh202, and Rnh203. The function of each subunit in catalysis and the role of RNase H2 in Okazaki fragment processing have not been completely clarified yet. In this study, we have attempted to establish a structure-function relationship among the subunits of the RNase H2 complex and its genetic interactions with other genes involved in Okazaki fragment processing. By reconstituting a functional complex from individual subunits and/or heterodimeric sub-complexes isolated from *Escherichia coli*, we found that neither Rnh201, the catalytic subunit alone nor any combination of two subunits (Rnh201 and Rnh202, Rnh201 and Rnh203, or Rnh202 and Rnh203) displayed any enzymatic activity. Rnh203 acted as a bridge molecule to tether Rnh201 and Rnh202 in the complex since it could form a complex with either Rnh202 or with Rnh201. Rnh201 and Rnh202 failed to form a complex in vitro. This confirms that all three subunits are essential for enzymatic activity of the yeast RNase H2 complex. Mutational analyses showed that both Rnh201 and Rnh202 possessed substrate binding activity, contributing to overall substrate binding of the RNase H2 complex to its substrate. The substrate binding activity of both subunits is critical particularly for cleavage of embedded ribonucleotides. In addition, we found that mutant RNase H2 genes that affected complex formation, substrate binding, or catalytic function showed synergistic growth defects in combination with mutations in FEN1 or DNA2, strengthening the importance of RNase H2 activity in Okazaki fragment processing.

The structure of the mammalian RNase H2 complex provides insight into RNA-DNA hybrid processing to prevent immune dysfunction

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The mammalian RNase H2 ribonuclease complex has a critical function in nucleic acid metabolism to prevent immune activation with likely roles in processing of RNA primers in Okazaki fragments during DNA replication, in removing ribonucleotides misinserted by DNA polymerases, and in eliminating RNA-DNA hybrids during cell death. Mammalian RNase H2 is a heterotrimeric complex of the RNase H2A, RNase H2B, and RNase H2C proteins that are all required for proper function and activity.
Mutations in the human RNase H2 genes cause Aicardi-Goutières syndrome.We have determined the crystal structure of the three-protein mouse RNase H2 enzyme complex to better understand the molecular basis of RNase H2 dysfunction in human autoimmunity. The structure reveals the intimately interwoven architecture of RNase H2B and RNase H2C that interface with RNaseH2Ain a complex ideally suited for nucleic acid binding and hydrolysis coupled to protein-protein interaction motifs that could allow for efficient participation in multiple cellular functions. We have identified four conserved acidic residues in the active site that are necessary for activity and suggest a two-metal ion mechanism of catalysis for RNase H2. An Okazaki fragment has been modeled into the RNase H2 nucleic acid binding site providing insight into the recognition of RNA-DNA junctions by the RNase H2. Further structural and biochemical analyses show that some RNase H2 disease-causing mutations likely result in aberrant protein-protein interactions while the RNase H2A subunit-G37S mutation appears to distort the active site accounting for the demonstrated substrate specificity modification.

Localization of the known AGS mutations on the human RNase H2 structure

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Ribonuclease H2 (RNase H2) is the major nuclear enzyme involved in the degradation of RNA/DNA hybrids and removal of ribonucleotides misincorporated in genomic DNA. Mutations in each of the three RNase H2 subunits have been implicated in a human auto-inflammatory disorder, Aicardi-Goutières Syndrome (AGS). To understand how mutations impact on RNase H2 function we determined the crystal structure of the human heterotrimer. We achieved this using the murine RNase H2 structure as a search model for molecular replacement. Initially, limited refinement into the human electron density resulted in poor geometry and statistics. We therefore re-refined the murine structure against deposited structure factors, and propose several changes to the previously reported murine RNase H2 atomic model.

Overall, the murine and human RNase H2 complexes show similar structures, including an interwoven triple barrel fold involving all three subunits. We provide biochemical validation for our structural model and our results provide new insights into how the subunits are arranged to form an enzymatically active complex. In particular, we establish that the RNASEH2A C-terminus is a eukaryotic adaptation for binding the two accessory subunits, with residues within it required for enzymatic activity. This C-terminal extension interacts with the RNASEH2C C-terminus and both are necessary to form a stable, enzymatically active heterotrimer. Disease mutations cluster at this interface between all three subunits, destabilizing the complex and/or impairing enzyme activity. Altogether, we locate 25 out of 29 residues mutated in AGS patients, establishing a firm basis for future investigations into disease pathogenesis and function of the RNase H2 enzyme.

Localization of the known AGS mutations on the human RNase H2 structure

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We have determined a 3.1 Å structure of human RNase H2 complex using Molecular Replacement method and mouse RNase H2 complex as the search model. The asymmetric unit contains six copies of the RNase H2 complex, which were refined independently. This facilitates the validation of the tracing and also allowed us to better define conformational mobility of the complex. The catalytic subunit contains two domains – the N-terminal one adopts an RNase H fold and the smaller C-terminal one is composed of α-helices. Subunits B and C form a tightly intertwined dimer with a triple-barrel fold in the center. In the catalytic subunit the interface for binding of the auxiliary subunits is located on the side of the C-terminal helical domain. For auxiliary subunits the interactions involve almost exclusively subunit C – one of its helices and a portion of the triple-barrel.

Human and mouse complexes are overall quite similar, as expected from high sequence similarity, but there are significant differences in tracing of several important regions of subunits B and C. For example, we propose different tracing of the mid-part of subunit B (after residue 90). Another difference is in a loop in the middle of subunit C. We assign a much longer sequence to it, which is not visible in our electron density maps. Because of the different tracing, we are able to map all of the 29 reported point mutations discovered in AGS patients onto our structure. We can now use our data to propose the possible structural and biochemical consequences of these mutations.

RNASEH2B is required for the nuclear localisation of human RNase H2 and its presence at replication foci

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Aicardi-Goutières Syndrome (AGS) is a genetic autoimmune disorder, with variable onset in the first year of life. The clinical features show similarities to several autoimmune diseases and congenital viral infections. AGS can result from mutations in any of the three genes that encode the protein subunits of the RNase H2 enzyme. The RNase H2 complex contains the catalytic RNASEH2A subunit and the RNASEH2B and RNASEH2C subunits, which are thought to provide structural support and facilitate interactions between the complex and additional cellular proteins. RNase H2 can cleave the RNA strand of RNA:DNA hybrids as well as 5' of single ribonucleotides embedded in a DNA duplex. Little is known about the precise physiological role of RNase H2, however, its ability to act on these substrates suggests two potential roles. RNase H2 may have a role in both DNA replication (removal of RNA primers during lagging strand synthesis) and DNA repair (removal of mis-incorporated ribonucleotides from genomic DNA). Alternative or additional roles in other cellular processes (e.g. transcription) involving RNA:DNA hybrids are also possible.

As the cellular localisation may tell us something about the biological role of this nuclease, we examined the cellular localisation of the RNase H2 subunits. To investigate the mechanisms that localise RNase H2 within the cell, we tagged each of the three subunits with EGFP. EGFP-RNASEH2B localises to the nucleus, consistent with the presence of a predicted nuclear localisation signal (NLS). Indeed, we found this NLS to be necessary and sufficient for the nuclear localisation of RNASEH2B. In contrast, EGFP-RNASEH2A and EGFP-RNASEH2C were mostly cytoplasmic in localisation. Co-transfection with RNASEH2B resulted in nuclear localisation of both EGFP-RNASEH2A and EGFP-RNASEH2C, suggesting that they need RNASEH2B to gain entry to the nucleus. Within the nucleus, we find that the interaction with PCNA is required to localise EGFP-RNASEH2B to replication foci. In contrast, the nuclear portion of RNase H1 is absent from these sites of active replication, ensuring that RNase H2 is the dominant RNase H activity during DNA replication.

We therefore conclude that the RNASEH2B subunit plays an important role in the intracellular localisation of the RNase H2 complex. Furthermore, the presence of EGFP-RNASEH2B at replication foci strongly supports a role for RNase H2 in an aspect of DNA replication. These findings provide useful information into potential cellular functions for RNase H2 and will help to determine the cellular processes that underlie the pathology of AGS.

The phenotypic spectrum of systemic autoimmunity in Aicardi-Goutières syndrome suggests a role of RNASEH2 as modulator of the innate immune response

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Aicardi-Goutières syndrome (AGS) is an early-onset encephalopathy resembling congenital viral infection characterized by basal ganglia calcifications, brain atrophy, and elevation of the antiviral cytokine interferon-α in cerebrospinal fluid. AGS is an autosomal recessive disorder caused by mutations in at least 5 genes encoding the three subunits of the ribonuclease H2 (*RNASEH2A-C*), the DNA exonuclease (*TREX1*), and the SAM domain and HD domain-containing protein 1 (*SAMHD1*), respectively.

In 20 patients with molecularly proven AGS, clinical and laboratory investigations revealed a high prevalence of findings commonly seen in patients with the autoimmune disease systemic lupus erythematosus such as cytopenia, auto-antibodies, skin lesions, oral ulcers, and arthritis, indicating that inherited defects in nucleic acid metabolism could lead to systemic autoimmunity. To examine the effects of RNASEH2 mutations identified in AGS patients on subcellular localization and integrity of the RNASEH2 complex, we co-transfected all three subunits tagged at the N-terminus with different fluorophores (YFP-A, CFP-B, Cherry-C) into HeLa cells. Co-expression of YFP-A-wt, CFP-B-wt, and Cherry-C-wt showed that RNASEH2 localizes to the nucleus. Co-expression of YFP-A-G37S or CFP-B-A177T in the context of the corresponding wt subunits did not alter nuclear localization of the RNASEH2 complex, while co-expression of Cherry-C-D115fs led to cytosolic and nuclear distribution of both the mutant C and the wild type A subunit. Fluorescence resonance energy transfer/ fluorescence lifetime imaging (FRET-FLIM) was used to further examine spatial relationships of individual subunits in living cells. As expected, C-D115fs showed a strong loss of interaction with the A and the B subunit indicating that the C-terminus of the C subunit is important for complex integrity. Interaction of A-G37S with the B, but not with the C subunit, was slightly, but significantly altered. B-A177T did not affect interactions with either the A or the C subunit. Thus, loss of subcellular targeting or integrity of the RNASEH2 complex that may interfere with substrate recognition or interaction with other protein partners underlies defects in nucleic acid metabolism in AGS.

Catalytic properties of the RNase H2 subunit A mutations in Aicardi-Goutières Syndrome

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Mutations in the three RNase H2 genes have been linked to Aicardi- Goutières Syndrome (AGS). The heterotrimeric RNase H2 complex contains the catalytic RNase H2A subunit that interfaces with the intimately interwoven RNase H2B and RNase H2C subunits. Our previous work has shown that the G37S mutation in the catalytic RNase H2A subunit results in an active RNase H2 complex with altered specificity and decreased enzyme activity on some substrates. We have now determined the catalytic efficiencies of several AGS-causing RNase H2A subunit missense mutations including R108W, R186W, N212I, F230L, R235Q, T240M, and the R25R deletion mutant. All mutants were purified as stable three-subunit complexes further supporting a required three-subunit structure in mammalian RNase H2. The RNase H2 subunit A mutant complexes exhibit a wide spectrum of altered catalytic activities ranging from wild-type to greater than 103-fold reduction in activity. Perturbations in activity are attributed mostly to changes in kcat values with more modest changes observed in KM values. Measurements of RNase H2 catalytic efficiency (kcat/KM) using various RNA-DNA hybrid substrates reveal a wide substrate preference for the enzyme supporting a role for RNase H2 in multiple nucleic acid metabolic processes. Complex interactions of RNase H2 with different substrates are indicated in the varied kinetic parameters revealed in these analyses. Furthermore, the different RNase H2 subunit A mutants exhibit large variations in the kinetic parameters and overall catalytic efficiencies. Our results indicate that mutations in the RNase H2 subunit A affect catalytic and nucleic acid binding properties with relevance to AGS.

PCNA directs Type 2 RNase H activity in DNA replication and repair

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Ribonuclease H2 is the major nuclear enzyme degrading cellular RNA/DNA hybrids in eukaryotes and the sole nuclease known to be able to hydrolyze ribonucleotides misincorporated during genomic replication. Mutations in any of the three subunits of the human RNase H2 enzyme complex cause Aicardi-Goutières Syndrome (AGS), an autoinflammatory disorder that may arise from nucleic acid byproducts generated during DNA replication. Intriguingly, an interaction between type 2 RNase H and PCNA is conserved in eukaryotes and Euryarchaeota. The PCNA-interacting peptide (PIP) box motif resides in the accessory RNASEH2B subunit of the eukaryotic RNase H2 trimer, whereas the PIP-box motif of monomeric archaeal RNase HII is located in a C-terminal tail that extends from its core.

We determined the crystal structures of *Archaeoglobus fulgidus* RNase HII in complex with PCNA, and that of a C-terminal peptide from human RNASEH2B bound to PCNA. In the archaeal structure, three binding modes are observed as the enzyme rotates about a flexible hinge while anchored to PCNA by its PIP-box motif. Using biochemical analysis, we show that PCNA promotes RNase HII activity in a hinge and PIP-box dependent manner. It enhances both cleavage of ribonucleotides misincorporated in DNA duplexes, and the comprehensive hydrolysis of RNA primers that need to be removed during Okazaki fragment maturation. In addition, PCNA imposes strand specificity on RNase H2 cleavage and guides RNase H2 to sites of active replication in vivo. Our findings provide insights into how Type 2 RNase H activity is directed during genome replication and repair, and suggest a mechanism by which RNase H2 may suppress generation of endogenous inflammatory nucleic acids.

Determining the trajectory of nucleic acids bound to HIV-1 reverse transcriptase by site-specific chemical footprinting

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Metal-dependent and –independent site-specific footprinting techniques have been used to determine the position of HIV-1 reverse transcriptase on its nucleic acid substrate, as well as its translocational equilibrium. Fe2+ footprinting involves the generation of reactive hydroxyl radicals that cleave the DNA template at the RNase H active site, or positions -17 and -18 upstream of the 3' primer end. KOONO footprinting provides a metal-free source of hydroxyl radicals at the RT residue C280, in close proximity to the DNA template and cleaves the template at positions -7 and -8. We describe here a second KOONO cleavage, this time on the DNA primer in the vicinity of the mutant RT residue T473C. Located in the RNase H primer grip motif, this cleavage occurs at positions -14 and -15. This allows the development of a novel method for directly measuring the trajectory of the nucleic acid. This new technique is highlighted by the use of methylphosphonate-modified DNA primers, which have modified trajectories relative to a "wildtype" DNA primer.

The position of HIV-1 reverse transcriptase on the template-primer is a determinant of primer mispair extension efficiency

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HIV-1 reverse transcriptase (RT) has a high propensity for misincorporation, which requires both dNTP misinsertion and the subsequent extension of the mispaired terminus thus formed. In general, a mismatched primer terminus is extended with low efficiency by DNA polymerases. However, HIV RT extends mismatches relatively efficiently compared to other polymerases, and extends some mismatched termini as efficiently as matched termini.

We have previously demonstrated that a residue that contacts the phosphate moiety of the incoming nucleotide, Lysine 65, plays a role in mismatch fidelity in addition to its recognized role in misinsertion fidelity. In that work, we precisely mapped the position of the reverse transcriptase on the template-primer, by using the RNase H active site to generate hydroxyl radicals. We demonstrated that on the template-primers tested, the wild type HIV-1 RT has a higher propensity to occupy an upstream position (-2 with respect to the templating base) on the mismatched primer, compared to position +1 on matched template-primer. The substitution of alanine for lysine 65 did not alter this change in position when presented with the mismatched primer-template, but rather reduced nucleotide utilization in the context of the mismatched terminus.

In order to further investigate the role of the RT position on mismatch extension efficiency, we have now analyzed extension by wild-type RT of a series of primers differing only in the terminal base-pair. The particular nucleotide present at the primer terminus, or the complementary template nucleotide, had little, or no, effect on the position of RT at a matched terminus. However, we show that with a mismatched primer terminus the occupancy of the upstream –2 position varies depending on the primer and template sequence. The rate (kcat) of mismatched primer extension correlated with the proportion of enzyme at the –2 position, but there was no correlation between the position and Km. This data suggests that the enzyme bound at the -2 position is non-productive, and we have demonstrated that the RT must move from this position in order for extension to take place. This non-productive binding position of the RT appears to contribute to the discrimination against extension of a mismatched terminus.

Effects of RNase H-inactivating mutations on the fidelity of HIV-1 group O reverse transcriptase

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Phylogenetic analysis of HIV-1 sequences led to the identification of four major clades: Group M (main), O (outlier), N (non-M/non-O) and P. Group M-subtype B strains (e.g., HXB2, BH10, NL4-3, etc…) have been widely used as a reference in virological studies aimed at the identification and development of antiretroviral drugs. HIV-1 group O shares approximately 65% nucleotide sequence identity with other groups of HIV-1. Amino acid sequence differences between the reverse transcriptases (RTs) of group M (subtype B) and group O HIV-1 variants can represent as much as 21% of the entire sequence. HIV-1 RTs are devoid of proofreading activity and exhibit low fidelity in comparison with eukaryotic DNA polymerases. The higher inaccuracy of HIV-1 (group M-subtype B) RT has been attributed to its strong tendency to produce -1 frameshifts (i.e., single-base deletions at nucleotide runs).
Previously, we compared the fidelity of DNA-dependent DNA synthesis catalyzed by HIV-1 group O and group M-subtype B (BH10 strain) RTs, by using gel-based pre-steady-state kinetic assays and an M13mp2 lacZα forward mutation assay. We demonstrated that the wild-type (WT) HIV-1 group O RT was about 2.5 times more faithful than the WT HIV-1 BH10 RT (Álvarez et al. J. Mol. Biol. 2009; 392: 872-884). Interestingly, unlike the WT BH10 RT, the WT HIV-1 group O RT showed a very low error rate for frameshifts, based on the analysis of the mutational spectra obtained with the M13mp2 lacZα template. A mutant HIV-1 group O RT bearing the amino acid substitution V75I also showed a very low error rate for frameshifts and a 4.7-fold increased fidelity relative to WT BH10 RT in forward mutation assays.
In this work, we have used the M13mp2 lacZα forward mutation assay to compare the effects of D443N and E478Q on the mutational spectra generated during reverse transcription in the presence of a DNA template. Both amino acid substitutions rendered enzymes devoid of RNase H activity that were around 3-4 times more faithful than the parental WT HIV-1 group O RT. Unexpectedly, their mutational spectra revealed a remarkable proportion of frameshifts (47.8% of all mutations for mutant D443N, and 47.1% for mutant E478Q). Similar results were obtained with the double-mutants V75I/D443N and V75I/E478Q. Thus, in the presence of V75I, RNase H-inactivating mutations increased fidelity while rendering enzymes with a higher tendency to generate frameshifts (>30% in both cases). Biochemical studies aimed towards understanding the molecular basis of these intriguing differences are currently underway.

N348I in HIV-1 Reverse Transcriptase Can Counteract the Nevirapine-mediated Bias toward RNase H Cleavage during Plus-Strand Initiation

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Drug resistance-associated mutations in HIV-1 reverse transcriptase (RT) can affect the balance between polymerase and ribonuclease H (RNase H) activities of the enzyme. We have recently demonstrated that the N348I mutation in the connection domain causes selective dissociation from RNase H-competent complexes, whereas the functional integrity of the polymerase-competent complex remains largely unaffected. N348I has been associated with resistance to the non-nucleoside RT inhibitor (NNRTI), nevirapine; however, a possible mechanism that links changes in RNase H activity to changes in NNRTI susceptibility remains to be established. To address this problem, we consider recent findings suggesting that NNRTIs may affect the orientation of RT on its nucleic acid substrate and increase RNase H activity. Here we demonstrate that RNase H-mediated primer removal is indeed more efficient in the presence of NNRTIs; however, the N348I mutant enzyme is able to counteract this effect. Efavirenz, a tight binding inhibitor, restricts the influence of the mutation. These findings provide strong evidence to suggest that N348I can thwart the inhibitory effects of nevirapine during initiation of (+)-strand DNA synthesis, which provides a novel mechanism for resistance. The data are in agreement with clinical data, which demonstrate a stronger effect of N348I on susceptibility to nevirapine as compared with efavirenz.

The N348I mutation of HIV-1 RT causes nevirapine resistance by an RNase H-independent mechanism that is based on decreased inhibitor binding (cancelled)

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The N348I mutation at the connection subdomain (CS) of HIV-1 reverse transcriptase (RT) confers clinically significant resistance to both nucleoside (NRTIs) and non-nucleoside RT inhibitors (NNRTIs) by mechanisms that are not well understood. We used transient kinetics to characterize the enzymatic properties of N348I RT and determine the biochemical mechanism of resistance to the NNRTI nevirapine (NVP). We demonstrate that changes distant from the NNRTI-binding pocket decrease inhibitor binding (increase $K_{d\text{-}NVP}$) by primarily decreasing the association rate of the inhibitor ($k_{on\text{-}NVP}$). We characterized RTs mutated in either p66 ($p66_{N348I}/p51_{WT}$), p51 ($p66_{WT}/p51_{N348I}$), or both subunits ($p66_{N348I}/p51_{N348I}$). Mutation in either subunit causes NVP resistance during RNA-dependent and DNA-dependent DNA polymerization. Mutation in p66 alone ($p66_{N348I}/p51_{WT}$) causes NVP resistance without significantly affecting RNase H activity, whereas mutation in p51 causes NVP resistance and impairs RNase H, demonstrating that NVP resistance can occur independently from defects in RNase H function. Mutation in either subunit improves affinity for nucleic acid and enhances processivity of DNA synthesis. Surprisingly, mutation in either subunit decreases catalytic rates (k_{pol}) of $p66_{N348I}/p51_{N348I}$, $p66_{N348I}/p51_{WT}$, and $p66_{WT}/p51_{N348I}$ without significantly affecting affinity for deoxynucleotide substrate ($K_{d\text{-}dNTP}$). Hence, in addition to providing structural integrity for the heterodimer, p51 is critical for fine-tuning catalytic turnover, RNase H processing, and drug resistance. In conclusion, connection subdomain mutation (CSM) N348I decreases catalytic efficiency and causes *in vitro* resistance to NVP by decreasing inhibitor binding.

(Substitute)

A small step for RT, a giant step for HIV: conformational dynamics of tRNA-primed initiation of reverse transcription

Stuart F. J. Le Grice et al.

Alizarine derivatives as new dual inhibitors of the HIV-1 reverse transcriptase(RT)-associated DNA polymerase and Ribonuclease H (RNase H) activities effective also on the RNase H activity of non-nucleoside resistant RTs

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The HIV-1 reverse transcriptase (RT) has two associated activities, DNA polymerase and Ribonuclease H (RNase H), both essential for viral replication and validated drug targets. In this report several alizarine derivatives were synthesized and tested on both HIV-1 RT-associated activities. Alizarine analogues K-49 and KNA-53 showed IC50 values for both RT-associated functions around 10 μM. When tested on the K103N RT both derivatives equally inhibited the RT-associated functions, while when tested on the Y181C RT, only KNA-53 inhibited the RNase H function but was inactive on the polymerase function. Mechanism of action studies showed that these derivatives do not intercalate into DNA and do not chelate the divalent cofactor Mg2+. Kinetic studies demonstrated that they are non-competitive inhibitors, they do not bind to the RNase H active site or to the classical NNRTI binding pocket, even though efavirenz binding negatively influenced K-49/KNA-53 binding and vice versa. This behavior gave us an indication that the alizarine derivatives binding site could be close to the NNRTI binding pocket. Docking experiments and molecular dynamic simulation confirmed the experimental data and the ability of these compounds to occupy a binding pocket close to the NNRTI site.

N-Hydroxynaphthyridone inhibitors of HIV Ribonuclease H

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The HIV-encoded enzyme ribonuclease H (RNase H) is required for correct reverse transcription of the single-stranded HIV-1 genomic RNA into double stranded DNA and is therefore a target for antiviral agents. RNase H is a magnesium dependent phosphohydrolase which is mechanistically related to other phosphotransferases and phosphohydrolases (including polymerases, transposases, integrases, and endonucleases) for which several classes of potent and selective inhibitors which act by binding to active site magnesium ions have been identified. Here, we describe the identification and characterization of novel N-hydroxynaphthyridinone inhibitors of HIV RNase H. These compounds block all biologically relevant RNase H activities tested including cleavage of the RNA strand in RNA-DNA hybrid duplexes, generation of the polypurine tract (PPT) primer, and cleavage of the PPT primer from nascently generated DNA during reverse transcription. The best compound was potent and selective in biochemical assays and showed antiviral efficacy in a single cycle viral replication assay. Crystal structures demonstrate that this class of compounds bind to the HIV RNase H active site via two metal ions that are coordinated by the catalytic site residues D443, E478, D498, and D549. The orientation of the N-hydroxynaphthyridinone pharmacophore is restricted by the ordering of D549 and H539 in the RNase H domain. These studies provide a means for structurally guided design of novel RNase H inhibitors.

In Vitro Combination Studies between Nonnucleoside Reverse Transcriptase Inhibitors and Ribonuclease H Inhibitors against HIV-1 Reverse Transcriptase

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Nonnucleoside reverse transcriptase inhibitors (NNRTIs) are an important therapeutic class of drugs that are used in first-line and salvage combination antiretroviral therapies to treat HIV-1 infection. The NNRTIs bind to a hydrophobic pocket in HIV-1 RT - termed the NNRTI-binding pocket - that is near to but distinct from the DNA polymerase active site of the enzyme. Although this binding pocket is distal (> 60 Å) from the ribonuclease H (RNase H) active site of the enzyme, NNRTI binding to RT can significantly impact the RNase H cleavage activity. For example, our group and other groups have demonstrated that NNRTIs can accelerate the RNase H cleavage of HIV-1 RT (Radzio & Sluis-Cremer, Mol Pharmacol 2008,73:601; Shaw-Reid et al, Biochemistry 2005, 44:1595). Based on these findings, we hypothesized that NNRTIs may impact on the activity of inhibitors targeted to the RNase H active site of HIV-1 RT. Accordingly we carried-out in vitro combination studies between efavirenz (an NNRTI) and beta-thujaplicinol derivatives (RNase H active site inhibitors) against HIV-1 RT. We demonstrate on several different template/primer substrates that efavirenz can diminish the ability of the beta-thujaplicinol derivatives to inhibit the RNase H activity of HIV-1 RT. Similarly, we show that the beta-thujaplicinol derivatives can counteract the ability of efavirenz to stimulate the RNase H activity of RT. Pre-steady-state kinetic experiments are currently underway to explore the associated underlying mechanisms. Taken together, these studies suggest an unfavorable interaction between NNRTIs and RNase H inhibitors.

Small molecule inhibitors of the RNase H function of HIV-1 reverse transcriptase.

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Two metal chelator chemotypes were designed as inhibitors of the RNase H function of HIV-1 RT. These molecules can coordinate to two divalent metal ions in the RNase H active site. Inhibition of RNase H activity was measured in a biochemical assay, but no anti-viral effect was observed. Binding was demonstrated via a solid state structure with examples of both chemotypes bound to the RNase H active site of the truncated p15Ec domain of HIV RT in the presence of two manganese II ions.

A 2.7 Å Resolution X-ray Crystal Structure for the Inhibitor Manicol Bound at the RNase H Active Site of HIV-1 Reverse Transcriptase

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Many anti-AIDS drugs target the DNA polymerase activity of HIV-1 reverse transcriptase (RT), a dual-function enzyme that is vital for the viability of the virus. Due to the rapid emergence of HIV variants that are resistant to these drugs, therapeutic agents are needed that act against new targets. There are no inhibitors used in the clinic that act against the RNase H (RNH) function of RT. RT converts viral genomic single-stranded RNA to double-stranded DNA that can be integrated into the host genome. The RNH activity degrades viral RNA after it has been copied into DNA, removes the tRNA used to initiate minus-strand DNA synthesis, and generates and removes the polypurine tract (PPT) primer used to initiate plus-strand DNA synthesis. We have previously reported a structure for RT in complex with b-thujaplicinol, a tropolone derivative that inhibits the RNH by binding to its active site. We have now solved a structure for HIV-1 RT in complex with a second tropolone derivative, manicol, to a resolution of 2.7 Å. Both compounds exhibit submicromolar inhibition of the RNH activity of RT. Like b-thujaplicinol, manicol coordinates two divalent cations at the RNH active site. Unlike b-thujaplicinol, manicol-protein contacts are dominated by interactions with the imidazole ring of His539, a conserved residue at the RNH catalytic site. What we learn from analysis of these structures may be useful for development of drugs that inhibit the RNH activity of HIV-1 reverse transcriptase.

Research efforts on RNase H in the Arnold lab have been supported by NIH grants R37 AI 27690 MERIT Award (to EA) and U19 AI073975 program project (Michael Parniak, program director).

The 3.0 Å resolution crystal structure of BTDBA, a diketo acid RNase H inhibitor, in complex with full length HIV-1 reverse transcriptase

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Emerging resistance to current AIDS therapies has led to increased interest in developing RNase H inhibitors as novel therapeutics. Out of the 25 drugs in the clinic that are approved to treat AIDS, none target the RNase H domain of reverse transcriptase (RT). The enzymatic activity of the RNase H domain is dependent on a two divalent cation mechanism which is targeted by a number of inhibitors. Members of the diketo acid class of molecules have been shown to inhibit the RNase H activity of reverse transcriptase. Although biochemical data suggests that diketo acid inhibitors bind specifically to the RNase H domain, no structure of a member of the diketo acid class in complex with reverse transcriptase has been solved to date. We have solved the 3.0 Å RESOLUTION crystal structure of the diketo acid inhibitor 4-[5-(benzoylamino)thien-2-yl]-2,4-dioxobutanoic acid (BTDBA) in complex with full length HIV-1 reverse transcriptase. The BTDBA diketo acid moiety sequesters two Mn^{2+} ions that are crucial to the RNase H catalytic mechanism. Additionally, an amide carboxylate from BTDBA makes hydrogen bonding contacts with the backbone nitrogen of Y501 and the side-chain hydroxyl of S499. Further interactions are made between the benzyl ring of the inhibitor and the Q500 side chain. These interactions define a pocket in which BTDBA interacts with reverse transcriptase and can be used as a basis for designing inhibitors with greater potency and selectivity.

Research efforts on RNase H in the Arnold lab have been supported by NIH grants R37 AI 27690 MERIT Award (to EA) and U19 AI073975 program project (Michael Parniak, program director). We are grateful to Daria Hazuda and Jay Grobler, Merck, for providing a sample of BTDBA.

Sensitivity of Xenotropic Murine Leukemia Virus-Related Retrovirus Reverse Transcriptase to α-Hydroxytropolone-Derived Ribonuclease H Inhibitors

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The natural product hydroxytropolone inhibitors β-thujaplicinol and manicol, derived from the bark of the western cedar Thuja plicata and the root bark of the Guyanan tree Dulacia guianensis, respectively, are potent and selective inhibitors of the ribonuclease H (RNase H) activity of human immunodeficiency virus reverse transcriptase (HIV RT). Recent crystallographic studies have indicated that β-thujaplicinol inhibits hydrolysis through chelation of catalytically critical magnesium ions in the RNase H active center. Based on the structural similarity of retroviral RNase H domains, we reasoned that hydroxytropolones, and derivatives thereof, would inhibit the RNaseH activity of related retroviruses. Using manicol as a scaffold, we report here activity of several novel α-hydroxytropolones against the RT-associated RNase activity of xenotropic murine leukemia virus-related retrovirus (XMRV), a novel gammaretrovirus that has been associated with both prostate cancer and chronic fatigue syndrome. In addition, we demonstrate that the nucleoside RT inhibitor AZT, which was shown to effectively inhibit XMRV replication, displays synergy with selected hydroxytropolone RNase H inhibitors, which would be beneficial to future combination antiviral therapy. Finally, unlike HIV-1 RT, XMRV RT exhibits negligible pyrophosphorolysis activity, and thus is insensitive to stimulation by eliminating RNase H activity. Taken together, these observations suggest an NRTI/RNase H inhibitor combination would be an effective XMRV combination therapy.