Functional Investigation of the ATPase/Helicase Domain of Human Dicer

A PhD thesis submitted in fulfilment of the requirements for the degree of doctor rerum naturalium (Dr. rer. nat.) in

Institute of Biochemistry and Molecular Biology, Department of Chemistry, Faculty of Mathematics, Informatics and Natural Sciences, University of Hamburg, Germany

By

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Disputation on 09.01.09

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This work was carried out from October 2005 to December 2008 in the laboratory of Prof. Uli Hahn at the Institute of Biochemistry and Molecular Biology, Department of Chemistry, Faculty of Mathematics, Informatics and Natural Sciences, University of Hamburg, Germany

This work is dedicated to my late beloved dad, Chief Ide Patrick Nwawulu Obi

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List of abbreviations

A/Hel	ATPase/Helicase
bp	Base pair
BSA	Bovine serum albumin
°C	Degree centigrade
CIAP	Calf intestinal alkaline phosphatase
DNA	Deoxyribonucleic acid
dNTP	2´-deoxynucleoside-5´-triphosphate
dsRNA	Double-stranded RNA
dsRBD	Double-stranded RNA binding domain
E. coli	*Escherichia coli*
EJC	Exon-junction-complex
FBS	Fetal bovine serum
FRET	Fluorescence resonance energy transfer
g	Gram
h	Hour
hDicer	Human Dicer
IPTG	Isopropyl β-D-thiogalactoside
K_{cat}	Turnover number
kDa	Kilo Dalton
K_m	Michaelis constant
LB	Luria Broth
M	Molar
MBP	Maltose binding protein
mM	Millimolar

min	Minute
mg	Milligram
µM	Micromolar
mRNA	Messenger ribonucleic acid
miRNA	Micro ribonucleic acid
Ni-NTA	Nickel nitrilotriacetic acid
NPC	Nuclear-pore complex
OD_{600}	Optical density at 600nm
OPMNPV	*Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus
PACT	Protein activator of protein kinase
P-bodies	Processing bodies
PCR	Polymerase chain reaction
P_i	Inorganic phosphate
PNK	Polynucleotide kinase
PNPase	Polynucleotidedephosphorylase
PTGS	Post transcriptional gene silencing
PVDF	Polyvinylidenefluoride
RHA	RNA helicase A
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNase	Ribonuclease
rpm	Revolution per minute
s	Second
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SF	Super family
siRNA	Small interfering ribonucleic acid

snRNA	Small nuclear ribonucleic acid
snRNP	Small nuclear ribonucleoprotein
TGS	Transcriptional gene silencing
TRBP	Tar RNA binding protein
V	Volts
v/v	Volume/volume
w/v	Weight/volume
wt	Wild-type

1 Introduction

The gene expression process is of fundamental importance for all living organisms. In eukaryotes, most genes reside in the chromosomes located in the cell nucleus and are expressed in the nucleus or cytoplasm. Regulation of gene expression in the eukaryotic cell occurs at many levels including synthesis, maturation, and degradation of protein-coding messenger RNAs (mRNAs). Differences in initiation as well as efficiency of translation are essential to the regulation of diverse genes (Dever *et al*, 2002).

In recent years, the control of gene expression by small non-coding RNA molecules has emerged as a major new mechanism for gene regulation (Levine *et al*, 2007). This gene regulation mechanism is called RNA-interference (RNAi), which involves small RNAs such as small interfering RNAs (siRNAs) and microRNAs (miRNAs). These small RNA molecules play a central role in cellular mechanisms that control the expression and degradation of mRNA. Many proteins are involved in the RNAi pathway, of which Dicer is one of the essential components. It is believed that Dicer has broad functions in the RNAi pathway. Thus, understanding the functions of Dicer will be essential for the elucidation of the RNA-mediated pathways of gene expression.

1.1 RNA interference

RNAi is an evolutionarily conserved process by which double-stranded RNA (dsRNA) induces highly specific gene silencing, first discovered by Andrew Fire and Craig Mello in 1998 (Fire *et al*, 1998). They reported the fascinating observation that injection of dsRNA into the worm *Caenorhabditis elegans* resulted in a specific silencing of homologous gene expression. This silencing was more potent than that obtained by injection of the antisense or

sense RNA alone, and the researchers earned a Nobel Prize for their work in 2006. Prior to the discovery of RNAi, a phenomenon called gene silencing was described in plants. Around the year 1990 experimenters noted that a transgene could not induce or stimulate gene activity but could inhibit the expression of homologous sequences (Napoli *et al*, 1990, Van der Krol *et al*, 1990). The inhibition of gene activity could take place at the transcriptional level (transcriptional gene silencing (TGS)), or at the posttranscriptional level (posttranscriptional gene silencing (PTGS)). A PTGS-like process called quelling was also established in the fungus *Neurospora crassa* (Cogoni and Macino, 1999).

Until now, the RNAi process has been found only in eukaryotes, where it is active in organisms ranging from yeast to humans. It is believed to play a fundamental role in diverse eukaryotic functions including: viral defence, silencing of transposons, chromatin remodelling, genome rearrangement, developmental timing, brain morphogenesis and stem cell maintenance (MacRae *et al*, 2006, Carmell *et al*, 2004).

1.1.1 Mechanism of RNAi

The RNAi process is initiated when dsRNA is processed into 21 - 23 nucleotide long dsRNA fragements called siRNAs by the ribonuclease (RNase) III enzyme, Dicer. dsRNA can be formed in cells by DNA- or RNA-dependent synthesis of complementary RNA strands or introduced into cells by viral infection or artificial expression (Filipowicz *et al*, 2005). The siRNAs are then incorporated into an RNA-Induced Silencing Complex (RISC), which mediates the degradation of mRNAs with sequences fully complementary to the siRNA (Figure 1.1). RISC is comprised of many proteins, of which representatives of the Argonaute family are the major component. The degradation of the complementary mRNAs is carried out by the Argonautes, which have endonuclease activity.

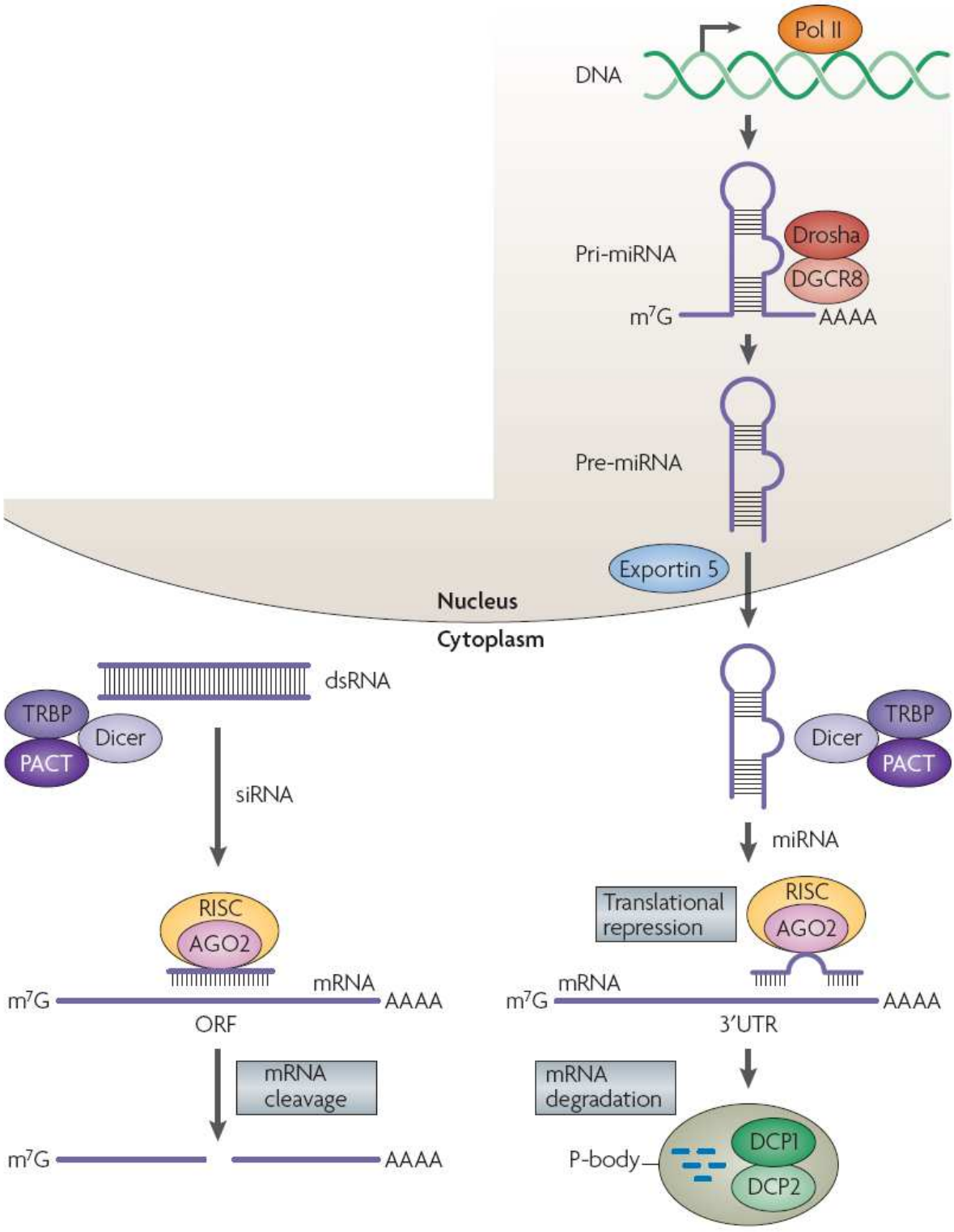


Figure 1.1: Post-transcriptional gene regulation by siRNAs and miRNAs (modified from Kim and Rossi, 2007). In mammals, Dicer participates in siRNA and miRNA pathways. Cleavage of dsRNA and precursor miRNA by a complex consisting of Dicer, TAR RNA-binding protein (TRBP) and protein activator of protein kinase (PACT) occurs in the cytoplasm. In a coordinated manner, the siRNA or miRNA is transferred to RISC, where a complementary mRNA is degraded or its translation repressed. Repressed mRNAs are degraded in P-bodies.

miRNAs are a second class of small RNA molecules that can induce silencing by targeting mRNA (Matzke and Birchler, 2005). They are processed from precursor molecules, which correspond to transcripts of independent miRNA genes (Kim, 2005). Maturation of miRNAs occurs in two steps, both catalysed by enzymes of the RNase III family- Drosha and Dicer (Figure 1.1). The mature miRNAs are incorporated into a RISC-like complex and, depending on their degree of complementarity to the target mRNA, elicit either translational repression or mRNA cleavage. When a miRNA has complete sequence complementarity with a target mRNA, it directs cleavage of the mRNA through RISC activity. In a case of partial sequence complementarity between the miRNA and the target mRNA, a translational repression of the mRNA occurs. The translation-repressed mRNAs are transported to cytoplasmic compartments known as processing bodies (P-bodies), where the mRNAs are degraded.

1.1.2 The Dicer enzyme

Dicer is a large multi-domain ribonuclease responsible for processing dsRNA to approximately 20-base pairs (bp) long siRNAs, which act as effectors during RNAi (Kolb *et al*, 2005). Dicer also catalyses the excision of miRNA from stem-loop precursors (pre-miRNAs). This ribonuclease was first identified in 2001 by Bernstein and colleagues, who were working with *Drosophila* S2 cells (Bernstein *et al*, 2001). Because of its ability to digest dsRNA into uniformly sized small RNAs, they named this enzyme Dicer. Dicer proteins have been found in all eukaryotes studied to date, with the exception of *Saccharomyces cerevisiae* (Kolb *et al*, 2005). Vertebrates and *Caenorhabditis elegans* encode only one Dicer, which can process both dsRNA and miRNA precursors (Filipowicz *et al*, 2005). However, genomes of many other organisms encode from two (e.g., *Drosophila melanogaster* and *Neurospora*

crassa) to four (*Arabidopsis thaliana*) Dicer proteins. In fly cells, there is a division of labour as the two Dicers have clearly different functions in RNA silencing. Dcr-2 is the major siRNA-producing enzyme in RNAi, while Dcr-1 is vital in miRNA-triggered gene silencing (Lee *et al*, 2004).

Dicer is a member of the RNase III protein family. This protein family is divided into three classes: Class 1, 2, and 3 are represented by *E. coli* RNase III, Drosha and Dicer, respectively (Blaszczyk *et al*, 2001, Nicholson *et al*, 2003). Class 1 enzymes have one RNase III domain, whereas class 2 and 3 enzymes have two RNase III domains.

Dicers are large proteins of ~200 kDa, containing typically an N-terminal ATPase/Helicase domain (A/Hel), a domain of unknown function (DUF283), a PIWI/Argonaute/Zwille domain (PAZ), two RNase domains (RNase IIIa and RNase IIIb), and a C-terminal dsRNA binding domain (dsRBD; Figure 1.2). These domains are highly conserved in Dicers from different organisms. The A/Hel domain is found in almost all Dicer proteins, except in the Dicer enzyme from *Giardia intestinalis*. This emphasizes the evolutionary conservation of this domain.

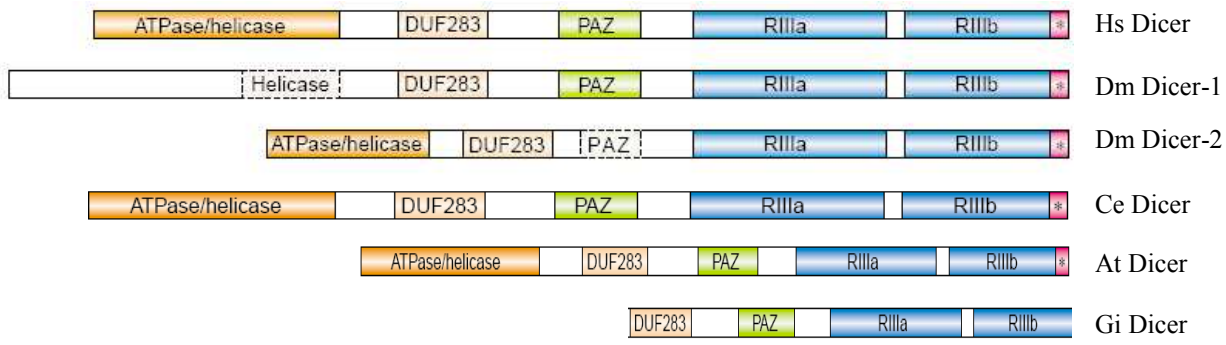


Figure 1.2: Domain structures of Dicer (modified from Filipowicz *et al*, 2005). Dicer belongs to the RNase III family of proteins and contains two RNase III catalytic domains (RIIIa and RIIIb) and a double-stranded RNA binding domain (denoted by the asterisk in the diagram). In addition, Dicers have an N-terminal ATPase/Helicase domain (A/Hel), followed by a domain of unknown function (DUF283) and a PIWI/Argonaute/Zwille domain (PAZ). Dicer from *Giardia intestinalis* lacks the N-terminal A/Hel domain and the C-terminal dsRBD. Abbreviations are: Hs: *Homo sapiens*; Dm: *Drosophila melanogaster*; Ce: *Caenorhabditis elegans*; At: *Arabidopsis thaliana*; Gi: *Giardia intestinalis*.

The functions of the A/Hel and DUF 283 domains are yet not been proved (Hammond *et al*, 2005, Filipowicz *et al*, 2005, Pellino *et al*, 2005, Tabara *et al*, 2002). Using a computational

approach, the DUF 283 domain has been predicted to function in dsRNA binding (Dlakic *et al*, 2006). Structural and mutagenesis studies have indicated that the PAZ domain is involved in binding of 3′-protruding ends of either siRNA or dsRNA substrates (Lingel *et al*., 2003, Song *et al*., 2003, Yan *et al*., 2003, Zhang *et al.*, 2004). The PAZ domain is also found in the Argonaute protein family involved in RNAi effector complexes and is named after three founding Argonaute proteins, Piwi, Argonaute and Zwille (Hammond *et al*, 2001). The RNase domains are involved in the cleavage of phosphodiester bonds of dsRNA into siRNA, while the dsRBD is involved in dsRNA binding (Zhang *et al*, 2004).

Sequence similarity of Dicer RNase III-like domains to the bacterial and fungal RNase III suggests that Dicer cleaves dsRNA through a similar mechanism. Early models predicted that Dicer forms a dimer on the substrate and performs four cleavage reactions (Zamore *et al*, 2001). However, recent data favour a model whereby Dicer acts as a monomer containing a single processing centre formed through intramolecular dimerization of the two RNase III domains of the same Dicer molecule (MacRae *et al*, 2006, Hammond *et al*, 2005 Zhang *et al*, 2004) (Figure 1.3).

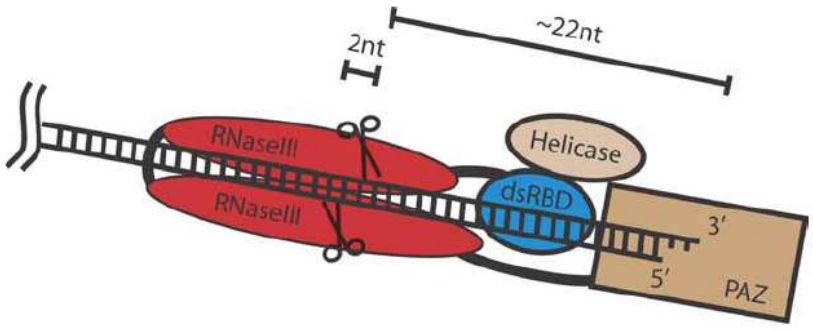


Figure 1.3: Model of Dicer catalysis (Hammond *et al*, 2005). The enzyme contains a single dsRNA cleavage centre with two independent catalytic sites. The centre is formed by the RNaseIIIa and RNaseIIIb domains of the same Dicer molecule and processes the dsRNA into ~20 bp RNA from its termini. The binding site of the dsRBD is not defined. The function of the A/Hel domain is not known.

The centre contains two independent catalytic sites, each cutting one RNA strand of the duplex and generating products with 2-nucleotide 3′overhangs. The crystal structure of a

primitive form of Dicer from *Giardia intestinalis* confirmed that two RNase III domains of Dicer are capable of forming an intramolecular heterodimer (MacRae *et al*, 2006). Additionally, the recently solved crystal structure of the C-terminal RNase III domain (RNase IIIb) of human Dicer (hDicer) revealed that the RNase IIIb domain can form a tightly associated homodimer, which is similar to the dimers of the bacterial RNase III domains and the two RNase III domains of *Giardia intestinalis* Dicer (Takeshita *et al*, 2007). Studies using dsRNA with blocked termini suggested that Dicer preferentially cuts dsRNA at their termini and also excises miRNAs from the end of pre-miRNA hairpins produced by Drosha (Carmell *et al*, 2004, Zhang *et al*, 2002). Additionally, these studies showed that the cleavage reaction was significantly delayed, if the enzyme could not initiate processing from the end and was forced to process internally. The authors deduced this to be a result of less efficient internal binding which caused a lag in processing.

1.1.2.1 Binding partners of Dicer

Several proteins interacting with Dicer have been identified. In *Drosophila,* Dcr-2 forms a complex with a dsRNA-binding protein called R2D2 (Liu *et al*, 2003). In the absence of R2D2, Dcr-2 can still process dsRNA efficiently, but the resulting siRNA are not effectively channelled into RISC. A physical interaction between the RISC-component Ago2 and Dcr-1 in co-immunoprecipitation experiments in *D. melanogaster* S2 cell extracts has been reported (Hammond *et al*, 2001). Similarly, the Ago protein RDE-1 has also been found to co-immunopurify with Dicer in *C. elegans* (Tabara *et al*, 2002). Direct interactions between hDicer and human Ago proteins have been demonstrated to occur through an RNase III domain of Dicer and the PiWi domain of the Ago proteins (Tahbaz *et al*, 2004). Dicer was also found to be a core component of RISC, essential for mRNA target cleavage (Lee *et al*,

2004, Pham *et al*, 2004, Tomari *et al*, 2004). Recently, some studies have revealed TRBP and PACT as important Dicer-interacting proteins in mammalian systems (Chendrimada *et al*, 2005, Haase *et al*, 2005, Gregory *et al*, 2005, Lee *et al*, 2006). Both proteins, which are structurally related and contain three dsRBD, have been previously identified as regulators of interferon-induced dsRNA-activated protein kinase (PKR) function (Gupta *et al*, 2003, Peters *et al*, 2001). Currently, a study has disclosed RNA helicase A (RHA) as a new and important partner of Dicer in human cells (Robb and Rana, 2007). RHA also interacts with siRNA, Ago2 and TRBP, functioning in the RNAi pathway.

1.2 Helicases

RNA and DNA helicases are considered to be enzymes that catalyse the separation of double-stranded nucleic acids in an energy dependent manner (Cordin *et al*, 2006). Since the discovery of the first helicase from *E. coli* more than 30 years ago (Abdel-Monem and Hoffman-Berling, 1976), dozens of these fascinating enzymes have been identified and characterised, and dozens more have been proposed to possess helicase activity based on sequence homologies. Although these proteins are highly conserved on the structural level, different helicases perform diverse functions that range from unwinding thousands of DNA bp during bacterial replication to destabilizing RNA helices with less than 10 bp during processes such as precursor mRNA splicing and ribosome biogenesis. Most helicases show little or no processivity when assayed *in-vitro* (Cordin *et al*, 2006). This could be a result of the assay conditions or the lack of interacting partners, or it could be that processive unwinding is merely shared by a specialized subset of the members of the helicase family. Some helicases have been shown to remove proteins from nucleic acids even without unwinding of DNA or RNA secondary structure (Fairman *et al*, 2004, Jankowsky *et al*, 2001,

Schwer, 2001). The universal presence of helicases in prokaryotes, eukaryotes and viruses reflects their fundamental importance in DNA and RNA metabolic processes, including: replication, recombination, DNA repair, transcription, translation and RNA splicing (Schmid and Linder, 1992). Consistent with their essential roles in nucleic acid metabolism, helicases have been implicated in a growing list of human genetic disorders including: Werner syndrome, Bloom syndrome and *Xeroderma pigmentosum* (Ellis *et al*, 1997).

Helicases are encoded by a large part of the eukaryotic and prokaryotic genomes (Silverman *et al*, 2003). Gorbalenya and Koonin (1989) classified helicases into five super families (named SF1 to SF5) depending on the occurrence and characteristics of conserved motifs in the primary sequence (Table 1.1).

Table 1.1: Characteristics of the five helicase super families (Singleton *et al*, 2007).

Superfamily	**Conserved Motifs**	**Examples**	**Source**	**Oligomeric state**	**Unwinding polarity**
SF1	I, Ia, Ib, II, III, IV, V, VI	PcrA, Rep, UvrD	All kingdoms	Monomer, dimer	$3' \rightarrow 5'$ $5' \rightarrow 3'$
SF2	I, Ia, Ib, II, III, IV, V, VI	HCV NS3, RecG, RecQ	All kingdoms	Monomer, dimer	$3' \rightarrow 5'$ $5' \rightarrow 3'$
SF3	A, B, C	SV40-LTag	RNA and DNA viruses	Hexamer	$5' \rightarrow 3'$
SF4	1, 1a, 2, 3, 4	DnaB	Eubacteria, bacteriophages	Hexamer	$5' \rightarrow 3'$
SF5	n.d.	Rho	Eubacteria	Hexamer	$5' \rightarrow 3'$

Abbreviation: n.d: not determined

All helicases possess the Walker A and B motifs, which are nucleotide triphosphate (NTP) binding motifs that are also found in many NTPases (Walker *et al*, 1982). As a result they can

bind and hydrolyse NTPs. SF1 and SF2 are comprised of a large number of DNA and RNA helicases from archaea, eubacteria, eukaryotes and viruses. They are known to unwind duplexes in a 3′ to 5′direction as well as 5′ to 3′ direction. A third super family (SF3) includes small putative helicase domains of ~100 amino acid residues that are found in DNA and RNA viruses. This family has only three conserved motifs, including the two classical ATP-binding motifs. A fourth family (SF4) consists of helicases that are related in sequence to the *E. coli* DnaB protein. These proteins have five motifs, unwind DNA in the 5′ to 3′ direction, and generally form hexameric ring structures. Finally, the last family (SF5) is recognized as a family with sequence similarity to the β-subunit of proton-translocating ATPases. This family is exemplified by the transcription termination factor Rho.

Helicases can also be sub-divided on the basis of mechanistic differences. Enzymes with specificity for DNA, RNA, or DNA-RNA hybrids have all been identified. The oligomeric state of many SF1 and SF2 enzymes has been the subject of considerable debate, though they are considered to be primarily monomeric or dimeric. The other superfamilies are hexameric rings formed from six individual RecA folds. However, the three-dimensional fold of the ATP binding domains is similar in the helicases of different families (Singleton *et al*, 2000).

1.2.1 DExD/H RNA helicase family

According to the classification of Gorbalenya and Koonin, 1993, the DExD/H RNA helicase family is a member of SF2 helicases (Gorbalenya and Koonin, 1993, Tanner and Linder, 2001, Caruthers *et al*, 2002). This protein family contains the largest group of enzymes in eukaryotic RNA metabolism (Jankowsky and Bowers, 2006). The protein family is highly conserved from viruses over bacteria to humans (Bleichert *et al*, 2007). Members of

this family share eight conserved sequence motifs that are located in two different domains (Figures 1.4 and 1.5).

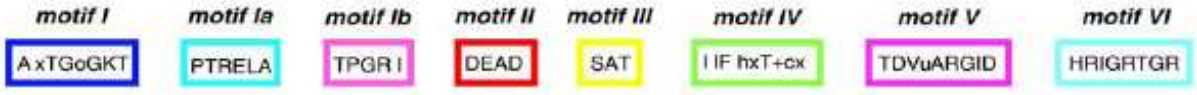


Figure 1.4: Conserved motifs in the DExD/H-box families. Abbreviations used are as follow: c: D, E, H, K, R; h: A, F, G, I, L, M, P, V, W, Y; o: S; T; I: I, L, V; x: any residue; u: A, G; +: H, K, R. Consensus sequences were taken from Cordin *et al*, 2006.

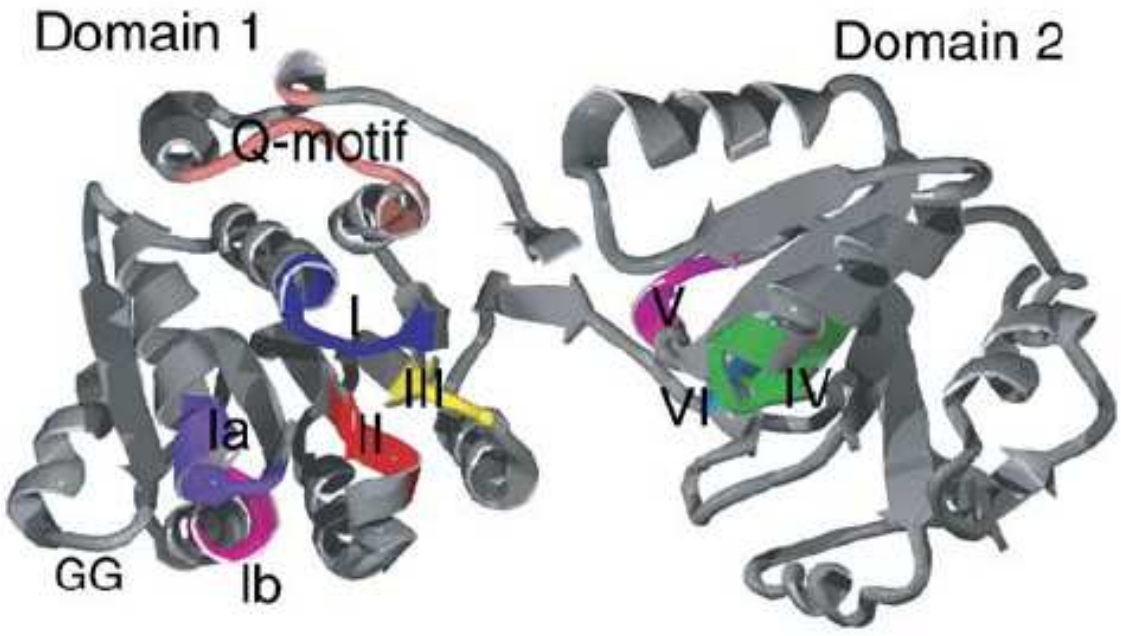


Figure 1.5: Crystal structure of the SF2 helicase eIF4A (Cordin *et al*, 2006). The conserved helicase motifs are closely associated in the tertiary structure of the protein and form a large functional domain. Motif I is colored in blue, Motif Ia in purple, Motif Ib in pink, Motif II in red, Motif III in yellow, Motif IV in green, Motif V in light purple and Motif VI in cyano.

Motifs I, Ia, Ib, II and III are found in domain 1, while Motifs IV, V and VI are in domain 2. The name of the family is derived from Motif II given as DExD/H, where x can represent any amino acid. Motifs I and II are NTP binding motifs first identified by Walker *et al*. (1982). They are also known as the Walker A and B motifs respectively. Motif I forms a loop structure, also called the P loop, that forms a pocket to bind the phosphates of NTP. Mutations of the first alanine residue, the conserved lysine or the last threonine of Motif I abolish ATPase activity by reducing the affinity for and the rate of ATP hydrolysis (Caruthers *et al*,

2002, Cordin *et al*, 2004). Motif II forms interactions with β and γ phosphates of ATP through a coordinated Mg^{2+} (Fry *et al*, 1986). The carboxyl of the aspartic acid coordinates the Mg^{2+} ion of MgATP/MgADP through outer sphere interactions, whereas the glutamic acid is suggested to act as a catalytic base in ATP hydrolysis. Mutations within Motif II decrease or abolish ATPase and helicase activities without altering RNA binding (Pause and Sonenberg, 1992, Iost *et al*, 1999). Motif III was proposed to participate in linking ATPase and helicase activities. Mutations in Motif III of elf4A and in related DEAH-box proteins cause a dramatic loss of helicase activity, but have only minimal effects on binding and hydrolysis of ATP and RNA binding (Pause and Sonenberg 1992, Schwer and Meszaros, 2000). Motifs Ia, Ib and IV have been poorly studied in DExD/H proteins. Motifs Ia and Ib are part of domain 1, whereas Motif IV is found at the bottom of domain 2. Motif V is proposed to be an RNA-binding motif in association with Motifs Ia, Ib and IV (Cordin *et al*, 2006). Nevertheless, a role in ATPase activity and in coupling the ATPase and helicase activities cannot be excluded (Caruthers *et al*, 2000). Motif V is part of a loop at the interface between domains 1 and 2 that points towards the RNA-binding region. Mutations in Motif V of the DEAD-box protein Prp28 have a detrimental effect on yeast growth, indicating that the conserved arginine and the last aspartate play important roles in the *in vivo* activity of Prp28 (Chang *et al*, 1997). Motif VI has been shown to be important for ATPase activity and RNA binding (Pause, *et al*, 1993). Several helicases exhibited nucleic acid-binding defects when Motif VI residues were altered (Pause and Sonenberg, 1992). Changing the basic residues, histidine or arginine, to the uncharged glutamine abolishes RNA binding and reduces ATP hydrolysis, which also results in reduced helicase activity. X-ray crystallographic studies have suggested that the conserved helicase motifs are closely associated in the tertiary structure of the protein and may form a large functional domain (Hall and Matson, 1999).

1.2.1.1 ATPase/Helicase domain of Dicer

Based on sequence alignments, the putative A/Hel domain of hDicer is related to the DExD/H RNA helicase family (Figure 1.6). Most Dicer orthologs contain a DExH-type ATP-dependent RNA helicase domain at their amino termini. In Dicer, the amino acid sequence of the conserved motif II is given by DECH. Most notably, Dicer from *Giardia intestinalis* lacks the amino-terminal DExH A/Hel domain present in Dicers from human, fly, worm, plant and fission yeast. Interestingly, in *Drosophila*, Dcr-1 lacks a functional helicase domain, while Dcr-2 possesses a complete A/Hel domain. Although the presence of A/Hel domain from Dicer is broadly conserved among higher eukaryotes, no function has yet been assigned to this domain. Possible activities of the A/Hel domain of Dicer include: unwinding of siRNA, translocating of Dicer along dsRNA substrates, and facilitating the handoff of substrates or products with other components of the RNAi machinery (MacRae *et al*, 2006).

	Motif I	Motif Ia	Motif Ib	Motif II	Motif III	Motif IV	Motif V	Motif VI
Consensus DEAD-box	AxTGoGKT	PTRELA	TPGRI	DEAD	SAT	IIFhxT+cx	TDVuARGID	HRIGRTGR
elf4A	AQSGTGKT	PTRELA	TPGRV	DEAD	SAT	VIFCNTRR	TDLLARGID	HRTGRGGR
Ski2	AHTSAGKT	PIKALS	TTEIL	DEVH	SAT	VFVFSKKR	TETFAMGLN	QLTGRAGR
hDicer	LNTGSGKT			DECH	TAS	IIFVERRY	TSIVEEGVD	SYVQSKGR
Dcr1	MRTGAGKT			DECH	TAS	VIFVERKA	TAVAEEGID	QYVQSRGR

Figure 1.6: Alignments of the conserved motifs of RNA helicases. Sequences of the conserved motifs are from elf4A and Ski2 from *S. cerevisiae*, hDicer and Dcr1 from *S. pombe*. Abbreviations used are as follow: c: D, E, H, K, R; h: A, F, G, I, L, M, P, V, W, Y; o: S; T; I: I, L, V; x: any residue; u: A, G; +: H, K, R. Consensus sequences were taken from Cordin *et al*, 2006.

1.2.2 Models for helicase activity

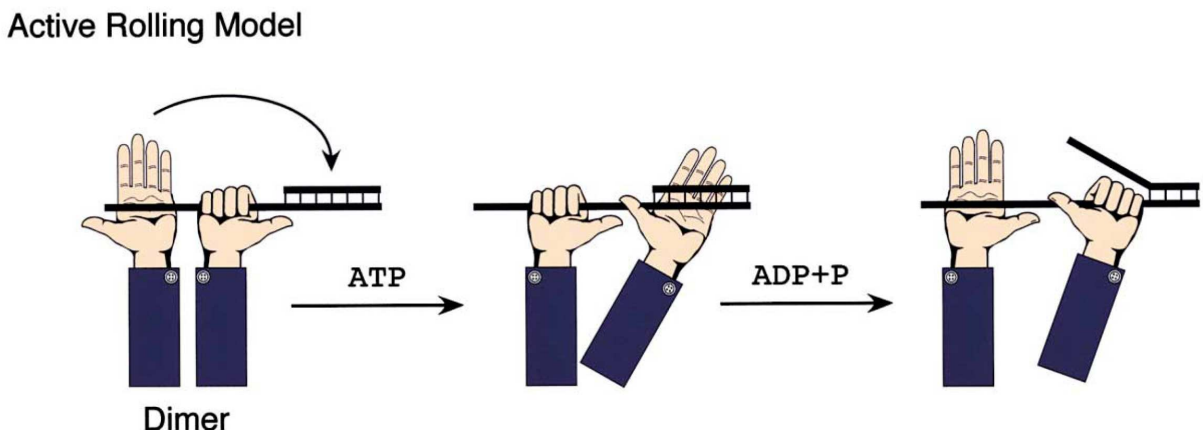
The basic activity of a helicase is to couple ATP binding and hydrolysis to conformational changes that result in bp separation and/or translocation along a nucleic acid substrate. The detailed molecular mechanism of dsDNA or dsRNA unwinding by helicases is

still not known. However, there are certain features of unwinding and translocation that are probably common to all helicases. Resulting from these similarities, a number of models have been proposed to explain helicase activity. The two widely accepted models are the active rolling and the inchworm models (Tanner and Linder, 2001, Rocak and Linder, 2004, Lohman *et al*, 1996, Soultanas and Wigley, 2000, Cordin *et al*, 2006). The two models are consistent with various experimental observations, but neither is able to explain the biophysical mechanism of all helicases.

The active rolling model requires at least a dimeric helicase. In this model, the two subunits are in two different conformational states (Figure 1.7A). One state has a high affinity for ssRNA and the other has a higher affinity for dsRNA. During the cycle of binding and hydrolysis of ATP, and release of products, the two proteins exchange their conformations and affinities for RNA. This leads to the movement along the RNA, which results in unwinding of the RNA.

In the inchworm model, the protein takes advantage of the opening and closing of the cleft between domains 1 and 2 to track along a ssRNA and to displace obstacles in front of it (Cordin *et al*, 2006) (Figure 1.7B). The relative movement between domain 1 and 2 is associated with the binding and hydrolysis of NTP (Tanner and Linder, 2001). Both domains bind the RNA substrate. The tracking mechanism may also apply to the ribonucleoproteinase (RNPase) or protein displacement activity described for Ded1 (Fairman *et al*, 2004). The inchworm mechanism works for both monomers and oligomers.

A



B

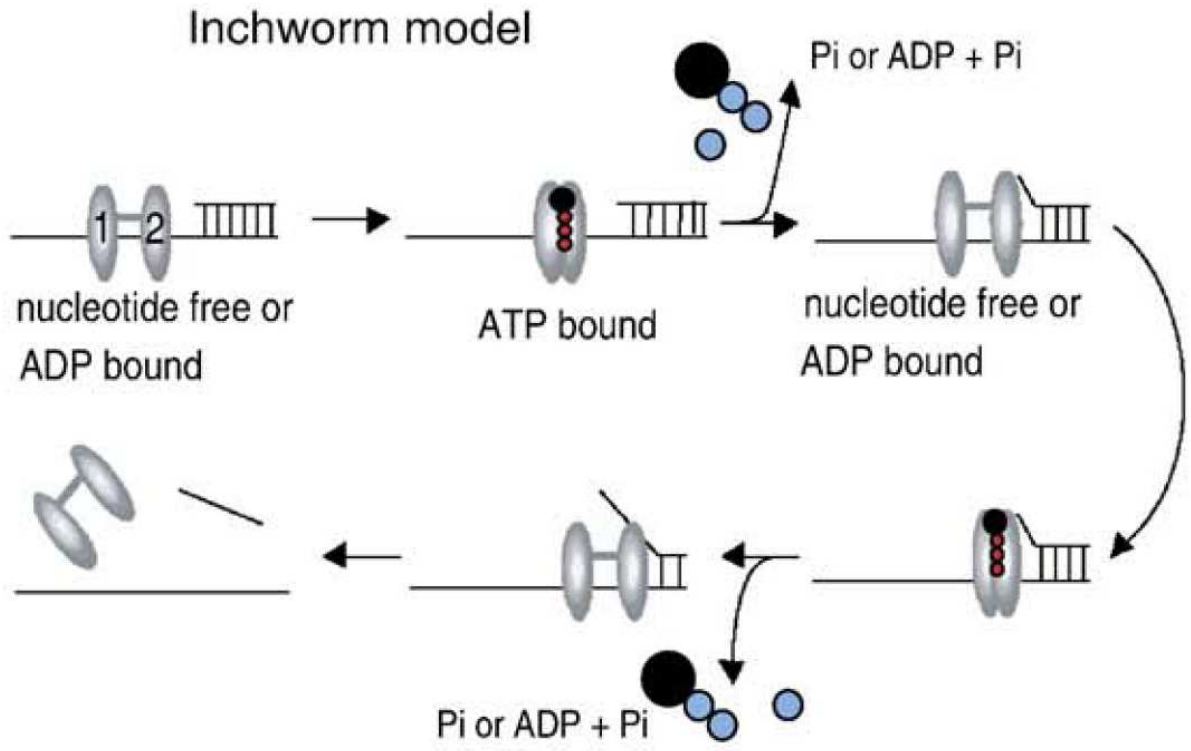


Figure 1.7: Models for helicase activity. (A) Active rolling model of helicase activity (Tanner and Linder, 2001). The active rolling model requires a dimerized helicase in which each monomer has a different conformational state and affinity for ssRNA and dsRNA. These conformations vary with NTP binding and hydrolysis. (B) Inchworm model of helicase activity (Cordin *et al*, 2006). The inchworm model works for both monomers and oligomers. In this model, the distance between domains 1 and 2 vary with NTP binding and hydrolysis. Specific amino acids form non-specific interactions with the sugar-phosphate backbone or stack or intercalate against the bases. Helicase domains 1 and 2 are labelled as shown. Phosphates of ATP are colored in red and phosphates of ADP and Pi are in blue.

1.2.3 Different functions of RNA helicases

RNA helicases are ubiquitous and as a result, they are essential for most aspects of cellular RNA metabolism. These include: transcription, ribosome biogenesis, pre-mRNA editing and splicing, RNA export to the cytosol, translation initiation and termination, RNA degradation and virus propagation (Cordin *et al*, 2006, Bleichert *et al*, 2007, de la Cruz *et al*, 1999, Rocak and Linder, 2004). General functions have been assigned to most RNA helicases through combinations of genetic and biochemical experiments.

1.2.3.1 Transcription

Only a few RNA helicases are found to be associated with transcription whereby their exact functions are not known. The function of RNA helicases in transcriptional regulation is likely to be dependent on interacting transcription factors. For most of the RNA helicases involved in transcription, the regions interacting with transcription factors are located in the N- or C-terminal domains outside the conserved helicase core. p68 and p72 helicases have been found to function as co-repressors and co-activators of transcription and to interact with different transcription factors and nuclear receptors (Watanabe *et al*, 2001, Wilson *et al*, 2004). However, the ATPase or helicase activity of p68 is not required for the transcriptional and repressional activities.

1.2.3.2 Ribosome biogenesis

Most RNA helicases are involved in ribosome biogenesis. Ribosome biogenesis is a multi-step process that involves several RNA species, many ribosomal proteins and a variety of trans-acting factors. Genetic depletion studies of some RNA helicases have identified pre-rRNA processing defects and assigned those helicases to the biogenesis of either the small or large ribosomal subunits, and to specific processing steps (Bernstein *et al*, 2006, Linder, 2006). In *S. cerevisiae*, 14 out of the 24 known DEAD-box RNA helicases are required for the formation of 40S or 60S ribosomal subunits (Kressler *et al*, 1999), while three of the five DEAD-box RNA helicases found in *E. coli* seem to be involved in ribosome biogenesis. Although genetic and biochemical interactions of some RNA helicases with ribosome biogenesis factors have been identified, the precise role of the RNA helicases during ribosome biogenesis is not known.

1.2.3.3 Splicing

Pre-mRNA splicing is a nuclear event that is essential for removing introns from the mRNA precursor. Although the trans-esterification reactions in the splicing process are energetically neutral, it has been known for a long time that splicing requires energy from NTP hydrolysis. This NTP hydrolysis has been attributed largely to the activity of RNA helicases. A substantial part of the energy consumption is used for structural rearrangements in the stepwise assembly of the spliceosome, which involves small nuclear RNAs (snRNAs) and over 70 proteins. Several RNA helicases have been shown to play crucial roles at key steps of splicing, although the exact role of the different RNA helicases in spliceosome assembly is not known. It is generally believed that they are required for the unwinding of

short RNA-RNA duplexes that are formed between the different snRNAs or pre-mRNA molecules. Additionally, recent genetic experiments also suggest that RNA helicases alter RNA-protein interactions as well (Cordin *et al*, 2006). Prp5, a DExD/H RNA helicase, is required at the early step of pre-spliceosome assembly and is thought to use the energy released by ATP hydrolysis to rearrange local RNA-RNA or RNA-protein interactions in order to allow U2 small nuclear ribonucleoprotein (snRNP) to join the complex (Xu *et al*, 2004). The RNA helicase p68 seems to be required for the dissociation of U1 snRNA from the 5´ splice site in an ATP-dependent fashion (Liu *et al*, 2002).

1.2.3.4 RNA Export

Export of mRNA through the nuclear pore requires proteins that bind mRNA. Hence, a nuclear RNA helicase might be necessary to alter the conformation and/or composition of RNP, which will transit through the nuclear-pore complex (NPC). Similarly, a cytoplasmic RNA helicase might re-structure or unpack the RNP when it emerges from the NPC. Additionally, a cytoplasmic RNA helicase could help to remove shuttling proteins from the exported RNP to allow them to return to the nucleus. Studies have shown that Dbp5, a DEAD-box RNA helicase accumulates around the nuclear envelope and is involved in mRNA export. Dbp5 binds RNA co-transcriptionally and interacts functionally with the transcription machinery (Estruch and Cole, 2003). Sub2/UAP56, a DECD helicase, is also required in the early steps of RNA transport and associates with spliced mRNAs carrying an exon-junction-complex (EJC) (Gatfield *et al*, 2001).

1.2.3.5 Translation

Eukaryotic translation initiation of most cellular mRNAs starts first with the association of the 40S ribosomal subunit and proceeds with scanning for the initiation codon. Because extensive secondary structures in the 5′ untranslated regions of mRNAs decrease translational efficiency, the initiation process probably requires RNA-helicase activity (Pause *et al*, 1993). DEAD-box RNA helicases, for example eIF4A and DedI from *S. cerevisiae* and their homologues in higher eukaryotes, have been found to be essential for translation initiation (Linder, 2003). Experimental data suggest that eIF4A, which forms part of the cap-binding complex, unwinds or rearranges RNA-duplex structures at the 5′ end of eukaryotic mRNA to prepare it for scanning by the small ribosomal subunit (Svitkin *et al*, 2005). DedI has also been shown to be necessary for translation initiation, but its precise role during this process is not known (Chuang *et al*, 1997).

1.2.3.6 RNA decay

RNA helicases are also required for the degradation of RNA molecules. The most studied case is RhlB helicase from *E. coli*, which forms a complex with RNase E and polynucleotidedephosphorylase (PNPase). A minimal degradosome that consists of RNase E, PNPase and the RhlB helicase has been shown to have ATP-dependent activity. The minimal degradosome lacking the RhlB helicase was shown to be incapable of degrading structured RNA molecules (Coburn *et al*, 1999). This suggested that RhlB is required for unwinding the substrate to allow degradation to occur. Similarly, Ski2 and Dob1 RNA helicases have been found in eukaryotic exosomes, where they may be involved in unwinding structured RNA designated for degradation.

1.3 Aim of this thesis

RNAi is a complex process that uses small RNAs of lengths of 21 - 23 nucleotides to guide sequence-specific silencing of genetic information both at transcriptional and post-transcriptional levels (Rossi, 2005). Intensive biochemical studies are underway to elucidate the pathway of RNA silencing that leads to the formation of the RISC in human cells. Important steps of the pathway are the generation of siRNA and miRNA by Dicer and the probable unwinding of the siRNA and miRNA by an unknown mechanism. hDicer is one of the major protein components of RNAi pathway and is known to contain a putative A/Hel domain. To date, the functions of this domain are not known (MacRae *et al*, 2007, Pellino *et al*, 2005, Filipowicz *et al*, 2005, Hammond *et al*, 2005).

The aim of this thesis was to clone and produce the A/Hel domain of hDicer recombinantly. Secondly, this work intended to functionally characterise the purified domain with special emphasis being laid on examination of the ATPase activity of the domain. Additionally, RNA binding, helicase and annealing activities of the domain were explored. The results of this study could provide an insight into the functions of hDicer and could contribute to our understanding of its mode of action.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

All common chemicals were obtained from Merck (Darmstadt), Roth (Karlsruhe), Biomol (Hamburg), Sigma (Taufkirchen), Fluka (Taufkirchen), Invitrogen (Karlsruhe), Millipore (Eschborn) and PeqLab (Erlangen), unless otherwise stated.

2.1.2 Enzymes, proteins and antibodies

Restriction enzymes, T4 DNA Ligase, Calf Intestinal Alkaline Phosphatase (CIAP), *Pfu* DNA Polymerase, T4 Polynucleotide Kinase (PNK), protein and DNA markers were purchased from MBI Fermentas (St. Leon Rot). Lysozyme and anti-mouse IgG were purchased from Sigma (Taufkirchen). Tetra-His antibody was purchased from Qiagen (Hilden). Maltose Binding Protein (MBP) and Anti-MBP monoclonal antibody were purchased from New England Biolabs (NEB) (Frankfurt). Anti-V5 and Anti-Xpress antibodies were purchased from Invitrogen (Karlsruhe), while Protease inhibitor was purchased from Roche (Mannheim).

2.1.3 Affinity columns and Kits

HisTrap Column was purchased from GE Healthcare (Munich), Amylose Resin was purchased from NEB (Frankfurt), while Micro Bio-Spin 6 columns were from Bio-Rad (Munich). PeqGold Cycle-Pure Kit and PeqGOLD Plasmid Miniprep Kit I were obtained from PeqLab (Erlangen). Quikchange Site Directed Mutagenesis Kit was purchased from Stratagene (Heidelberg), whereas Phusion Mutagenesis Kit was obtained from Finnzymes (Espoo, Finland). Big Dye Terminator Ready Reaction Kits were obtained from Applied Biosystems (Weiterstadt), whereas Coomassie Protein Assay Kit was purchased from Pierbio Science (Bonn).

2.1.4 Bacterial strains

The following *E. coli* strains were used in this work.

Strain	**Genotype**	**Source**
BL21(DE3)pLysS	F^- ompT $hsdS_B(r_B^-m_B^-)$ gal dcm (DE3) pLysS (Cam^R)	Invitrogen
Origami	F- ompT hsdSB(rB- mB-) gal dcm lacY1 aphC gor522::Tn10 trxB (KanR, TetR)	Novagen
TB1	F^- ara Δ(lac-proAB) [ϕ80dlacΔ(lacZ)M15] rpsL(Str^R) thi hsdR	NEB
Top10	F^- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL (Str^R) nupG	Invitrogen

2.1.5 Insect cell line

High Five insect cells, which originated from the ovarian cells of the cabbage looper, *Trichopulsia ni* were purchased from Invitrogen (Karlsruhe).

2.1.6 Plasmids

The pMAL-c2X plasmid was purchased from NEB (Frankfurt), while pRSET A and pIB/V5-His plasmids were purchased from Invitrogen (Karlsruhe). The pBS-Dicer vector was a gift from W. Filipowicz (Friedrich Miescher Institute for Biochemical Research, Basel, Switzerland).

2.1.7 Media

The following growth-media were used in this work. Where necessary, the media were autoclaved before use.

Media	**Description**	**source**
Express Five SFM	18 M sterile glutamine, 0.01 µg/L gentamycin, 1L Express Five SFM medium	Invitrogen
LB Agar	10 g/L tryptone, 5 g/L yeast extract, 171 mM NaCl, 15 g/L agar, pH 7.0	Roth
LB Broth	10 g/L tryptone, 5 g/L yeast extract/L, 171 mM NaCl, pH 7.0	Roth

SOC medium	20 g/L bactotrypton, 5 g/L bacto-yeast-extract, 10 mM NaCl, 10 mM $MgCl_2$, 2.5 mM KCl, 20 mM glucose	Invitrogen

2.1.8 Buffers and solutions

Amylose binding buffer	20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 1 mM EDTA
Amylose elution buffer	20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 1 mM EDTA, 10 mM maltose
Annealing buffer	50 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 1 mM DTT
BCIP stock solution	0.5 % (w/v) 5-brom-4-chlor-3-indolyphosphate in dimethylformamid (DMF)
Blocking buffer	0.5 % (w/v) bovine serum albumin (BSA) in Tris Buffered-Saline (TBS)
CAPS buffer	10 mM 3- cyclohexylamino-propyl sulfonic acid, 10 % (v/v) Methanol, pH 11.0
Coomassie blue staining solution	0.1 % (w/v) Coomassie brilliant blue R-250, 10 % (v/v) acetic acid, 45 % (v/v) methanol
Coomassie unstaining solution	45 % (v/v) methanol, 10 % (v/v) acetic acid
Detection buffer	0.1 M Tris-Hcl, pH 9.5, 4 mM $MgCl_2$
Detection solution	22.5 mL detection buffer, 2.5 ml NBT, 250 µL BCIP
Developing solution	263 mM Na_2CO_3, 0.06 % (v/v) formaldehyde
Dialysis buffer	20 mM Tris-HCl, pH 7.4, 100 mM NaCl
Electrophoresis buffer (5x)	125 mM Tris, 960 mM glycine, 0.5 % (w/v) SDS
HisTrap binding buffer	20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 30 mM imidazole

HisTrap elution buffer	20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 500 mM imidazole
Incubation solution	500 mM Sodium acetate, 14 mM $Na_2S_2O_3$, 25 % (v/v) ethanol, 0.5 % (v/v) glutardialdehyde
Loading buffer	100 mM Tris, pH 7.6, 1 % (w/v) SDS, 100 mM EDTA, 60 % (v/v) glycerol, 0.03 % xylene cyanol FF and 0.03 % (w/v) bromophenol blue
NBT stock solution	0.1 % (w/v) nitro-blue-tetrazoliumchloride in detection buffer
Phosphate buffered saline (PBS)	140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4
Reaction buffer	40 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 2 mM DTT
2x Sample buffer	125 mM Tris, 4 % (w/v) SDS, 20 % (v/v) glycerol, 0.002 % (w/v) bromophenolblue
Stacking gel buffer (4x)	500 mM Tris, pH 6.8, 0.4 % (w/v) SDS
Separation gel buffer (4x)	1.5 M Tris, pH 8.8, 0.4 % (w/v) SDS
Tris-Acetic acid-EDTA (TAE) buffer (50x)	2 M Tris, pH8.0, 5.7 % (v/v) acetic acid, 50 mM EDTA
Tris Buffered-Saline (TBS)	50 mM Tris, pH 7.5, 150 mM NaCl
Tris Buffered-Saline Tween (TBST)	50 mM Tris, pH 7.5, 150 mM NaCl, 0.5 % (v/v) tween 20
Tris-Boric acid-EDTA (TBE) buffer (5x)	450 mM Tris, 440 mM boric acid, 10 mM EDTA

2.1.9 Oligonucleotides

DNA oligonucleotides were purchased from Invitrogen (Karlsruhe), while RNA oligonucleotides were purchased from Ambion (Cambridgeshire, UK). Below is a list of oligonucleotides used in this work.

DNA Oligonucleotide	**Sequence 5´-3´**
Hel-fw	AAGGTCGACATGAAAAGCCCTGCTTTGCAACCC
Hel-rev	GAGGTACCGCGGCCGCTCATGTATCCGCTAACATTA TATAATTAGA
H-pMAL-mutagen-fwd	ATAATGTTAGCGGATACATTAGCGGCCGC
H-pMAL-mutagen-rev	GCGGCCGCTAATGTATCCGCTAACATTAT
His-fwd	GGCCGCACATCATCACCATCACCATTGAGTCGACA
His-rev	AGCTTGTCGACTCAATGGTGATGGTGATGATGTGC
Hel-Ins-fw	ATAAGCTTATGAAAAGCCCTGCTTTGCAACCCCTC
Hel-Ins-rev	AATCTAGAGGTGTATCCGCTAACATTATATAATTAGA
Hel-400-fw	ACAAAAGAGAGATGGAACCA
Hel-566-fw	TTATGAAGCTCTGTGAAAAT
Hel-1230-fw	GTCATGGAGTGATTCTGAGG
pMAL-seq-fw	GGTCGTCAGACTGTCGATGAAGCC
OpIE2-fw	CGCAACGATCTGGTAAACAC
OpIE2-rev	GACAATACAAACTAAGATTTAGTCAG
DECH-mut-fwd	CTTTTGGTGTTTGCTGAGTGTCATCTT
PH-DECH-mut-rev	GTTAATGTCTGACAGTGATAAGTAACC
GKT-mut-fwd	ACTGGCTCAGGGGCGACATTTATTGCAGTA
PH-GKT-mut-rev	GTTTAAACAGACGATGGTATTATGATCCAG

RNA Oligonucleotide	**Sequence 5´-3´**
GAPDH 16 sense RNA	UGAGAACGGGAAGCUU
GAPDH 21 sense RNA	GGCUGAGAACGGGAAGCUUTT
GAPDH 21 antisense RNA	AAGCUUCCCGUUCUCAGCCTT
Cy3-21R-	Cy3-ACUGCUAGAGAUUUUCCACAU
Cy5-21R+hp	Cy5-UUAUGUGGAAAAUCUCUAGCAGUGGGUUUAU GGCUGUUCGCCAUUU
21R+	AUGUGGAAAAUCUCUAGCAGU
70R	GAGUCCGGGGCUCUAGUGCCGCUCGACUAGAGCCCU GUAAUGGUACAGACAUAGAUUACUGUGUCCGUGC

2.1.10 Software

The following softwares were used in this work.

Clone-Manager	Scientific& Educational Software, Cary, USA
Gel-Pro	Intas, Göttingen
GraphPad Prism 4.03	GraphPad Software, La Jolla, USA
Ms Office 2003	Microsoft, Redmond, USA
Vector NTI Advance 10	Invitrogen, Karlsruhe

2.1.11 Instruments

Äkta Purifier	GE Healthcare, Munich
Centrifuges: -Sorvall Discovery 90SE -Sorvall RC 5B Plus -Table centrifuge 5804 R -Table centrifuge 5417 R	 Kendro Laboratory Products, Lagenselbold Kendro Laboratory Products, Lagenselbold Eppendorf, Hamburg Eppendorf, Hamburg
Electrophoresis-apparatus for: -Agarose gels -Polyacrylamide gels	 Bio-Rad, Munich GE Healthcare, Munich
Electrophoresis Power Supply Pac 100	Bio-Rad, Munich
Electroporator *E. coli* pulser	Bio-Rad, Munich
Electroporation-cuvettes	Molecular BioProducts, San Diego, USA
ELISA-Reader	Tecan, Crallsheim
Exposure Cassette	Bio-Rad, Munich
Gel documentation system	Intas, Göttingen
Incubator	Bio-Rad, Munich
Incubator Shaker Innova 4330	New Brunswick Scientific, Nürtingen
Mastercycler personal	Eppendorf, Hamburg
Personla Molecular Imager FX	Bio-Rad, Munich
pH meter	WTW, Weilheim
Photometers -NanoDrop ND-1000 -Varian Cary 50 UV-Vis	 PeqLab, Erlangen Varian, Darmstadt

SoniPrep 150	Haake, Karlsruhe
TE Series Transfer Electrophoresis Unit	GE Healthcare, Munich
Weighing Machine Lab-scale BL 1500 S	Sartorius, Göttingen

2.2 Methods

2.2.1 Molecular biological methods

2.2.1.1 Polymerase chain reaction (PCR)

PCR is a method used for *in-vitro* amplification of small amounts of DNA (Mullis and Faloona, 1987). PCR primers for cloning of the coding sequence of A/Hel domain of hDicer (nucleotides 1 – 1698) were designed using Clone-Manager (Scientific & Educational Software, Cary, USA). The *A/Hel* gene was amplified from the pBS-Dicer vector using a *Pfu* DNA Polymerase. In general, a 100 µL PCR contained 200 µM mix of each of the four dNTPs, 1x *Pfu* buffer, 2 mM $MgSO_4$, 1 µM forward and reverse primers, and approximately 1 ng of template DNA. The following PCR program (Table 2.1) was used. PCR products were purified using PeqGold Cycle-Pure Kit (PeqLab, Erlangen) according to the manufacturer´s protocol.

Table 2.1: PCR-program.

Step	Duration	Temperature
1. Primary denaturation	1 min	94 °C
2. Denaturation	10 s	94 °C
3. Annealing	30 s	50 °C
4. Elongation	3 min	72 °C
5. 29 cycles (steps 2-4)		
6. Terminal elongation	5 min	72 °C

2.2.1.2 Preparation of plasmid DNA

Plasmid DNA was isolated and purified using the PeqGOLD Plasmid Miniprep Kit I (PeqLab, Erlangen) according to the protocol of the manufacturer.

2.2.1.3 Site-directed mutagenesis

To mutate the guanine of the stop codon (TGA) at the end of *A/Hel* gene fragment into thymin, the H-pMAL-mutagen-fwd and H-pMAL-mutagen-rev primers were used. The point mutation was introduced using the Quikchange Site Directed Mutagenesis Kit (Stratagene, Heidelberg) according to the manufacturer´s manual.

For the cloning of gene fragments coding for A/Hel variant proteins (K70A and D210A), mutations were introduced using Phusion Mutagenesis Kit (Finnzymes, Espoo, Finland) following the protocol of the manufacturer. GKT-mut-fwd and PH-GKT-mut-rev primers were used for the K70A variant, while DECH-mut-fwd and PH-DECH-mut-rev primers were used for the D210A variant.

2.2.1.4 Restriction cleavage and dephosphorylation

DNA was digested using the corresponding restriction endonucleases and buffers (MBI Fermentas, St. Leon Rot) as recommended by the manufacturer. Where necessary, digested plasmid DNA was additionally treated with CIAP to avoid religation.

2.2.1.5 Ligation

For ligation, a 1:1 to 1:5 molar- ratio of linearized vector to digested DNA fragment was used. The ligation was performed using T4 DNA Ligase (MBI Fermentas, St. Leon Rot) following the instructions of the manufacturer.

2.2.1.6 Agarose gel electrophoresis

Agarose gel electrophoresis was used for the analysis of restriction and PCR products, and the preparative purification of DNA fragments. DNA was separated in a horizontal 1 % agarose/1x TAE gel containing 1 µg/mL ethidium bromide at 130 V (analytic gel electrophoresis) or 90 V (preparative gel electrophoresis). Before loading, DNA samples were mixed with 6x loading dye. λ-DNA/Eco130I was used as DNA marker. DNA was visualized using a standard ultraviolet transilluminator (Intas, Göttingen).

2.2.1.7 DNA sequencing

DNA sequencing was performed based on the dideoxynucleotide chain termination method (Sanger *et al*, 1977). 500 ng of plasmid DNA, 10 pmol of corresponding primer, and 2.5 µL of Half-Term buffer were mixed and filled with dH_2O to a volume of 17 µL. After a pre-incubation at 96 °C for 5 min, 3 µL Big Dye Terminator (Applied Biosystems, Weiterstadt) was added. The sequencing reaction was performed in a thermocycler using the following program (Table 2.2). After the sequencing reaction, the DNA was precipitated with 10 µL of sodium acetate, pH 5.2 and 250 µL 96 % ethanol and centrifuged at 15,000 rpm at room temperature for 30 min. After the addition of 400 µL of 70 % ethanol, the DNA was again centrifuged and the supernatant was discarded. The DNA was left to dry at 37 °C for 30 min. Separation and evaluation of sequenced DNA was performed at the Institute for Pathology, Universitätkrankenhaus Eppendorf.

Table 2.2: sequencing program.

Step	Duration	Temperature
1. Denaturation	30 s	94 °C
2. Annealing	15 s	50 °C
3. Elongation	4 min	60 °C
4. 25 cycles (steps 1-3)		
6. Terminal elongation	5 min	72 °C

2.2.1.8 RNA labelling

Lyophilized RNAs were resuspended (0.1 nmol/µL) in nuclease-free water and stored at –20 °C. RNAs were 5´-labelled with ^{32}P-γ-ATP (GE Healthcare, Munich) using T4 Polynucleotide

Kinase. Labelled strands were purified from unincorporated radioactive nucleotides by gel filtration on Micro Bio-Spin 6 columns (Bio-Rad, Munich) following the protocol of the manufacturer.

2.2.1.9 Preparation of competent cells

To prepare transformation competent *E. coli* cells, 100 mL of LB medium was inoculated with an overnight culture of the *E. coli* cells. The cells were grown at 37 °C to an OD_{600nm} of 0.5. After cooling on ice for 20 min, the cells were centrifuged at 3,000 rpm at 4 °C for 20 min. The cell pellet was washed twice with cold 1 mM HEPES buffer, pH 7.0 and once with cold 10 % glycerol. After re-suspension in 10 % glycerol, the cells were aliquoted and flash-frozen in liquid nitrogen. The competent cells were stored at –80 °C.

2.2.1.10 Transformation

Transformation of competent *E. coli* cells was performed using the electroporation method. 50 µL of competent cells (OD_{600nm} of 0.5) were mixed with 2 µL plasmid DNA (20-100 ng/µL) on ice. The mixture was transferred to a pre-cooled electroporation cuvette on ice and the introduction of the plasmid DNA into the bacterial cells was performed using *E. coli*-pulser (Bio-Rad, Munich). After the addition of 500 µL SOC medium, the cells were grown at 37 °C for 1 h. Cells were plated on LB agar plates containing the respective antibiotics and incubated at 37 °C overnight.

2.2.1.11 Culturing of High Five insect cell line

High Five cells were cultivated in Express Five SFM as a monolayer in culture-flasks at 27 °C. For subculturing, cells were passaged at a density of 9.0 x 10^6/75 cm^2 every 3-4 days by diluting at 1:5 (volume of cells: final volume of medium) in order to maintain log phase growth. Cell viability was assessed regularly by staining with trypan-blue, and examination of cells under the microscope.

2.2.1.12 Storage of High Five insect cell line

For storage, cells were harvested at 80-90 % confluency and more than 90 % viability. After dislodging cells from the surface of a culture-flask, the cell suspension was centrifuged at 500 rpm for 10 min at room temperature and the supernatant was discarded. Cells were resuspended at a density of 3 x 10^6 cells/ml in a freezing medium consisting of 42.5 % conditioned Express Five SFM, 42.5 % fresh Express Five SFM, 10 % Dimethyl Sulfoxide (DMSO) and 5 % Fetal Bovine Serum (FBS). 1 mL of the cell suspension was transferred to sterile cryovials and placed at –20 °C for 1 h and then at –80 °C for 24 h. Afterwards cells were stored in liquid nitrogen.

2.2.1.13 Transfection

For transfection experiments, 2 x 10^6 cells in 2.5 mL High Five SFM were seeded in a 25 cm^2 culture-flask and incubated at 27 °C for 1 h. 2 µg DNA and 10 µL Insect GeneJuice Transfection Reagent (Merck, Darmstadt) were diluted with 100 µL Express Five SFM

respectively. The diluted DNA was slowly added to the diluted Insect GeneJuice Transfection Reagent and mixed gently. The DNA/Insect GeneJuice Transfection Reagent mixture was incubated at room temperature for 15 min, after which 800 µL Express Five SFM was added to the transfection mixture. Express Five SFM was carefully removed from the cells without disrupting the monolayer and the transfection mixture was added to the cells. Cells were incubated then at 27 °C for 3 days.

2.2.1.14 Stable transfection

In order to increase the expression of the recombinant A/Hel protein, generation of stable insect cells constitutively expressing the recombinant protein was performed. 72 h post-transfection, transfected cells were subcultured for a period of 4 weeks in the presence of 50 µg/mL blasticidin (Cayla-Invivogen, Toulouse, France). Surviving clones were selected and tested for the expression of A/Hel protein using Western blotting.

2.2.2 Proteinchemical methods

2.2.2.1 Protein expression in *E. coli* cells

E. coli cells (BL21(DE3)pLysS, Origami and TB1) were transformed with H-pMAL-c2X plasmid DNA carrying the *A/Hel* gene. Cells were grown at 37 °C in 1 L of LB medium to an OD_{600nm} of 0.7. Isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 0.5 mM and growth continued for 3 hours. Cells were harvested by centrifugation at 4 °C and stored at –20 °C. Cells were lysed under non-denaturing conditions in the presence of

lysozyme (1 mg/mL) and protease-inhibitor. After sonication (5 × 1 min), cell-lysate was centrifuged at 10,000 rpm at 4 °C for 80 min and the soluble extract was obtained for purification of the A/Hel protein.

2.2.2.2 Protein expression in High Five insect cells

High Five insect cells were transfected with H-pIB/V5-His plasmid DNA carrying the *A/Hel* gene. Stable transfected cells (0.5 x 10^6 cells in 100 mL of Express Five SFM) were continuously cultivated in a shaker at 27 °C and at 100 rpm in a 250 mL Corning Erlenmeyer flask (Omni Life Science, Hamburg). Cells were passaged every 3 - 4 days by diluting at 1:5 (volume of cells: final volume of medium) in order to maintain log phase growth. Harvested cells were stored at –80 °C prior to lysis. To prepare cell extract, insect cells were lysed under pressure at 1,000 psi in HisTrap binding buffer for 30 min using pressure gauge (Ashcroft, Costa Mesa, USA). Cell lysate was centrifuged at 58,000 rpm and 4 °C for 1 h and the soluble extract was obtained for purification of the A/Hel protein.

2.2.2.3 Protein purification

Recombinant MBP-A/Hel-His-tag fusion protein produced in *E. coli* was purified using immobilized metal ions and amylose affinity chromatographies. Soluble protein extract was applied to a 1 mL HisTrap-column equilibrated with 20 mL HisTrap binding buffer at a flow rate of 1 mL/min. The column was extensively washed with 20 mL of the buffer. Bound proteins were eluted with HisTrap elution buffer using one-step and linear gradients. The protein-containing fractions were pooled and applied to a 600 µL amylose column

equilibrated with 20 mL amylose binding buffer at a flow rate of 1 mL/min. The column was extensively washed with 20 mL of the amylose binding buffer and the protein finally eluted with amylose elution buffer using a one step gradient. The purity of the preparations was ascertained by SDS-PAGE, followed by Coomassie blue staining.

Recombinant A/Hel-His protein produced in insect cells was purified using immobilized metal ion chromatography, following the same procedure used for the purification of recombinant MBP-A/Hel-His-tag fusion protein produced in *E. coli.*

2.2.2.4 SDS-polyacrylamide gel-electrophoresis (SDS-PAGE)

Protein samples were analysed on 9 % SDS polyacrylamide gels according to Laemmli (1970) using a Minigel-unit. Before loading, samples were mixed with 2x loading dye and boiled for 5 min. Gels were stained with Coomassie blue solution for at least 1 h and destained with the Coomassie unstaining solution.

2.2.2.5 Silver staining of proteins

Silver staining of proteins is based on the formation of complexes between Ag^{2+} ions and glutamate, aspartate and cysteine residues of proteins. This method is very sensitive and has a detection limit of 50 - 100 pg (Heukeshoven and Dernick, 1988). Subsequent to SDS-PAGE, polyacrylamide gel was incubated in dH_2O/ethanol/acetic acid solution (55:30:15) for 30 min and then in incubation solution for 1 – 12 h. After washing three times in dH_2O for 10 min, the gel was stained in 6 mM $AgNO_3$, 0.06 % (v/v) formaldehyde. Afterwards, the gel was washed shortly in dH_2O and incubated in developing solution, until protein bands are detected. To stop the reaction, the gel was transferred in 50 mM EDTA solution.

2.2.2.6 Western blotting

After SDS-PAGE, the electrophoretic transfer of proteins to an Immobilon-P Polyvinylidenefluoride (PVDF) membrane (Millipore, Eschborn) was performed using a TE Series Transfer Electrophoresis Unit (GE Healthcare, Munich). Prior to use, the membrane was activated in methanol and subsequently rinsed in dH_2O and CAPS-buffer for 5 min. Whatman-papers, sponges and SDS-polyacrylamide gels were also equilibrated in CAPS-buffer for 5 min. After equilibration, the materials were stacked in a sandwich comprising of sponges, polyacrylamide gel, filterpaper and membrane. The protein transfer was carried out at 50 V at 4 °C for 1 h.

2.2.2.7 Immunodetection

The PVDF membrane was rinsed twice for 10 min in TBS buffer and was then incubated in blocking buffer for 1 h at room temperature or overnight at 4 °C to block non-specific sites. After washing twice in TBST buffer, the membrane was exposed to primary antibodies (diluted 1:2,000 - 1:10,000 in blocking buffer) specific for the protein-tags of interest for 1 h. Subsequently, the membrane was washed twice for 10 min in TBST buffer and then incubated with a specific secondary antibody (anti mouse-IgG coupled to alkaline phosphatase diluted 1:20,000 in blocking buffer) for 1 h at room temperature. After washing twice in TBST buffer and once in detection buffer, the membrane was incubated in detection solution until the corresponding bands were detected. The membrane was then rinsed shortly in dH_2O and dried using filter paper.

2.2.2.8 Determination of protein concentration

Protein concentration was determined using the Coomassie Protein Assay Kit (Pierbio Science, Bonn) following the instructions of the manufacturer. Different dilutions of BSA were used as standards.

2.2.2.9 ATPase activity assay

Colorimetric assay based on Biomol Green Reagent (Biomol, Hamburg) was performed (Tanaka and Schwer, 2005). Reactions were in 200 µL total volume containing 113 nM recombinant A/Hel protein and 0.5 mM ATP in a reaction buffer. Following incubation at 37 °C, 25 µL reaction-aliquots were taken at 0, 2, 4, 6, 10 and 15 min and transferred into a 96 well microtiter plate. Reactions were stopped by adding 25 µL 83 mM EDTA (pH 8.0). 100 µL Biomol Green Reagent was then added to each well and the absorbance was measured at 620 nm on a microtiter-plate reader after incubation at 22 °C for 20 min. The amount of released phosphate was calculated by interpolation of the OD_{620nm} values to a phosphate standard curve. For the ATPase activity assays in the presence of RNA or RNase A, 1µM GAPDH RNA, 200 ng/µL total yeast RNA or 0.2 mg/mL RNase A was added.

2.2.2.10 Enzyme kinetics

To determine the kinetic parameters for ATPase activity, the colorimetric assay based on Biomol Green Reagent, which measured the amount of released phosphate from hydrolysis of ATP was used. Reactions were in 200 µL total volume containing 113 nM recombinant A/Hel

protein in reaction buffer. The concentrations of ATP were varied between 0.06 and 1 mM. Reactions were incubated at 37 °C and 25 µL aliquots were taken at 0, 2, 4, 6, 10 and 15 min. After stopping the reactions with 25 µL 83 mM EDTA (pH 8.0), 100 µL Biomol Green Reagent was added and the absorbance was measured at 620 nm on a microtiter-plate reader after incubation at 22 °C for 20 min. The amount of released phosphate was calculated by interpolation of the OD_{620nm} values to a phosphate standard curve. Kinetic parameters were determined by linear and non-linear regression plots using GraphPad Prism 4.03 (GraphPad Software, La Jolla, USA) and Excel (Microsoft, Redmond, USA).

2.2.2.11 RNA binding assay

Binding of A/Hel protein to RNA was carried out in reaction buffer in the presence of 2 mM ATP. Radioactive-labelled 21 or 70 nucleotide long ssRNA (6 nM) or siRNA (13 nM) and A/Hel protein (247 nM) were incubated at 37 °C for 30 min. Samples were electrophoresed at 100 V for 1 h at 4 °C on 8 % native TBE gels. Gels were exposed to storage phosphor screens and imaged on a Personal Molecular Imager FX (Bio-Rad, Munich).

2.2.2.12 NTPase and dNTPase activity tests

The reaction mixtures (25 µL), which contained 113 nM recombinant A/Hel protein in reaction buffer were incubated with 0.5 mM of each NTP or dNTP at 37 °C for 15 min. Reactions were stopped by adding 25 µL 83 mM EDTA (pH 8.0). 100 µL Biomol Green Reagent was added and the absorbance measured at 620 nm after incubation at 22 °C for 20

min. Autohydrolysis of the NTPs and dNTPs was determined in respective control samples lacking the recombinant protein.

2.2.2.13 Unwinding assay

To prepare a 16+21 RNA duplex, GAPDH 16 sense RNA was annealed to a ^{32}P-labelled GAPDH 21 antisense RNA to produce a 16-bp duplex region with a 3´single-stranded overhang. Similarly, a GAPDH 21 sense RNA was hybridised to the ^{32}P-labelled GAPDH 21 antisense RNA to produce a siRNA. Reactions were performed in 20 µL volume consisting of 113 nM A/Hel protein in reaction buffer, 2 mM ATP and 5 nM RNA duplex. Additionally, 100 nM unlabelled GAPDH 21 antisense RNA was added to prevent the reannealing of the oligonucleotides, which could take place under the reaction conditions. Reactions were incubated for 1 h at 37 °C and stopped by transferring on ice and addition of 4 µL of loading buffer. Samples were analysed by electrophoresis on an 18 % polyacrylamide gel at 4 °C for 3 h at 100 V using TBE running buffer. The radiolabelled samples were detected using Personal Molecular Imager FX.

2.2.2.14 Combined fluorescence RNA annealing and strand displacement assay

The combined fluorescence RNA annealing and strand displacement assay was performed in collaboration with the laboratory of Rene Schroeder at the University of Vienna, Austria. For the fluorescence RNA annealing assay, 5 nM each of two fluorescence-labelled short RNAs (Cy3-21R and Cy5-21R+hp) were annealed in the presence of various concentrations of A/Hel protein for 180 s at 37 °C in annealing buffer (Rajkowitsch and Schroeder 2007) (Figure 2.1).

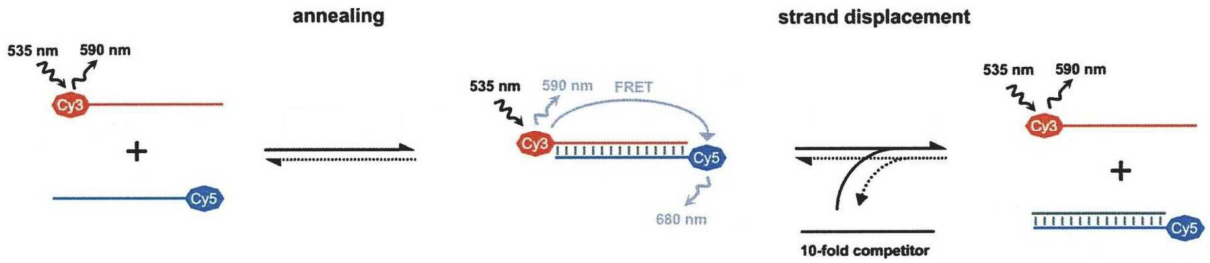


Figure 2.1: Fluorescence-based assay for RNA annealing and displacement activities (Rajkowitsch and Schroeder 2007). Annealing of two fluorophore-labelled RNAs yields a FRET signal, which is measured every second in a microplate reader. The strand displacement assay begins with the addition of 10-fold excess of a non-lablled complementary competitor RNA. In the presence of displacement activity, the pre-hybridised double-labelled dsRNA dissociates and the non-labelled competitor RNA hybridises with a complementary Cy5-labelled RNA. This will lead to a decrease in FRET.

Fluorescence resonance energy transfer (FRET) occurs upon annealing of the two RNAs that are 5´ tagged with Cy3 or Cy5 fluorophores. The two fluorophores are close enough for FRET to occur, with the increase in energy transfer being proportional to the fraction of dsRNA. The excited donor dye transfers energy onto the acceptor dye that emits light of a longer wavelength. Upon excitement of Cy3, the fluorescence emissions of Cy3 and Cy5 were monitored in a microplate reader once every second. The FRET index was calculated as F_{Cy5}/F_{Cy3} and normalized between 0 - 1. Using Prism 4.03 software, the resulting time-resolved curve was fitted with the second-order reaction equation for equimolar initial reactant concentrations $y = A (1 – 1 / (k_{ann} t + 1))$; k_{ann} = observed annealing reaction constant and A = maximum reaction amplitude. Average values were derived from three individually fitted measurements.

For the fluorescence strand displacement assay, a second phase was initiated by the injection of a 10-fold excess (50 nM final concentration) of non-labelled 21R+ RNA. Without strand displacement activity, RNA annealing continued. In the presence of a strand displacement-active protein, competitor injection resulted in a decrease in FRET, since the dissociated Cy3-21R- RNA was readily annealed to the non-labelled competitor The resulting graphs were fitted with $y = y_0 + A (1 – \exp(-k_{ann2} t))$ for signal increase and with $y = y_0 + A$

exp(-k_{SD} t) for signal decay; k_{ann2} and k_{SD} = observed annealing reaction constants and A = maximum reaction amplitude.

3 Results

3.1 Recombinant expression of A/H domain of hDicer

To be able to investigate the functions of the A/H domain of hDicer, it was very important to produce the recombinant protein in adequate amount. Production of recombinant A/Hel protein was carried out in *E. coli* and insect cells.

3.1.1 Recombinant expression of A/H domain of hDicer in *E. coli*

3.1.1.1 Amplification and cloning of A/Hel domain of hDicer

The coding sequence of A/Hel domain of hDicer (nucleotides 1 - 1698) was amplified from the pBS-Dicer vector using the Hel-fwd and Hel-rev primers. Using the SalI/XhoI and KpnI restriction sites, the *A/Hel* gene fragment was cloned into a pRSET A vector to yield H-pRSET A (Figure 3.1).

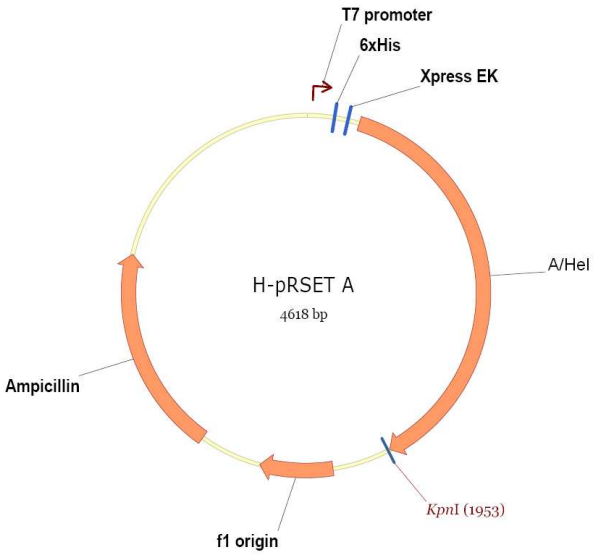


Figure 3.1: Vector-map of H-pRSET A. The amplified coding sequence of the *A/Hel* gene fragment was cloned into the pRSET A plasmid. The vector contained: a *T7* promoter, which provides dose-dependent regulation of heterologous gene expression; a *f1* origin, which allows single strand rescue of DNA; an ampicillin resistance gene, which allows selection of transformed *E. coli* cells; a polyhistidine sequence, which permits purification of recombinant fusion protein on metal-chelating resins; Xpress epitope sequences, which allows detection of the fusion protein by the Xpress antibody. The vector map was designed using Vector NTI Advance 10 (Invitrogen, Karlsruhe).

To be able to produce an MBP-A/Hel fusion protein, the *A/Hel* DNA fragment was subcloned into a pMAL-c2X vector using the BamHI and HindIII restriction sites contained in the pRSET A vector. The pMAL-c2X vector contains a *malE* gene, encoding MBP, upstream of its multiple cloning site, resulting in the expression of an MBP fusion protein. To allow for the expression of a His-tag coding region, which was later cloned downstream of the *A/Hel* fragment, the stop codon (TGA) at the end of the *A/Hel* gene fragment was mutated into TTA using the H-pMAL-mutagen-fwd and H-pMAL-mutagen-rev primers. By hybridization of the His-fwd and His-rev primers, cloning of the His-tag sequence downstream of the *A/Hel* fragment was performed using the NotI and HindIII restriction sites. The resulting H-pMAL-c2X-His vector encoded the A/Hel domain of hDicer fused to an N-terminal MBP and a C-terminal His-tag (Figure 3.2).

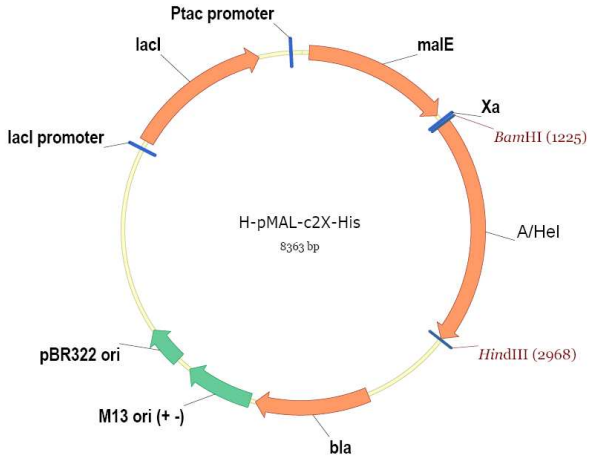


Figure 3.2: Vector-map of H-pMAL-c2X-His. The *A/Hel* gene fragment was inserted downstream from the *malE* gene in pMAL-c2X vector. The vector contained a *Ptac* promoter for the regulation of heterologous gene expression; an ampicillin resistance gene (*bla*); a hexahistidine sequence; a *malE* gene, which encodes maltose-binding protein; a *lacI*, which encodes for the Lac repressor; *lacI* promoter for the regulation of *lacI* gene; Xa sequencce, which encodes recognition site for Factor Xa; *M13 ori*, which allows the production of ssDNA by infection of *E. coli* cells bearing the plasmid with a helper phage; *pBR322 ori* for a high copy replication in *E. coli*. The vector map was designed using Vector NTI Advance 10 (Invitrogen, Karlsruhe).

3.1.1.2 Production of A/Hel domain of hDicer in *E. coli* cells

E. coli cells (BL21(DE3)pLysS) were transformed with an H-pRSET A vector, containing the cDNA of hDicer (nucleotides 1 to 1698) and an N-terminal His-tag sequence. Expression of this construct in *E. coli* cells produced an insoluble A/Hel domain of hDicer. Western blot analysis of a cell extract from the transformed *E. coli* cells cultivated at 37 °C and 220 rpm, and induced at 0.5 mM IPTG showed the presence of a protein with a molecular size of 67 kDa in the cell pellet. This protein was identified as A/Hel protein using anti-Xpress antibody. However, no A/Hel protein was seen in the supernatant of the cell extract, signifying that the protein was insoluble in *E. coli* cells under these conditions (Figure 3.3). Changing the expression parameters by performing the protein production in Origami *E. coli* strain, at lower temperatures (18 °C, 25 °C), at different IPTG concentrations (0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM), or at a lower revolution (180 rpm) did not result in a soluble A/Hel protein (data not shown).

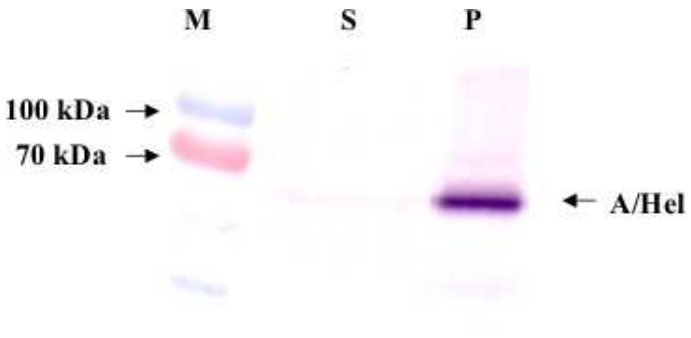
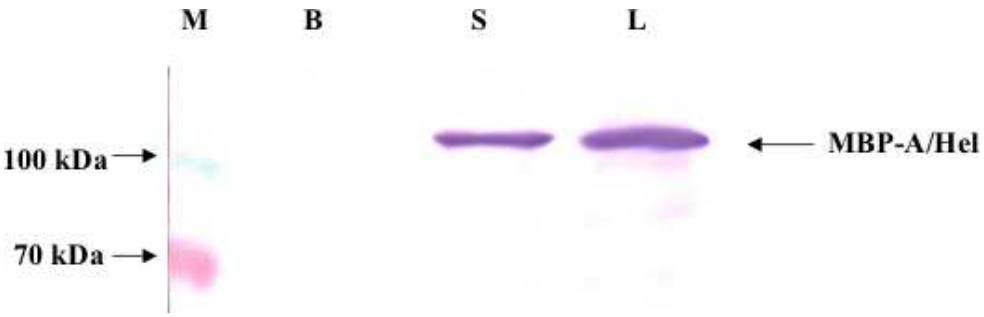


Figure 3.3: Production of recombinant A/Hel protein from H-pRSET A vector in *E. coli* cells (BL21(DE3)pLysS). Protein expression was performed at 37 °C, 0.5 mM IPTG and 220 rpm. Cells were lysed and separated into cell pellet and supernatant by centrifugation. After separation on a 9 % SDS-polyacrylamide gel, proteins were transferred to a PVDF membrane. Identification of recombinant A/Hel protein was performed using anti-Xpress antibody. M: protein marker, S: supernatant, P: cell pellet.

Since the expression of recombinant A/Hel protein from H-pRSET A vector in *E. coli* cells led to an insoluble protein, considerations were made to fuse the A/Hel protein to MBP produced from *E. coli*, because MBP and other such protein tags are known to enhance the solubility of their fusion partners (Nallamsetty and Waugh, 2006, Tsunoda *et al*, 2005, Wonderling *et al*, 1995). Expression of H-pMAL-c2X-His vector in *E. coli* cells produced the A/Hel domain of hDicer as a soluble fusion protein with an N-terminal MBP and a C-terminal His-tag. Immunoblotting analysis using anti-His as well as anti-MBP antibodies confirmed the presence of A/Hel protein in the supernatant of the cell lysate (Figures 3.4A and 3.4B). A band of about 107 kDa corresponding to the molecular mass of the recombinant A/Hel protein was detected, demonstrating that this approach was successful.

A



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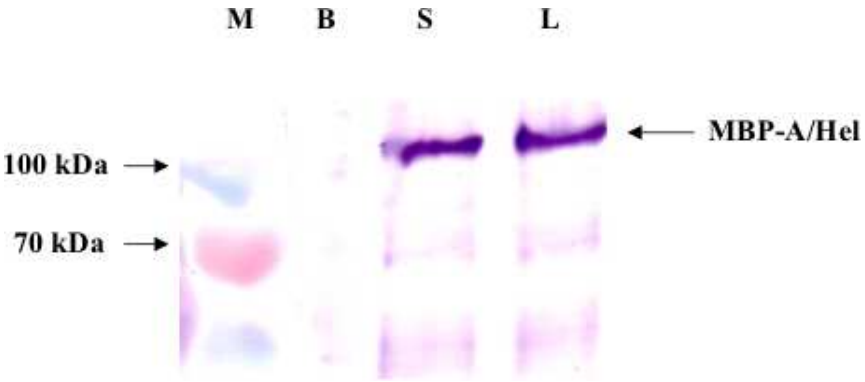


Figure 3.4: Western-blot to monitor the production of recombinant A/Hel protein from H-pMAL-c2X-His vector in *E. coli* (TB1). Protein expression was performed at 37 °C, 0.5 mM IPTG and 220 rpm in *E. coli* cells (TB1). Cells were lysed and separated into cell pellet and supernatant by centrifugation. After 9 % SDS-PAGE, proteins were transferred to a PVDF membrane. Identification of recombinant A/Hel protein was performed using anti-His antibody (A) and anti-MBP antibody (B). M: protein marker, B: cell lysate before IPTG induction, S: supernatant after IPTG induction, L: cell lysate after IPTG induction.

The production of the A/Hel protein variants (K70A and D210) was analogous to the A/Hel wild-type (wt) protein production. Both variant proteins were also fused to an N-terminal MBP and a C-terminal His-tag. The identity of the variant proteins was confirmed using anti-His antibody (Figure 3.5).

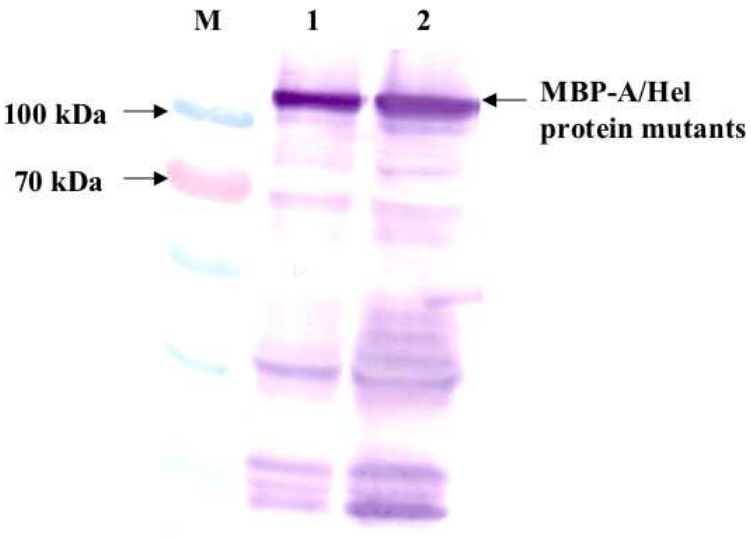
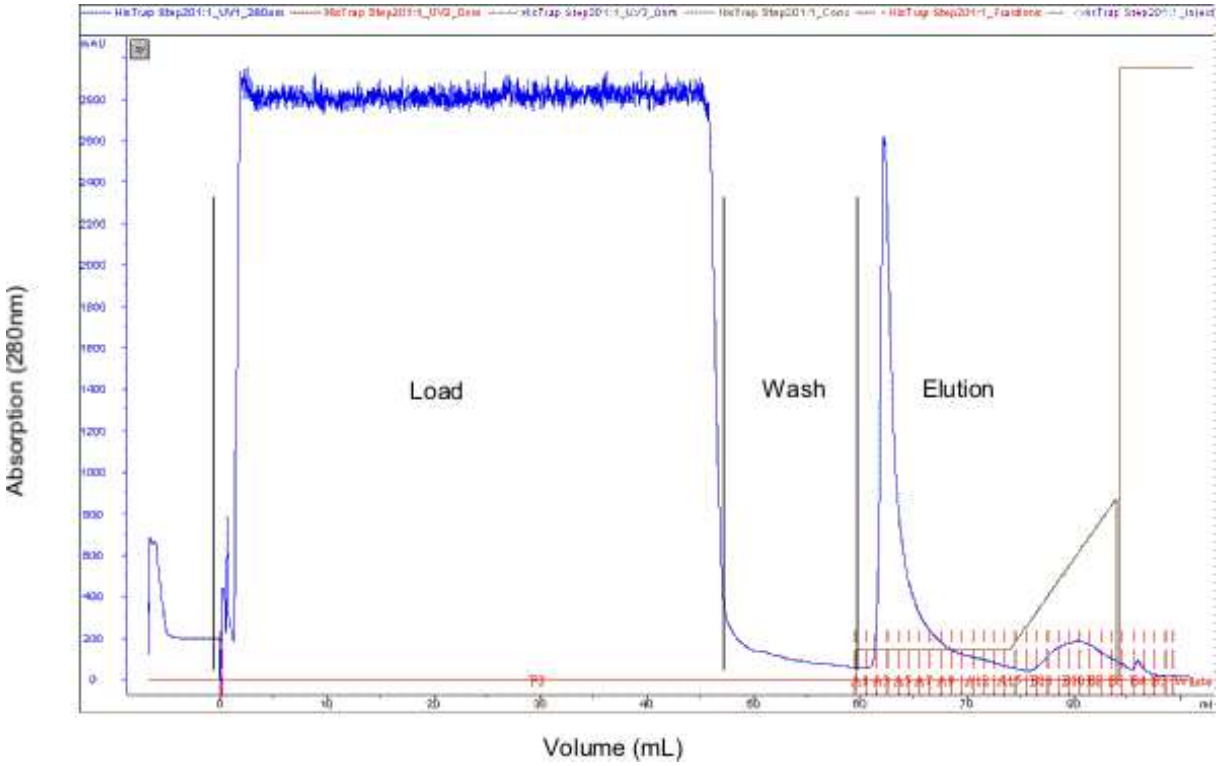


Figure 3.5: Western-blot to demonstrate the production of recombinant K70A and D210A A/Hel variants in *E. coli* cells (TB1). Protein expression was performed at 37 °C, 0.5 mM IPTG and 220 rpm in *E. coli* cells (TB1). Cells were lysed in sample buffer. After 9 % SDS-PAGE, proteins were transferred to a PVDF membrane. Identification of recombinant K70A and D210A A/Hel protein variants was performed using anti-His antibody. M: protein marker, 1: cell lysate of *E. coli* cells expressing K70A A/Hel variant protein, 2: cell lysate of *E. coli* cells expressing D210A A/Hel variant protein.

3.1.1.3 Purification of recombinant A/Hel domain of hDicer.

In order to examine the functions of the A/Hel domain of hDicer, the recombinant protein had to be purified to near homogeneity. Since the recombinant A/Hel protein was fused to a His-tag and MBP, the purification of the protein was performed using nickel-nitrilotriacetic (Ni-NTA) and amylose affinity chromatographies. To purify the recombinant A/Hel protein, cell-lysate from *E. coli* cells producing the protein was loaded on a HisTrap column. After washing, proteins bound specifically to the column were eluted with an imidazole gradient (Figure 3.6A). The elution of bound proteins was dependent on their binding strength. Proteins that bound less tightly to the column eluted before the strong-binding proteins. To ascertain the purity of the eluate fractions and which fractions of the eluates contained the recombinant A/Hel protein, protein-containing fractions were analysed on a 9 % SDS-polyacrylamide gel (Figure 3.6B).

A



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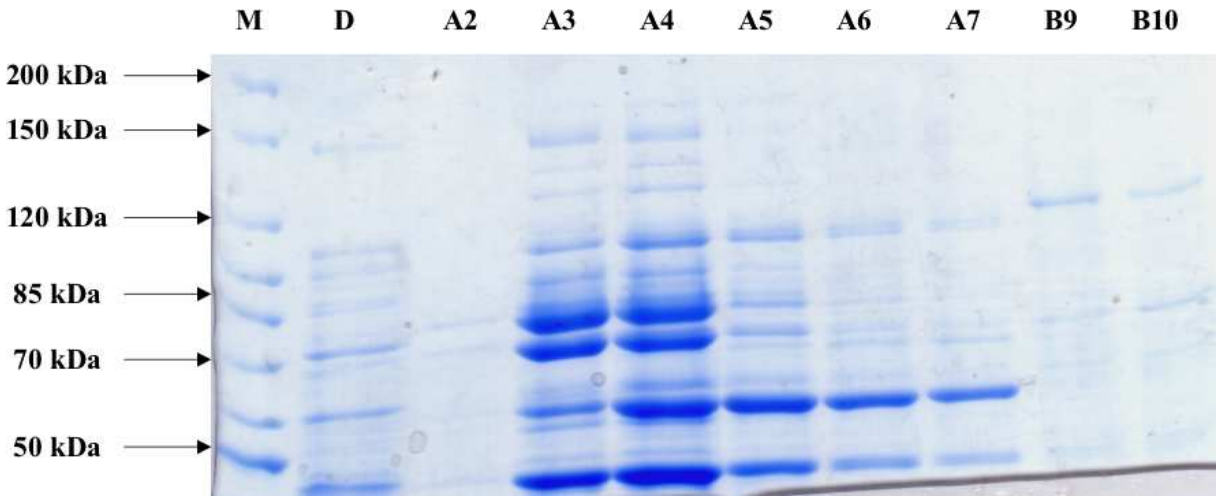
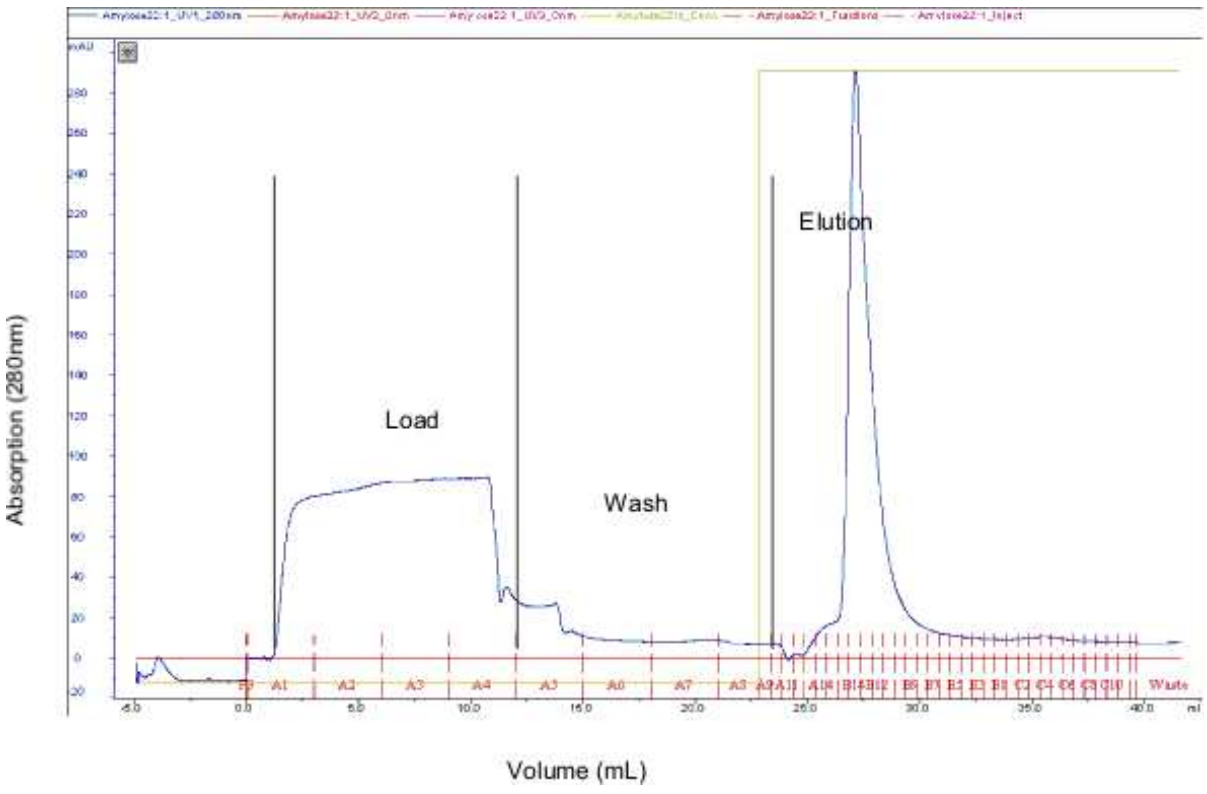


Figure 3.6: Purification of recombinant A/Hel protein using Ni-NTA affinity chromatography. (A) Elution profile. *E. coli* cells (TB1) expressing A/Hel protein were lysed and the soluble cell extract was applied to a HisTrap column at a flow rate of 1 mL/min. After washing out non-specific bound proteins, elution was performed with an imidazole gradient. (B) SDS-PAGE. The flow-through (D) and fractions (A2 – A7, B9 and B10) were analysed on a 9 % SDS-polyacrylamide gel. Proteins were stained using Coomassie-blue.

The result showed that one of the bands in fractions B9 and B10 was a band of about 107 kDa corresponding to the molecular weight of the recombinant A/Hel protien. As a result, the A/Hel protein-containing fractions were pooled and further purified using the amylose affinity chromatography.

To increase the purity and the concentration of the A/Hel protein, the pooled A/Hel protein-containing fractions obtained from Ni-NTA affinity purification was applied on an amylose column. After washing out unbound proteins, the A/Hel protein was eluted with 10 mM maltose (Figure 3.7A). The elution profile showed a narrow, symmetrical peak indicating an efficient purification of the A/Hel protein. Analysis of eluates from the amylose affinity purification on a 9 % SDS-polyacrylamide gel showed a single band of about 107 kDa corresponding to the molecular mass of the recombinant A/Hel protein (Figure 3.7B). The presence of the presumably recombinant A/Hel protein was confirmed using an anti-His antibody (Figure 3.7C). The typical yield was about 0.2 mg of purified A/Hel protein per litre of bacterial culture. The purified protein was stored in 25 % glycerol at -20 ^{o}C.

A



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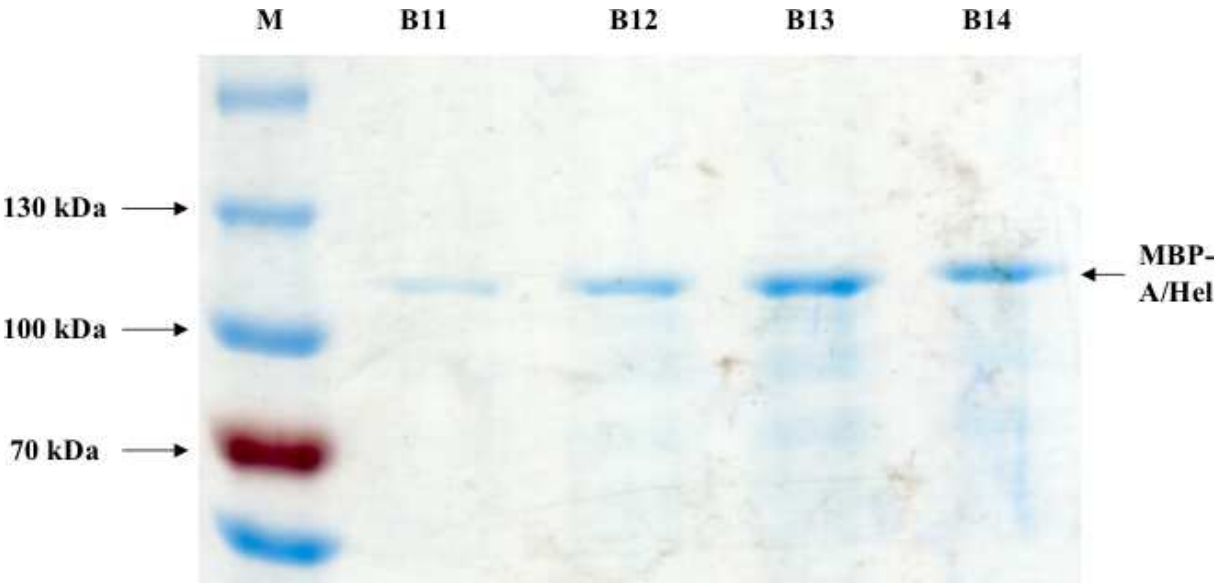

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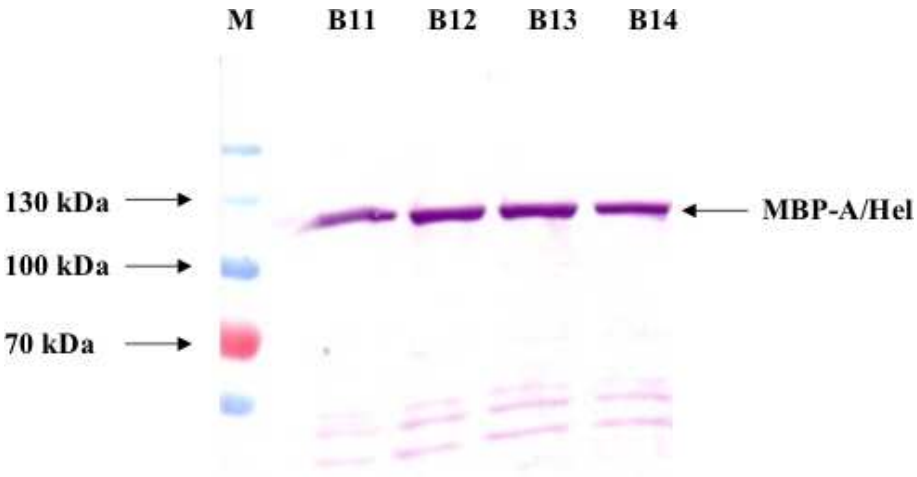

Figure 3.7: Purification of recombinant A/Hel protein using amylose affinity chromatography. (A) A/Hel protein-containing fractions from the Ni-NTA affinity purification were pooled and applied to an amylose column at a flow rate of 1 mL/min. (B) Eluates (B11 – B14) from amylose affinity chromatography were analysed on a 9 % SDS-polyacrylamide gel and were stained with Coomassie blue. (C) Eluates from the amylose affinity chromatography were electrophoresed on a 9 % SDS-polyacrylamide gel, and proteins were transferred to a PVDF membrane. Identification of recombinant A/Hel protein was performed using anti-His antibody.

3.1.2 Recombinant production of A/H domain of hDicer in High Five insect cells

3.1.2.1 Cloning and production of A/Hel domain of hDicer in insect cells

For the cloning of the coding sequence of A/Hel domain of hDicer into a pIB/V5-His vector, the *A/Hel* gene fragment was PCR-amplified from the pBS-Dicer vector. The amplified gene fragment was cloned into the pIB/V5-His vector using HindIII and XbaI restriction sites to yield H-pIB/V5-His. The resulting H-pIB/V5-His vector encoded the A/Hel domain of hDicer fused to a C-terminal V5 epitope and a His-tag (Figure 3.8).

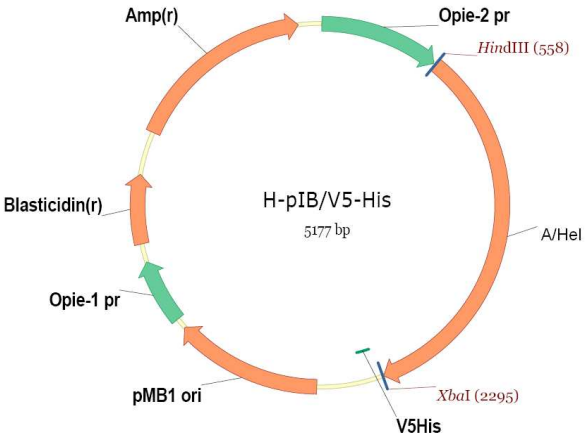

Figure 3.8: Vector map of H-pIB/V5-His. The coding sequence of A/Hel domain of hDicer was cloned into pIB/V5-His plasmid. The vector contained: an *OpIE2* promoter for high level, constitutive expression of the gene of interest; an *OpIE1* promoter for expression of the blasticidin resistance gene; a blasticidin resistance gene for selection of stable cell lines; an ampicillin resistance gene; a V5 epitope for detection of the recombinant protein using anit V5 antibody; hexahistidine sequences. The vector map was designed using Vector NTI Advance 10 (Invitrogen, Karlsruhe).

To produce the A/Hel domain of hDicer intracellularly, insect cells were transfected with the H-pIB/V5-His vector. Three days after transfection, cells were lysed and assayed for the production of the A/Hel domain of hDicer. Transfected cells produced the recombinant A/Hel protein (Figure 3.9).

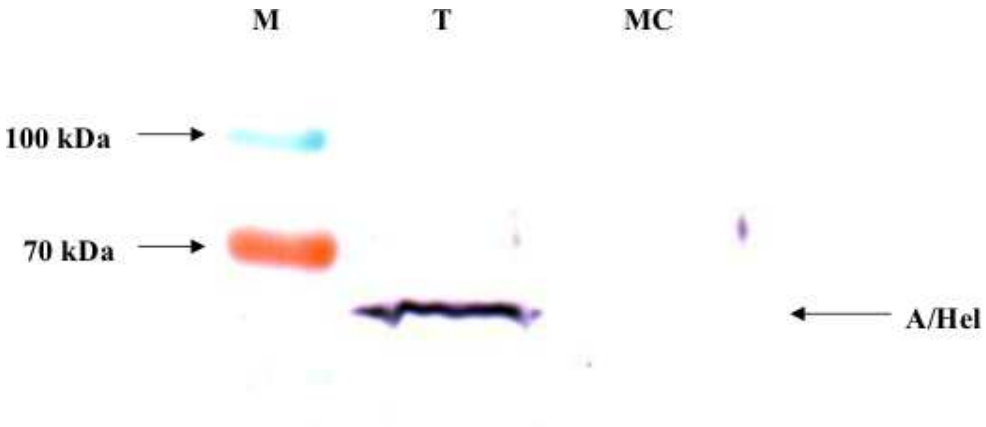

Figure 3.9: Western-blot to examine the production of A/Hel protein in insect cells. High Five insect cells were transfected with the H-pIB/V5-His vector. Transfected cells were lysed, the cell-lysate was separated on a 9 % SDS-polyacrylamide gel, and proteins were transferred to a PVDF membrane. Identification of recombinant A/Hel protein was performed using anti-V5 antibody. Mock (untransfected) cells were used as a negative control. M: protein marker, T: cell lysate from transfected cells, MC: cell-lysate from mock cells.

Expression of the recombinant protein was verified by Western blotting using an anti-V5 antibody. A band of about 67 kDa corresponding to the molecular weight of A/Hel protein was detected in cell lysate of the transfected cells. Mock (untransfected) cells, which served as a negative control, showed no production of the recombinant A/Hel protein.

3.1.2.2 Purification of recombinant A/Hel protein from insect cells

Since the recombinant A/Hel protein was fused with a His tag, the recombinant protein was purified using Ni-NTA affinity chromatography. Soluble cell extract from transfected insect cells was applied to a HisTrap column. Bound proteins were eluted step-wise with various imidazole concentrations (Figure 3.10A). Protein-containing fractions were selected and analysed for purity and presence of the recombinant A/Hel protein using SDS-PAGE and Western blotting. Results obtained from the SDS-PAGE analysis showed that there were still many protein contaminants in the different fractions (Figure 3.10B). None of the analysed fractions contained the A/Hel protein in high purity. Insect cells are known to contain

histidine-rich proteins. These proteins tend to bind to the HisTrap column and as a result, make the purification of the recombinant A/Hel protein very difficult. Western blot analysis of the elution fractions revealed the presence of the recombinant A/Hel protein in fractions of the latter eluates (C2 - D8) (Figure 3.10C). The identification of the recombinant A/Hel protein was performed using an anti-V5 antibody since the recombinant protein was fused with a V5 epitope.

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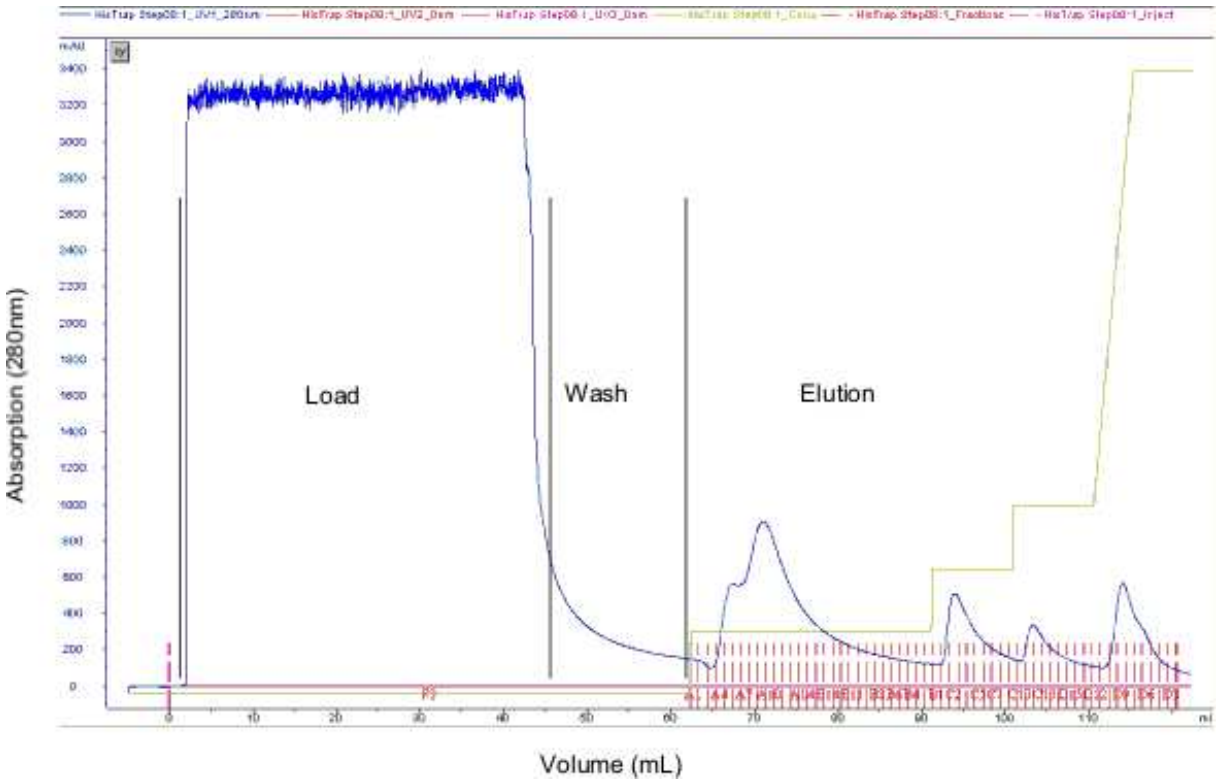

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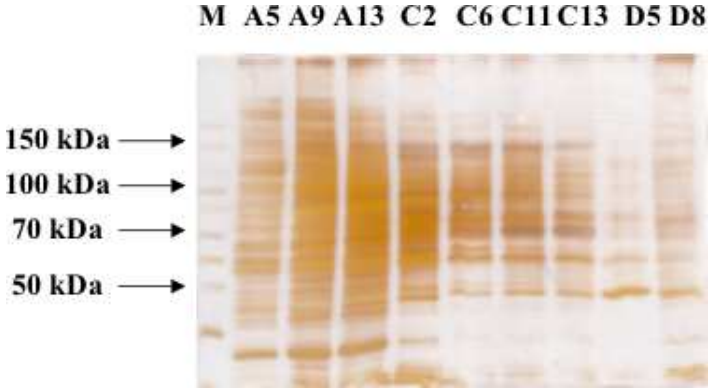

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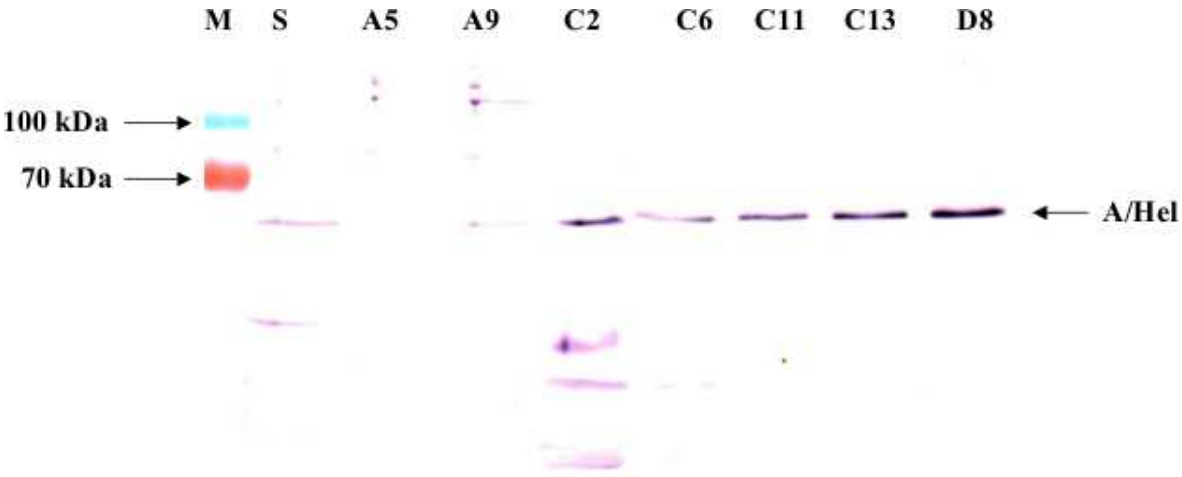


Figure 3.10: Purification of recombinant A/Hel protein from insect cells using Ni-NTA affinity chromatography. (A) High Five insect cells producing the A/Hel protein were lysed and the soluble cell extract was applied to a HisTrap column at a flow rate of 1 mL/min. Bound proteins were eluted with various concentration of imidazole. (B) The eluates (A5, A9, A13, C2, C6, C11, C13, D5 and D8) were analysed on a 9 % SDS-polyacrylamide gel. Proteins were stained using silver ions. (C) Soluble extract (S) and eluates (A5, A9, C2, C6, C11, C13 and D8) were loaded on a 9 % SDS-polyacrylamide gel. After SDS-PAGE, proteins were transferred to a PVDF membrane. Identification of recombinant A/Hel protein was performed using an anti-V5 antibody.

In order to characterise the recombinant A/Hel protein produced in insect cells, the protein had to be of a high purity. Further purification of the protein using ion exchange chromatography was initiated. A/Hel protein-containing fractions from the Ni-NTA affinity purification were pooled and dialysed to reduce the high salt concentration in the original protein buffer. Upon dialysis in low or moderate-salt buffers (containing up to 150 mM NaCl), the proteins including the recombinant A/Hel protein precipitated out of solution. Changing the dialysis buffer from Tris to phosphate buffer or varying the pH of the buffer from 6.5 to 8.5 did not prevent precipitation. As a result, purification of the recombinant protein using ion exchange chromatography was aborted. Furthermore, purification of the recombinant A/Hel protein using size exclusion chromatography was also futile, since the concentration of the recombinant protein expressed in insect cells was very low.

3.2 Characterisation of recombinant A/Hel domain of hDicer

Since the recombinant A/Hel protein produced in *E. coli* was purified to near homogeneity and the concentration of the purified protein was sufficient for biochemical analysis, further characterisation of recombinant A/Hel protein was carried out using the recombinant protein produced from the bacteria.

3.2.1 Recombinant A/Hel protein has an ATPase activity

The presence of the DECH-box motif in the A/Hel domain of hDicer has led to the prediction that this protein may be an ATPase. To investigate whether the purified recombinant A/Hel protein possesses ATPase function, a colorimetric ATPase assay monitoring phosphate release was performed using 0.5 mM ATP in the presence of 2 mM $MgCl_2$ but in the absence of RNA. The recombinant protein catalysed the hydrolysis of ATP into ADP and inorganic phosphate (Figure 3.11). The ATPase activity was directly proportional to the incubation time. No hydrolysis of ATP was observed with negative controls containing no protein or only MBP. Additionally, to eliminate the possibility that the ATPase activity of the recombinant A/Hel protein may be a result of A/Hel interacting proteins from *E. coli*, A/Hel protein variants (D210A and K70A) purified by an identical procedure were examined for ATPase activity. There was about two-fold reduction in ATPase activity of the D210A variant, confirming the ATPase activity of the A/Hel protein (Figure 3.11). However, the K70A variant showed an ATPase activity similar to the wt A/Hel protein.

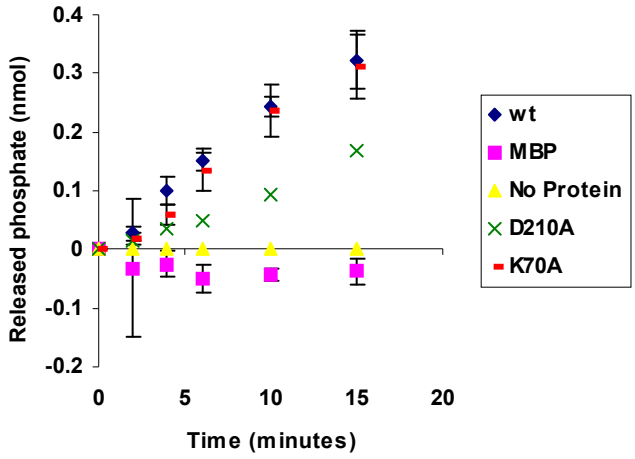


Figure 3.11: Hydrolysis of ATP by recombinant A/Hel protein. Proteins were incubated in reaction buffer in the presence of 0.5 mM ATP at 37 °C. Aliquots were taken at 0, 2, 4, 6, 10 and 15 min. Reactions were stopped by the addition of EDTA. Biomol Green Reagent was added and the absorbance was measured at 620nm. The amount of released phosphate was calculated by interpolation of the OD_{620nm} values to a phosphate standard curve. Each data point is the average from at least three independent measurements (mean ± s.d., n = 3).

Since hydrolysis of ATP by some proteins from the DExH/D-box family has been reported to be dependent on RNA as a cofactor (Iggo and Lane, 1989, Tanaka and Schwer, 2005), stimulation of ATPase activity of the recombinant A/Hel protein in the presence of 1 µM GAPDH 21 sense RNA was tested. However, the presence of the RNA did not lead to a stimulation of ATP hydrolysis by the recombinant protein (Figure 3.12). To exclude the possibility that the lack of RNA stimulation of the ATPase activity was due to RNA contamination of the recombinant protein, RNase A was added to the reaction. The same ATPase activity was observed in the reactions with or without RNase A (Figure 3.12), concluding that the ATPase activity of the A/Hel protein is independent of RNA. Furthermore, since hydrolysis of NTPs by helicases requires divalent metal cations, the effect of Mg^{2+} ions on ATPase activity of the recombinant A/Hel protein was investigated. Hydrolysis of ATP by the recombinant A/Hel protein was dependent on Mg^{2+} ions (Figure 3.12). In the absence of the metal ion, no ATP hydrolysis was observed. Negative controls

containing no protein or only MBP showed no hydrolysis of ATP. These results strongly suggest that the ATPase activity is linked directly to the recombinant A/Hel protein.

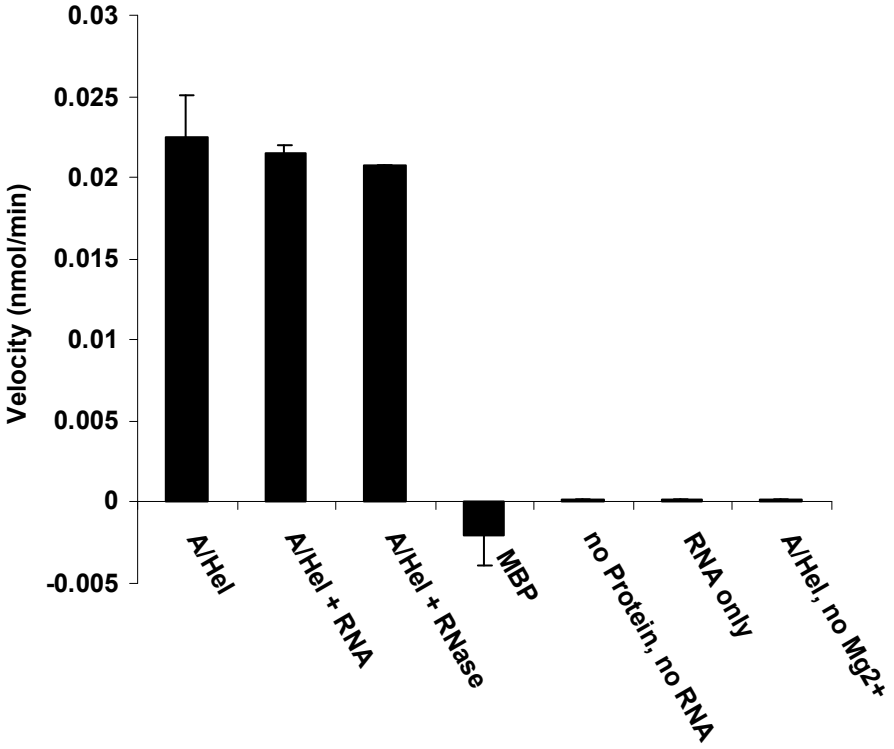


Figure 3.12: ATPase activity of recombinant A/Hel protein in the presence of RNA. Reactions were carried out in reaction buffer with 0.5 mM ATP and 113 nM A/Hel protein at 37 °C for 15 min in the presence of 1µM GAPDH 21 sense RNA or RNase A or in the absence of RNA. Reactions were stopped by the addition of EDTA. Biomol Green Reagent was added and the absorbance was measured at 620 nm. The amount of released phosphate was calculated by interpolation of the OD_{620nm} values to a phosphate standard curve. Each data point is the average from at least three independent measurements (mean $\pm$ s.d., n = 3).

3.2.2 Kinetic parameters for the ATPase activity of recombinant A/Hel protein

To determine the affinity of the recombinant A/Hel protein for ATP, hydrolysis of ATP was measured as a function of ATP concentration. Various concentrations of ATP were incubated with the recombinant A/Hel protein and the initial reaction velocities were measured. An increase in enzyme activity with increasing ATP concentrations was observed (Figure 3.13).

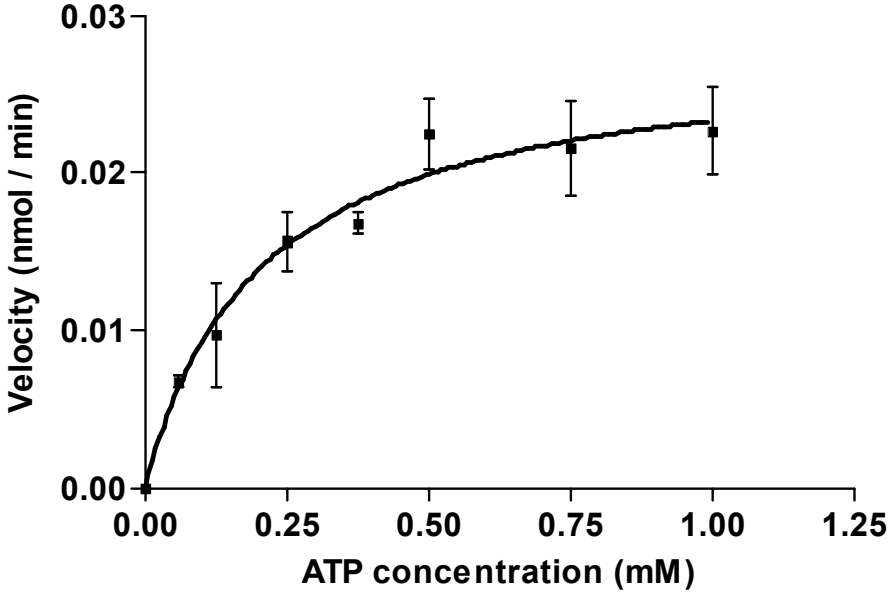


Figure 3.13: Kinetic measurements of the ATPase activity of recombinant A/Hel protein. Reactions were carried out in reaction buffer with 113 nM A/Hel protein and different concentrations of ATP at 37 °C for various incubation times. Reactions were stopped by the addition of EDTA. Biomol Green Reagent was added and the absorbance was measured at 620 nm. The amount of released phosphate was calculated by interpolation of the OD_{620nm} values to a phosphate standard curve. Kinetic parameters were determined by linear and non-linear regression plots using GraphPad Prism 4.03. Values depicted represent the average of at least three independent measurements (mean $\pm$ s.d., n = 3).

At 0.5 mM ATP, substrate saturation was achieved and no further increase in enzyme activity at higher ATP concentrations could be detected. The reaction kinetics followed the Michaelis-Menten model showing a Hill coefficient of about 1, which implies the absence of a cooperative effect. Therefore, the affinity of the A/Hel protein for a single ATP molecule is independent of whether or not other ATP molecules are already bound to the protein. The Michaelis-Menten constant for ATP ($K_{m\ ATP}$) was determined to be 207.5 µM, whereas the enzyme turnover number, k_{cat}, representing the maximal catalytic rate was determined to be 10 min^{-1}. The specificity constant (k_{cat}/K_m) for the recombinant A/Hel protein was 5 x 10^4 $min^{-1}M^{-1}$.

3.2.3 Hydrolysis of NTPs and dNTPs by recombinant A/Hel protein

Since the specificities of DExD/H proteins for a particular NTP and the extent of NTPase activity vary considerably among the different proteins (Uhlmann-Schiffler *et al*, 2006, Du *et al*, 2002), the nucleotide specificity of recombinant A/Hel protein was assessed using different NTPs and dNTPs. There was an effective hydrolysis of purine triphosphates (ATP, dATP, GTP, dGTP) by the recombinant A/Hel protein, while no hydrolysis of pyrimidine triphosphates (CTP, dCTP, UTP, dTTP) could be detected (Figure 3.14).

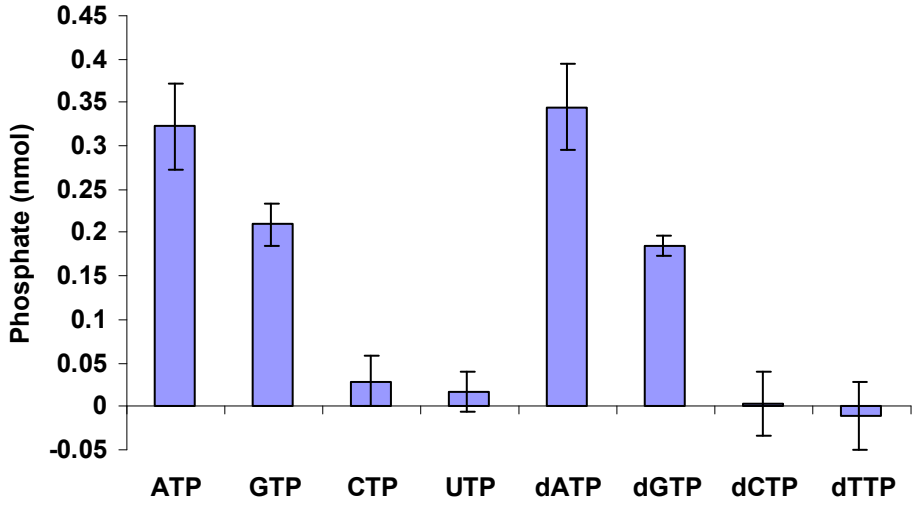


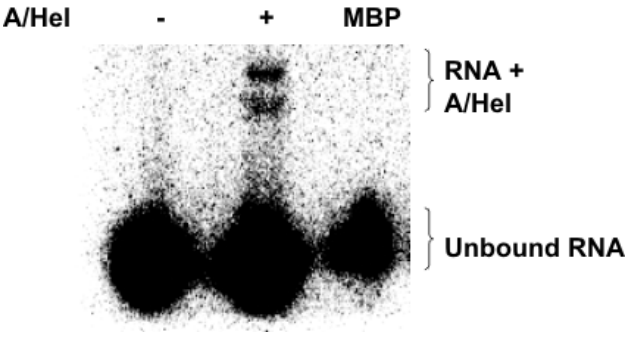
Figure 3.14: NTPase activity of recombinant A/Hel protein. The recombinant protein was incubated with 0.5 mM of each NTP or dNTP in a reaction buffer at 37 °C for 15 min. Reactions were stopped by the addition of EDTA (pH 8.0). Biomol Green Reagent was added and the absorbance measured at 620 nm after incubation at 22 °C for 20 min. The amount of released phosphate was calculated by interpolation of the OD_{620nm} values to a phosphate standard curve. Each data point is the average from at least three independent measurements (mean $\pm$ s.d., n = 3).

3.2.4 RNA binding

hDicer is known to form stable complexes with ssRNA, dsRNA and siRNA (Pellino *et al*, 2005, Provost *et al*, 2002, Zhang *et al*, 2002, Kini and Walton, 2007), though there are some conflicting reports existing for the binding of the protein to siRNA (Chendrimada *et al*,

2005). To study whether the A/Hel protein would also form stable complexes with ssRNA and siRNA, binding reactions were set up with 5′-^{32}P-GAPDH 21 sense RNA, or siRNA and A/Hel protein at 37 °C. The reaction products were analysed using a gel-shift assay. The results showed that A/Hel protein bound to the radiolabelled ssRNA in the presence of ATP and Mg^{2+} (Figure 3.15A). Two bands were seen in the binding of ssRNA to A/Hel protein, suggesting that different stoichiometries are possible. Negative controls containing MBP or no protein did not show any binding of the ssRNA. Furthermore, the binding of the recombinant protein to the ssRNA in the absence of Mg^{2+} and/or ATP indicated that the formation of A/Hel protein-ssRNA complex was independent of Mg^{2+} and ATP (Figure 3.15B). The same result as observed for A/Hel protein binding to the ssRNA in the presence of ATP and Mg^{2+} was also seen in the absence of Mg^{2+} and ATP. Thus, Mg^{2+} and ATP are not required for the binding of ssRNA by the recombinant protein. Additionally, binding of the wt and variant A/Hel proteins to a radiolabelled 70 nucleotide ssRNA in the absence of ATP and Mg^{2+} was investigated. Each of the three proteins was able to bind the ssRNA (Figure 3.15C).

A



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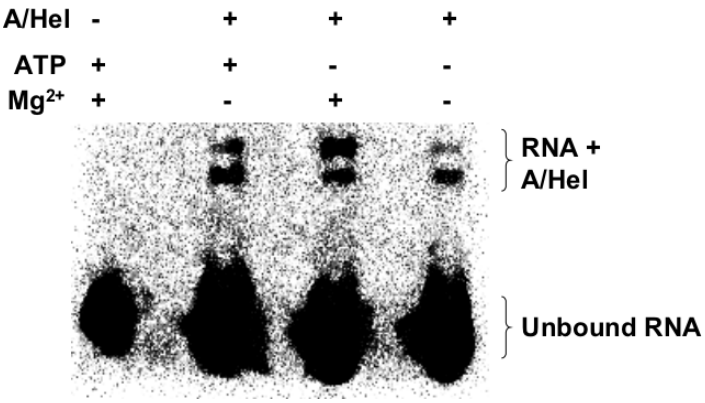

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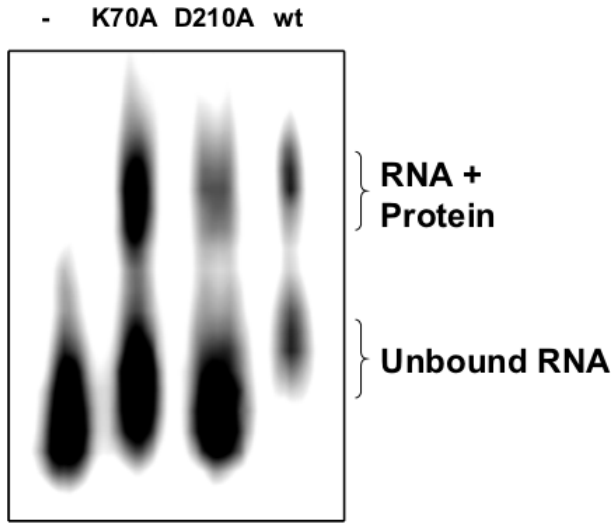

Figure 3.15: Binding of ssRNA by recombinant A/Hel protein. (A) Binding of recombinant A/Hel protein to a GAPDH 21 sense RNA in the presence of ATP and Mg^{2+}. (B) Binding of recombinant A/Hel protein to a GAPDH 21 sense RNA in the absence of ATP and/or Mg^{2+}. (C) Binding of the wt and variant A/Hel proteins to a 70 nucleotide ssRNA (70R). In a gel-shift assay, the recombinant proteins were incubated with ssRNA at 37 °C for 30 min in the presence or absence of ATP and/or Mg^{2+}. Samples were separated at 100 V for 1 h at 4 °C on an 8 % polyacrylamide gel. The gel was exposed to storage phosphor screens and imaged on a Personal Molecular Imager.

Additionally, association of A/Hel protein with siRNA in the presence or absence of Mg^{2+} and ATP was examined. No binding of the A/Hel protein to siRNA was detected under any conditions (Figure 3.16A and 3.16B).

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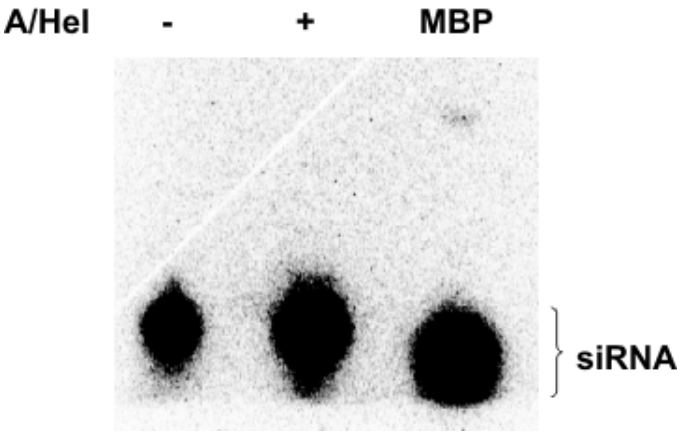

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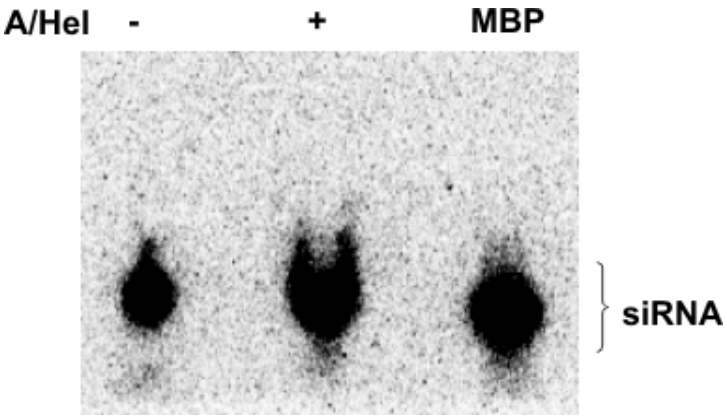

Figure 3.16: Recombinant A/Hel protein does not bind siRNA. (A) Binding-assay of recombinant A/Hel protein and siRNA in the presence of ATP and Mg^{2+} and (B) in the absence of ATP and Mg^{2+}. In a gel-shift assay, the recombinant A/Hel protein was incubated with a radiolabelled siRNA at 37 °C for 30 min in the presence or absence of ATP and/or Mg^{2+}. Samples were separated at 100 V for 1 h at 4 °C on an 8 % polyacrylamide gel. The gel was exposed to storage phosphor screens and imaged on a Personal Molecular Imager.

3.2.5 dsRNA unwinding

Since the conserved helicase motifs are present in the A/Hel domain of hDicer, unwinding activity of the recombinant A/Hel protein was investigated. RNA helicase activity was tested using siRNA and dsRNA substrates (a GAPDH 16 sense RNA hybridised to a radiolabelled GAPDH 21 antisense RNA to produce a 16-bp duplex region with a 3´single-strand overhang). Incubation of the RNA substrates with the recombinant A/Hel protein in the presence of Mg^{2+} and ATP did not result in the displacement of the radiolabelled 21-nucleotide long RNA, as analysed by PAGE followed by autoradiography (Figure 3.17). Hence no unwinding of the RNA duplexes by the recombinant A/Hel protein could be detected.

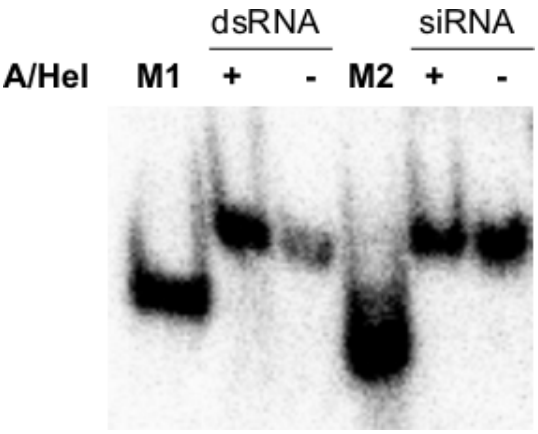

Figure 3.17: Recombinant A/Hel protein has no unwinding activity. Duplex RNA substrates were incubated with the recombinant protein at 37 °C for 1 h in the presence of ATP and Mg^{2+}. Unlabelled GAPDH 21 antisense RNA was added to prevent the reannealing of unwound RNA oligonucleotides, which could take place under the reaction conditions. Reactions were stopped by transferring on ice and addition of 4 µL of loading buffer. Samples were analysed by electrophoresis on an 18 % polyacrylamide gel at 4 °C for 3 h at 100 V using TBE running buffer. The radiolabelled samples were detected using Personal Molecular Imager. M1: ^{32}P-labelled GAPDH 16 sense RNA, M2: ^{32}P-labelled GAPDH 21 antisense RNA.

Furthermore, to confirm the result that A/Hel protein exhibited no unwinding activity, a recently developed assay that combines the monitoring of RNA annealing and strand displacement activities in a single set-up and detects dsRNA by FRET was employed (Rajkowitsch and Schroeder 2007). After formation of double-labelled dsRNA, which was

observed as an increase in FRET, the injection of non-labelled competitor RNA showed no decrease in FRET, which would be expected in the case of unwinding activity by A/Hel protein (Figure 3.18). This result emphasized that the A/Hel protein has no strand displacement activity on the dsRNA substrate.

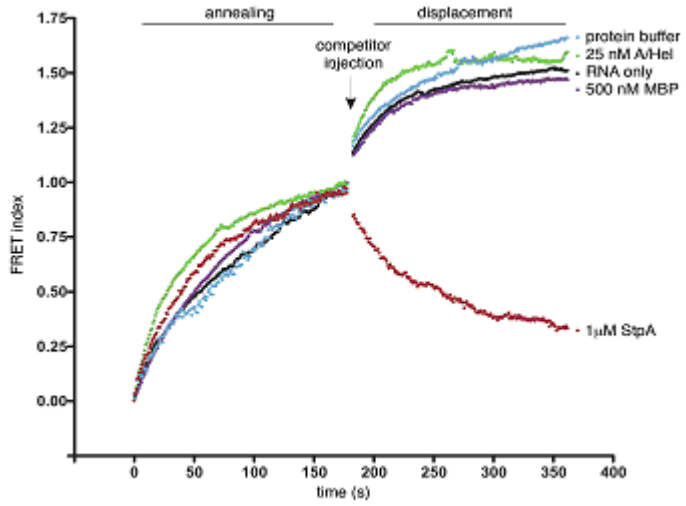

Figure 3.18: A/Hel protein has no unwinding activity but efficiently anneals complementary strands of RNA. In a combined RNA annealing and strand displacement assay, 25 nM A/Hel protein or 1 µM StpA, a known RNA chaperone from *E. coli*, which served as the positive control, was incubated with a Cy3-fluorescence labelled RNA (Cy3-21R-) and its complementary Cy5-fluorescence labelled RNA oligonucleotide containing a 3´ hairpin (Cy5-21R+hp) in phase I of the assay (annealing). The donor (Cy3) and acceptor (Cy5) fluorescence emissions were quantified every second for 180 s. The FRET index was calculated as F_{Cy5}/F_{Cy3} and normalized to 1 at $t_{180\,s}$. The second phase of the assay (displacement) was initiated by the injection of a 10-fold excess of non-labelled competitor RNA (21R+). Representative curves are shown. The decrease in FRET indicates that StpA induces strand displacement. Negative controls comprised of the fluorescence labelled RNA oligonucleotides, 500 nM MBP, or 50 % (v/v) protein storage buffer.

3.2.5 A/Hel protein exhibits RNA annealing activity

Several DExD/H-box proteins have been shown to catalyse RNA strand annealing (Cordin *et al*, 2006, Uhlmann-Schiffler *et al*, 2006, Jankowsky and Fairman, 2007). To test whether the recombinant A/Hel protein can enhance annealing of complementary RNA strands, RNA annealing activity of 25 nM A/Hel protein was examined using a Cy3-fluorescence labelled 21-nucleotide long RNA (Cy3-21R-) and its complementary Cy5-

fluorescence labelled RNA oligonucleotide containing a 3′ hairpin (Cy5-21R+hp). The recombinant A/Hel protein catalysed the annealing of the oligonucleotides in the absence of ATP (Figure 3.18). The short RNAs used in this study do not form stable intra-molecular secondary structures and as such, can anneal with each other inter-molecularly with an observed annealing rate constant k_{ann} of 0.009 sec^{-1}. The bimolecular rate for duplex formation was enhanced approximately four-fold by the recombinant A/Hel protein in comparison to negative controls without the recombinant protein (Table 3.1). As a positive control, StpA, a known RNA chaperone from *E. coli*, which has both RNA annealing and strand displacement activities was used (Zhang *et al*, 1995, Rajkowitsch *et al*, 2005, Rajkowitsch and Schroeder, 2007).

Table 3.1: Reaction constants of RNA annealing.

Sample	**K_{ann} (s^{-1})**
RNA only	0.009 ± 0.0005
Protein buffer	0.006 ± 0.0030
1 µM StpA	0.018 ± 0.0031
500 nM MBP	0.009 ± 0.0001
25 nM A/Hel protein	0.028 ± 0.0037

Values depicted represent the average of at least three independent measurements (mean ± s.d., n ≥ 3). K_{ann}: observed annealing reaction constant.

RNA annealing activity was also examined at different concentrations of the recombinant A/Hel protein. An increase in annealing activity was observed with increasing A/Hel protein concentration (Figure 3.19). Similar results were also obtained when two complementary RNA oligonucleotides that formed a blunt-ended substrate were used (data not shown). Hence, the hairpin of the former RNA substrate is not required for loading or binding by the recombinant A/Hel protein.

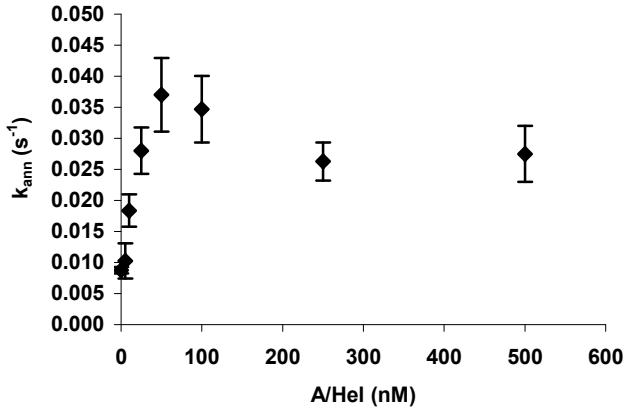

Figure 3.19: RNA annealing at different concentrations of A/Hel protein. Various concentrations of A/Hel protein (0, 5 nM, 10 nM, 25 nM, 50 nM, 100 nM, 250 nM and 500 nM) were incubated with Cy3-fluorescence labelled 21-nucleotide long RNA (Cy3-21R-) and its complementary Cy5-fluorescence labelled RNA oligonucleotide containing a 3´ hairpin (Cy5-21R+hp) in the absence of ATP. The donor (Cy3) and acceptor (Cy5) fluorescence emissions were quantified every second for 180 s. The FRET index was calculated as F_{Cy5}/F_{Cy3} and normalized to 1 at $t_{180\ s}$. The resulting time-resolved curve was fitted with the second-order reaction equation for equimolar initial reactant concentrations $y = A\ (1 - 1\ /\ (k_{ann}\ t + 1))$ using Prism 4.03 (GraphPad Software). k_{ann}: observed annealing reaction constant, A: maximum reaction amplitude. Average values were derived from three individually fitted measurements.

Furthermore, RNA annealing activity of the recombinant A/Hel protein was tested in the presence of ATP. Interestingly, addition of ATP reduced the ability of the recombinant protein to accelerate annealing (Figure 3.20A). In fact, at 5 mM ATP concentration, a complete inhibition of annealing activity of the recombinant protein was observed. A similar effect was also seen using other NTPs (Figure 3.20B). Addition of equimolar Mg^{2+} ions partly restored the annealing activity of the recombinant protein. Presumably, ATP binds to Mg^{2+}, which might be required for the RNA annealing by the recombinant A/Hel protein.

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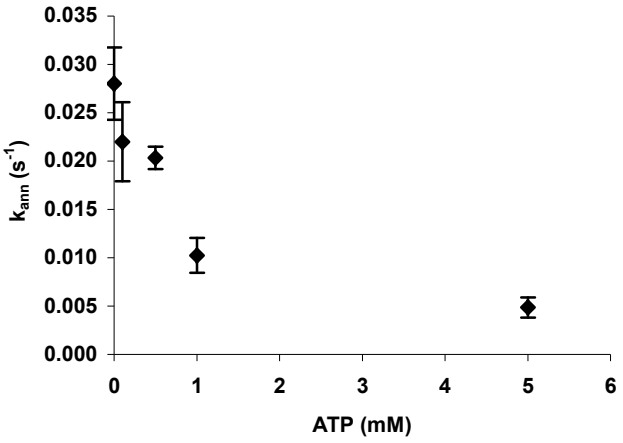

B

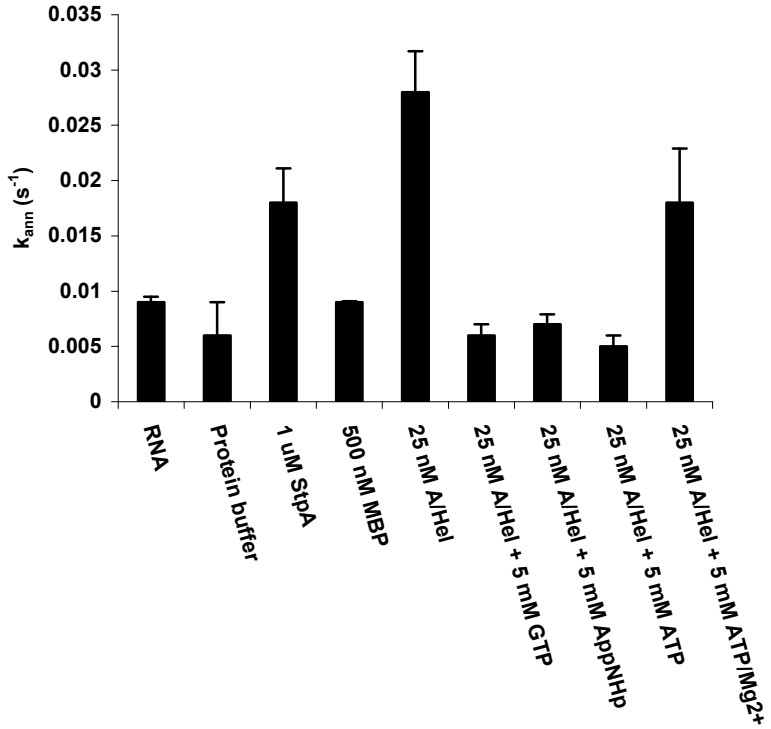

Figure 3.20: Effect of NTPs on the annealing activity of A/Hel protein. (A) 25 nM A/Hel protein was incubated with Cy3-fluorescence labelled RNA (Cy3-21R-) and its complementary Cy5-fluorescence labelled-RNA oligonucleotide containing a 3´ hairpin (Cy5-21R+hp) in the presence of 0.1 mM, 0.5 mM, 1mM and 5mM ATP. (B) 25 nM A/Hel protein was incubated with Cy3-fluorescence labelled RNA (Cy3-21R-) and its complementary Cy5-fluorescence labelled RNA oligonucleotide containing a 3´ hairpin (Cy5-21R+hp) in the presence of 5 mM GTP or 5 mM AppNHp. The donor (Cy3) and acceptor (Cy5) fluorescence emissions were quantified every second for 180 s. The FRET index was calculated as F_{Cy5}/F_{Cy3} and normalized to 1 at $t_{180\,s}$. The resulting time-resolved curve was fitted with the second-order reaction equation for equimolar initial reactant concentrations $y = A\,(1 - 1 / (k_{ann}\,t + 1))$ using Prism 4.03 (GraphPad Software). k_{ann}: observed annealing reaction constant, A: maximum reaction amplitude. Average values were derived from three individually fitted measurements.

Finally, the annealing activity of the variant A/Hel proteins was tested. Measurement of annealing activity up to a protein concentration of 400 nM showed that there was a more than two-fold reduction of the annealing activity of the variant proteins when compared with the wt A/Hel protein (Figure 3.21).

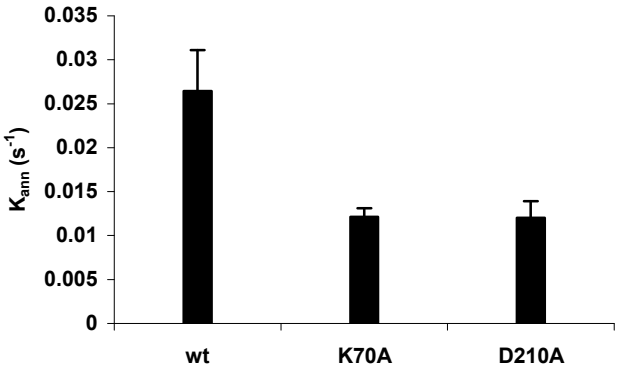

Figure 3.21: RNA annealing activity of A/Hel protein variants. 400 nM wt and variant A/Hel proteins were incubated with Cy3-fluorescence labelled RNA (Cy3-21R-) and its complementary Cy5-fluorescence labelled RNA oligonucleotide containing a 3´ hairpin (Cy5-21R+hp) in the absence of ATP. The donor (Cy3) and acceptor (Cy5) fluorescence emissions were quantified every second for 180 s. The FRET index was calculated as F_{Cy5}/F_{Cy3} and normalized to 1 at $t_{180\,s}$. The resulting time-resolved curve was fitted with the second-order reaction equation for equimolar initial reactant concentrations $y = A\,(1 - 1 / (k_{ann}\,t + 1))$ using Prism 4.03 (GraphPad Software). k_{ann}: observed annealing reaction constant, A: maximum reaction amplitude. Average values were derived from three individually fitted measurements.

4 Discussion and Outlook

RNAi occurs through a complex series of linked biochemical events involving a large number of protein components, many of which are still being identified (Rose *et al*, 2005, Rand *et al*, 2004). Although efforts to identify the different proteins involved in RNAi have been extensive, studies that characterise the exact biochemical function of any particular protein or complex in the RNAi pathway are limited. Most investigations have relied on crude cell extracts, and as a result, the roles of some proteins in the RNAi pathway are still unclear. This study investigated the functions of the A/Hel domain of hDicer and presented a comprehensive characterisation of the activity of the purified recombinant protein. Additionally, procedures for producing and purifying the recombinant A/Hel protein were established. This work provides the first evidence that the A/Hel domain of hDicer has an ATPase function. Moreover, it demonstrates a direct interaction of ssRNA with the aforementioned Dicer domain. Finally, this study demonstrates that the domain enhances RNA annealing.

4.1 Expression of A/Hel domain of hDicer

4.1.1 Production in *E. coli*

Production of recombinant proteins in *E. coli* is the oldest and most commonly utilized method for production of recombinant proteins. Prokaryotic production of recombinant proteins offers many advantages, which include: easy handling of culture, cost effectiveness, inducible expression using IPTG, high protein yield, and simple purification. Although

machineries for post-translational modifications of proteins are lacking in prokaryotes, there are many reports about an effective and successful technique for production of Dicer domains as well as other proteins involved in RNAi in *E. coli* (Takeshita *et al*, 2007, Rivas *et al*, 2005, Ma and Patel, 2004, Kawasaki *et al*, 2003).

Two different strategies were used for the expression of the recombinant A/Hel protein in *E. coli*. In the first approach, the recombinant protein was produced with an N-terminal His–tag, whereas the second approach was based on the production of the recombinant protein in fusion with an N-terminal MBP and a C-terminal His-tag. MBP and other protein tags are known to enhance the solubility of their fusion partners (Jacquet *et al*, 1999).

The production of the N-terminal His-tag A/Hel fusion protein was under the control of the strong T7 promoter. Protein production was detected using immunoblotting and analysis of the cell lysate showed that the recombinant A/Hel protein was found in the insoluble fraction as inclusion bodies. Inclusion bodies are major protein aggregates, which commonly occur in recombinant bacteria when the expression of plasmid-encoded genes is induced at high rates (Georgiou and Valax, 1996). These bodies are often seen in the production of heterologous proteins in *E. coli* (Carrio *et al*, 2001).

Many methods have been designed to improve the formation of soluble active proteins during production in *E. coli*. Coproduction of recombinant proteins with chaperones has sometimes proved to be effective in enhancing the solubility of proteins. Soluble and active recombinant human Argonaute 2 has been produced in *E. coli* cells, coproducing the HSP 90 chaperone (Rivas *et al*, 2005). Studies have shown that production of recombinant proteins in Origami *E. coli* cells, coproducing variants of thioredoxin reductase and glutathione reductase, which produce the oxidative cytoplasmic environment, yielded a 10-fold more soluble and active protein than in a standard *E. coli* host cell (Rabhi-Essafi *et al*, 2007, Saejung *et al*, 2006). However, production of the N-terminal His-tag-A/Hel fusion protein in these cells did not lead to the formation of a soluble recombinant A/Hel protein.

Another method for preventing inclusion body formation as well as increasing the solubility of proteins is to reduce the growth rate of the bacteria. Growth at lower temperatures is a well-known technique for facilitating correct folding (Schrodel and de Marco, 2005). The reason why a lower temperature favours the native state is related to a number of factors including: a decrease in the driving force for protein self-association, a slower rate of protein synthesis, and changes in the folding kinetics of the polypeptide chain. Lowering the amounts of the expression inducer (IPTG concentration) and the rate of revolution have often improved the yield of recombinant soluble proteins. Nevertheless, in all of the tested cases, the recombinant A/Hel protein was recovered as a precipitate in inclusion bodies.

Since the attempts to produce a soluble His-tag-A/Hel fusion protein in *E. coli* were unsuccessful, addition of a protein tag at the N-terminus of the recombinant A/Hel protein was initiated. An N-terminal MBP and a C-terminal His-tag was added to the recombinant A/Hel protein. MBP has been reported to be one of the best and most frequently used tags for protein solubilization in *E. coli* (Kapust and Waugh, 1999, Spangfort *et al*, 1996, Pryor and Leiting, 1997). In addition to its solubilizing property, MBP exhibits a high affinity to amylose and offers the opportunity to purify recombinant proteins using amylose affinity chromatography. The MBP-A/Hel-His fusion protein was placed under the control of a lac-promoter, and was produced as a soluble protein in *E. coli* cells. Using a two-step affinity chromatography comprising of Ni-NTA and amylose affinity chromatographies, the recombinant A/Hel fusion protein was purified to homogeneity. The identity of the protein was confirmed by Western blot analysis using anti-His and anti-MBP antibodies.

4.1.2 Expression in insect cells

The insect cell is an eukaryotic system able to carry out complex post-translational modifications. It also has the ability for proper folding of mammalian proteins and therefore, offers the best chance of obtaining a soluble protein when a protein of mammalian origin is being produced.

The coding sequence of the A/Hel domain of hDicer was cloned into a pIB/V5-His plasmid vector and transfected into High Five insect cells using lipid-mediated transfection. The expression of the recombinant protein was under the control of the *OpIE2* promoter, which is derived from the baculovirus *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (*OP*MNPV). The *OpIE2* promoter provides relatively high levels of constitutive expression. Many studies have reported the expression of full-length hDicer in insect cells using the baculoviral system (Provost *et al*, 2002, Zhang *et al*, 2002, Kolb *et al*, 2005). Expression of the recombinant A/Hel protein in insect cells produced a soluble protein fused with a C-terminal V5 epitope and a His-tag. The identity of the recombinant protein was confirmed using an anti-V5 antibody. Stable insect cell lines expressing the recombinant protein were selected using blasticidin S. This offered the opportunity for a continuous production of the recombinant protein by the cells. Since the recombinant protein contained a C-terminal His-tag, its purification was carried out using Ni-NTA affinity chromatography. This method provided a concentration of the recombinant protein, but failed to purify the protein to homogeneity. Many protein contaminants - histidine-rich proteins present in the cell extract of the insect cells - eluted with the recombinant protein during the affinity purification. The contaminating proteins bound with the same affinity to the Ni-NTA column as the recombinant A/Hel protein, making it difficult to purify the recombinant protein using this method. Further purification of the recombinant A/Hel protein from the contaminating

proteins using ion-exchange chromatography was unsuccessful, since the recombinant protein precipitated along with other proteins at low salt concentrations. Solubility of proteins at low ionic strength increases with salt concentration due to pairing of salt ions with charged groups of the protein allowing the stabilization of protein structures. The precipitation of the proteins at low salt concentration was irreversible, suggesting that the proteins were denatured. Purification of the recombinant A/Hel protein using size exclusion chromatography was not possible because the concentration of the recombinant protein was very low. Size exclusion chromatography is only suitable for purifying proteins when the protein of choice is present in a relatively high concentration.

4.2 Characterisation of recombinant A/Hel domain of hDicer

The recombinant A/Hel protein produced in *E. coli* was used for functional investigations since the protein was purified to homogeneity. Characterisation of a protein with unknown functions is often favorable when the protein is pure and in high concentration. This prevents the interference of its functions by contaminating proteins. Since sequence alignment grouped the A/Hel domain of hDicer to the DExD/H family of RNA helicases, characterisation of the domain was strongly based on features unique to the family.

4.2.1 Recombinant A/Hel protein has an ATPase activity

Sequence analysis revealed that the primary structure of the A/Hel domain of hDicer is related to that of the DExD/H family of RNA helicases and that the domain possesses six consensus sequence motifs of the RNA helicase family. Because many proteins of this family

have been previously characterised as ATPases (Gorbalenya *et al*, 1989, Schmid. and Linder, 1992), the hypothesis that the A/Hel domain of hDicer might share the same enzymatic property was made.

In vitro biochemical studies showed that the recombinant A/Hel protein had ATPase activity. Alteration of the conserved aspartate of Motif II to alanine in the A/Hel protein decreased ATPase activity. Similarly, reports have indicated that mutations within this motif in DExD/H proteins decrease or abolish ATPase activities without altering RNA binding (Pause and Sonenberg, 1992, Iost *et al*, 1999). These results indicate that the motif plays an important role in ATP catalysis. However, in contrast to some DExD/H proteins, changing the conserved lysine of Motif I to alanine had no significant effect on ATPase activity of the A/Hel protein. Motif I has been shown to be involved in NTP binding by forming hydrogen bonds with the pyrophosphates of the nucleotide (Caruthers and McKay, 2002).

Previous studies have suggested that the ATPase activity of some DExD/H proteins is stimulated in the presence of RNA (Iggo and Lane, 1989, Tanaka and Schwer, 2005). However, ATP hydrolysis by A/Hel protein was not dependent on RNA presence. There was no difference in the ATPase activity of A/Hel protein with or without RNA. These results are consistent with those obtained for the DEAD-box proteins Dbp9 from yeast and Xp54 from *Xenopus laevis*, which exhibit an RNA-independent ATPase activity (Ladomery *et al*, 1997, Kikuma *et al*, 2004). The ATPase activity of some DEAD-box proteins, for example eIF4A and DbpA from *E. coli*, is known to be greatly enhanced by their biological partners or specifically structured RNAs (Cordin *et al*, 2006). It is probable that the stimulation of the ATPase activity of the A/Hel domain of hDicer may require special RNA structures or cofactors *in vivo*, and that the other domains of the full-length hDicer may contribute to this.

All motor proteins that use ATP to fuel their movements require divalent metal cations, which coordinate the phosphate groups of ATP in the catalytic site (Frick *et al*, 2007). Similarly, the A/Hel protein exhibited ATPase activity only in the presence of Mg^{2+} ions. In

Walker-type ATP-binding sites, Mg^{2+} usually bridges ATP to a conserved aspartate that is near another acidic residue serving as a catalytic base to activate the water molecule that participates in the hydrolysis (Walker *et al*, 1982).

The recombinant A/Hel protein hydrolysed ATP with an affinity comparable to those of other DExD/H proteins. A/Hel protein has a $K_{m\ ATP}$ constant of 207.5 µM, which is within the range of $K_{m\ ATP}$ (80 µM to over 1 mM) reported for other DExD/H proteins (Lorsch and Herschlag, 1998, Wagner *et al*, 1998, Rocak *et al*, 2005). These values are still significantly below the cellular concentration of ATP (5 - 10 mM), indicating that A/Hel protein can bind and hydrolyse ATP in the cytoplasm. The catalytic reaction constant (k_{cat}) for ATP hydrolysis by the recombinant A/Hel protein was 10 min^{-1}. This value is comparable with k_{cat} values of other DExD/H helicases like elF4A from mouse (3 min^{-1}), Has1 from yeast (5.5 min^{-1}), An3 from *Xenopus* (6 min^{-1}) and SrnB from *E. coli* (1.2 min^{-1}), but is much lower than the k_{cat} measured for DbpA from *E. coli* (600 min^{-1}) or that of Prp22p from yeast (400 min^{-1}) (Barhoumi *et al*, 2006).

The discovery that the N-terminal domain of hDicer possesses an ATPase activity opens an important question about how this activity is coupled to the mode of action of hDicer. Dicer is known to process dsRNA substrate into small RNA fragments of discrete size using its two ribonuclease domains, which form an intramolecular dimer (MacRae *et al*, 2007). The double-strand specific ribonuclease activity of recombinant hDicer has been reported to be independent of ATP (Provost *et al*, 2002, Zhang *et al*, 2002), although the reports do not preclude the implication of a distinct ATP-dependent catalytic activity in the RNAi pathway. However, Dcr1 from fission yeast was recently shown to generate siRNAs solely in the presence of ATP requiring its conserved N-terminal putative helicase domain (Colmenares *et al*, 2007). Similarly, ATP is needed for efficient dsRNA cleavage by *Drosophila* extract and by recombinant *Drosophila* Dcr-2 (Lee *et al*, 2004). A role for ATP in siRNA formation, demonstrated for *Drosophila* and *C. elegans* extracts, has generally been

linked to the A/Hel domain of Dicer. Its hypothetical function is to either move the enzyme along the dsRNA or locally unwind the substrate, possibly required for cleavage (Bernstein *et al*, 2001, Ketting *et al*, 2001, Nykanen *et al*, 2001, Hutvagner *et al*, 2002). Thus, ATP appears to be necessary for dsRNA processing in fission yeast and insects, but not in mammals. The precise role of the ATPase activity of A/Hel domain in dsRNA processing remains unclear. It is possible that ATP hydrolysis by Dicer is more likely required for product release and/or a conformational change in the enzyme (Zhang *et al*, 2002), or even for other, still unknown functions of Dicer.

Though NTP binding and hydrolysis by DExD/H proteins is substantially based on the Walker A and B motifs, usage of NTPs by the different DExD/H proteins differs considerably (Du *et al*, 2002, Uhlmann-Schiffler *et al*, 2006). The A/Hel protein effectively hydrolysed purine triphosphates (ATP, dATP, GTP, dGTP), but failed to hydrolyse pyrimidine triphosphates (CTP, dCTP, UTP, dTTP). The different nucleotide specificities shown by recombinant A/Hel protein could be explained in terms of base selectivity. The imidazole ring of purine appears to be a critical determinant for nucleotide binding and hydrolysis. In the context of RNAi, the significance of the nucleotide specificity by the A/Hel protein is unclear.

4.2.2 Recombinant A/Hel protein binds ssRNA

In vitro association of hDicer with ssRNA, dsRNA and siRNA has been reported (Provost *et al*, 2002, Zhang *et al*, 2002, Pellino *et al*, 2005, Kini and Walton, 2007), although there are some contradicting reports existing for hDicer binding to siRNA (Provost *et al*, 2002, Chendrimada *et al*, 2005). The recombinant A/Hel protein binds to ssRNA independently of ATP and Mg^{2+} ions. One group has also shown that recombinant hDicer binds ssRNAs *in vitro*, independent of their sequence and structure (Kini and Walton, 2007).

However, contrary to results of this study, the group reported that recombinant hDicer did not stably bind ssRNA in the absence of Mg^{2+} ions. The difference could be that while those authors used a full-length dicer, this work solely examined the A/Hel domain of the Dicer enzyme. The data presented here strongly suggest that the A/Hel domain binds ssRNA and that the binding of ssRNA by hDicer may be solely dependent on this domain, although it has been speculated that the PAZ domain may partake in the interaction (Kini and Walton, 2007). It remains unclear whether binding of ssRNA by Dicer would occur *in vivo*, and if so, what the biological relevance of such an interaction would be. One possibility is that the binding of ssRNAs by Dicer would protect them from degradative RNases, thereby improving their half-life and chances to be loaded onto RISC.

The recombinant A/Hel protein did not associate with siRNA under any conditions tested in this study. Binding of siRNA by Dicer has been attributed to the PAZ domain, which is an RNA-binding module found in Dicer proteins and in the Argonaute protein family (Filipowicz *et al*, 2005, Kolb *et al*, 2005, MacRae *et al*, 2007). The apparent low RNase catalytic activity observed for Dicer proteins can be attributed to association of Dicer with its reaction product (siRNA) (Zhang *et al*, 2002). In *Drosophila*, Dcr-2 association with siRNA does not require ATP, whereas endogenous hDicer/siRNA interaction does require ATP (Pellino *et al*, 2005). It is yet unknown whether the formation of endogenous hDicer/siRNA complex is achieved through binding and hydrolysis of ATP by the A/Hel domain of hDicer.

4.2.3 RNA unwinding

No RISC protein component has been reported to unwind siRNA *in vitro*. Mature RISC formation requires the removal of the passenger strand and integration of the guide strand into RISC. Genetic data and immuno-precipitation experiments have identified several

RNA helicases that are necessary for/or associated with RNAi in a wide range of eukaryotic organisms. It is still unknown which of the RNAi-related helicases are the best candidates to melt the double-stranded siRNA or miRNA to facilitate RISC formation (Hutvagner *et al*, 2005). In flies, a genetic screen for embryonic axis specification combined with biochemical analysis revealed an RNA helicase, Armitage, whose role in RISC assembly is upstream of siRNA unwinding, but is required for RNAi *in vivo* and for RISC formation *in vitro* (Tomari *et al*, 2004).

The details of RISC activation in human cells are currently unknown (Robb and Rana, 2007). The recombinant A/Hel protein exhibited no *in vitro* unwinding activity, leaving unclear, which protein is responsible for the unwinding of siRNA. Moreover, *in vitro* characterisation of purified RNA Helicase A, a recently identified bona fide human RISC-associated helicase protein, showed that it failed to unwind siRNA-like structures with short 3´ overhangs (Robb and Rana, 2007). *In vitro* unwinding activity has been shown for only a subset of the DExD/H proteins (Cordin *et al*, 2006). This could be due to the assay conditions or the lack of interacting partners. *In vivo*, RNA helicase activities can be modulated due to complex formation with other cellular factors (Uhlmann-Schiffler *et al*, 2006). Unwinding of siRNA by *Drosophila* embryo lysate in the presence of ATP has been reported, although only a small percentage (≤ 5 %) of unwound siRNA was detected (Nykanen *et al*, 2001). The A/Hel protein alone may be insufficient to unwind an RNA duplex. It could also be possible that the A/Hel domain of hDicer has other functions in RNAi, rather than the unwinding of siRNA duplexes. These functions may include rearrangement of ribonucleoprotein complexes or folding of RNA substrates in order to generate siRNAs through subsequent RNase cleavage.

4.2.4 Recombinant A/Hel protein enhances RNA strand annealing.

Several DExD/H-box proteins have been shown to catalyse strand annealing even though strand annealing is not a general feature of RNA helicases (Yang and Jankowsky, 2005). The recombinant A/Hel protein promotes RNA annealing in the absence of ATP, consistent with reports of annealing activity for other DExD/H-box proteins, which either anneal RNA independently of ATP or decrease annealing activity with increasing ATP concentrations, as is the case with Ded1 (Cordin *et al*, 2006, Jankowsky and Fairman, 2007). A notable exception is the bacterial DEAD-box protein CrhR, which requires ATP for strand annealing (Cordin *et al*, 2006). Variation of the conserved lysine and aspartate to alanine in Motifs I and II of the A/Hel protein decreased annealing activity. The exact mechanisms by which ATP inhibits annealing are still unclear. In the presence of ATP, the RNA helicase could adopt conformations less favorable for the facilitation of strand annealing compared to the protein without ATP. The exact physiological significance of strand annealing activities by RNA helicases remains to be elucidated. In the context of Dicer, RNA annealing activity may be involved in the regulation of dicing activity.

4.3 Outlook

The establishment that the A/Hel domain of hDicer has *in vitro* ATPase, ssRNA binding, and RNA annealing activities opens the door to understanding its physiological function. It is noteworthy to perform *in vivo* experiments using the A/Hel protein variant (D210A) generated in this study. This variant has been shown to have a reduced ATPase activity. Physiological investigation of the A/Hel domain of hDicer will entail the incorporation of the same variation into the full-length hDicer protein and the examination of

effects of the alteration on the activity of the enzyme in the cell. Additionally, this strategy will help to elucidate the particular step in the RNAi pathway, where the ATPase activity of A/Hel domain of hDicer is required.

It is still unknown which proteins are responsible for the unwinding of siRNA. Identifying the interacting partners of A/Hel domain of hDicer will be of immense importance since a solo A/Hel domain failed to unwind siRNA. Finding its interacting partners will help to explain whether the A/Hel protein needs cofactors to perform this activity. Additionally, establishing the binding partners of A/Hel domain of hDicer will add to the increasing number of proteins that are important in the RNAi pathway and help to explain their respective roles in the RNAi pathway.

Many proteins of the DExD/H box family have been demonstrated to displace proteins from RNA. Investigating the A/Hel domain of hDicer for ribonucleoproteinase activity will be of great importance given that hDicer has been found to function in the context of complex ribonucleoprotein assemblies (RISC). Elucidating this activity could contribute in molecular detail how RNA-RNA and RNA-protein contacts are crafted and remodelled in RISC.

The finding that the A/Hel domain of hDicer can anneal RNA strands uncovers another activity of hDicer, apart from its ribonuclease and ATPase activities. However, it is still very pertinent to determine at which step of the pathway this activity is needed. It could be possible that the A/Hel domain partakes in the formation of precursor miRNA, which has a characteristic hairpin structure or the A/Hel domain could function in the hybridization of anti-sense strand of siRNA with a targeted mRNA before Ago2 cleavage. Discovering the answers to these questions will provide an insight into the functions of hDicer and contribute to our understanding of its mode of action.

Finally, the three dimensional structure of A/Hel domain of any Dicer enzyme is still unsolved. Recent crystal structure of Dicer from *Gardia intestinalis* explains a possible mode of action of Dicer, especially in relation to its ribonuclease activity. Dicer was reported to

function as an intramolecular dimer, whereby each of its two ribonuclease III domains cuts a phosphodiester bond of each strand of a dsRNA. However, the Dicer from *Gardia intestinalis* lacks an N-terminal A/Hel domain and thus, differs from Dicer of other organisms. Since solving the three dimensional structure of full-length hDicer has proved abortive at the moment, a good strategy to solve the whole structure of hDicer will be to focus on the individual domains of the protein. This strategy has been successfully used in determining the structure of ribonuclease IIIb domain as well as the PAZ domain of hDicer. A similar method can be applied to achieve the three dimensional structure of the A/Hel domain of hDicer.

5 Summary

The aim of this study was the recombinantly production, and characterisation of the ATPase/Helicase (A/Hel) domain of human Dicer (hDicer). The production of the A/Hel domain of hDicer as a His-tag-fusion protein in *E. coli* resulted in the formation of insoluble protein aggregates (inclusion bodies). However, the N-terminal fusion of maltose-binding protein (MBP) from *E. coli* to the A/Hel domain resulted in the production of a soluble A/Hel protein, which was purified to homogeneity using amylose and Ni-NTA affinity chromatographies. The A/Hel domain was also produced in High Five insect cells but the resultant protein could not be purified to homogeneity.

The A/Hel protein exhibited an ATPase activity independent of the presence of RNA but dependent on Mg^{2+} ions. The ATPase reaction followed Michaelis-Menten kinetics with a corresponding $K_{m\ ATP}$ constant of 207.5 µM and a turnover number, $k_{cat\ ATP}$, of 10 min^{-1}. The recombinant protein showed nucleotide specificity, hydrolyzing only purine and not pyrimidine triphosphates. Additionally, the protein did bind single-stranded RNA (ssRNA), but did not bind small interfering RNA (siRNA). Furthermore, gel-shift and Florescence Resonance Energy Transfer (FRET)-based assays used to investigate unwinding activity showed that the A/Hel protein was unable to unwind dsRNA substrates used in this study. Moreover, using the FRET-based assay, it could be demonstrated that the protein enhanced RNA annealing.

Future experiments should be based on the elucidation of the physiological significance of these results. Together with the outcome of the physiological investigations, the results of this study could provide an insight into the functions of hDicer and contribute to our understanding of its mode of action within RNA interference.

6 Zusammenfassung

Ziel dieser Arbeit war die rekombinante Produktion und Charakterisierung der ATPase/Helicase (A/Hel)-Domäne vom humanen Dicer (hDicer). Die Produktion der A/Hel-Domäne des hDicer als His-Tag-Fusionsprotein in *E. coli* ergab unlösliche Proteinaggregate (Inclusion Bodies). Die N-terminale Fusion des Maltose-Bindungsprotein (MBP) von *E. coli* mit der A/Hel- Domäne ergab die Produktion eines löslichen A/Hel-Proteins, welches mittels Affinitätschromatographie über Amylose und Ni-NTA Säulen aufgereinigt werden konnte. Zusätzlich wurde die A/Hel-Domäne in High-Five-Insekten-Zellen produziert. Das resultierende Protein konnte jedoch nicht mittels Affinitätschromatographie aufgereinigt werden.

Das A/Hel-Protein besaß eine ATPase-Aktivität, die unabhängig von der Anwesenheit von RNA, jedoch abhängig von Mg^{2+} Ionen war. Die ATPase-Reaktion folgte der Michaelis-Menten-Kinetik mit einer korrespondierenden $K_{m\ ATP}$-Konstante von 207,5 µM und einer Wechselzahl $k_{cat\ ATP}$ von 10 min^{-1}. Das rekombinante Protein zeigte Nukleotidspezifität, da es nur Purin-, nicht jedoch Pyrimidin-Triphosphate hydrolysierte. Zusätzlich band das Protein an einzelsträngige RNA (ssRNA), aber nicht an Small Interfering RNA (siRNA).

Des Weiteren wurden zur Untersuchung der Entwindungsaktivität Gel-Shift- und Fluorescence-Resonance-Energy-Transfer (FRET)-basierende Assays durchgeführt. Es zeigte sich, dass das A/Hel-Protein nicht dazu fähig war, die als Substrat dienende dsRNA zu entwinden. Es wurde jedoch mit Hilfe des FRET-Assays nachgewiesen, dass das Protein das Annealing an RNA verstärkt.

Auf diesen Ergebnissen sollten zukünftige Experimente für die Aufklärung der physiologischen Signifikanz basieren. Die Ergebnisse auf dieser Arbeit aufbauender physiologischer Untersuchungen können die Erkentnisse dieser Arbeit ergänzen und so zum

Verständnis über die Funktion des hDicer-Proteins und seiner Rolle im Mechanismus der RNA-Interferenz beitragen.

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8 Hazardous Materials (Gefahrstoffe) and Security Information (Sicherheitsdaten)

The following reagents and solvents have been provided with the following security advices according to the German hazardous material regulation "Gefahrstoffverordnung" §6.

Substance	**Verbindung**	**Gefahren Symbol**	**R-Sätze**	**S-Sätze**
Acrylamide	Acrylamid	T	45-46-20/21-25-36/38-43-48/23/24/25-62	53-45
Ammoniumpersulfate	Ammoniumpersulfat	O, Xn	8-22-36/37/38-42/43	22-24-26-37
Ampicillin	Ampicillin	Xn	36/37/38-42/43	22-24-26-37
Dimethylsulfoxide	Dimethylsulfoxid	Xi	36/38	26
Dithiothreitol	Dithiothreitol	Xi	36/37/38	36/37/39-22
Disodium-EDTA-dihydrate	Disodium-EDTA-dihydrat	Xn	22	-
Acetic acid	Eisessig	C	10-35	23.2-26-45
Ethanol	Ethanol	F	11	7-16
Ethidiumbromide	Ethidiumbromid	T+	22-26-36/37/38-40	26-28.2-36/37-45

Glutardialdehyde, 25%	Glutardialdehyd, 25%	Xn	42/43	22-45-36/37/39
HCl 37%	HCl 37%	C	34-37	26-36/37/39-45
Imidazole	Imidazol	F, Xn, N	11-38-48/20-51/53-62-65-67	9-16-29-33-36/37-61-62
2-Mercaptoethanol	2-Mercaptoethanol	T, N	22-24-34-51/53	26-36-37/39-45-61
Methanol	Methanol	T	61	26-36/37-39-45
NaOH	NaOH	F, T	11-23/24/25-39/23/24/25	7-16-36/37-45
Ni-NTA Agarose	Ni-NTA Agarose	O, C	8-35	8-27-39-45
NBT	NBT	Xn	10-22-40-42/43	13-26-36-46
NaCl	NaCl	F, Xi	11-36-67	7-16-24/25-26
2-Propanol	2-Propanol	T	24/25-34	28.6-45
SDS	SDS	C	34-37	26-36/37/39-45
TEMED	TEMED	Xn	22-36/38	22-24/25
Tetracycline	Tetracyclin	Xi	36/37/38	26-36
Tris	Tris	F, C	11-20/21/22-35	3-16-26-29-36/37/39-45

Xylene Cyanol FF	Xylen Cyanol FF	C	34	3-28-36/39-45

8.1 Gefahrensymbole

E	explosionsgefährlich
C	ätzend
F+	hochentzündlich
Xi	reizend
O	brandfördernd
F	leichtentzündlich
T	giftig
Xn	gesundheitsschädlich
N	umweltgefährlich

8.2 Gefahrenhinweise und Sicherheitsratschläge

R 8	Feuergefahr bei Berührung mit brennbaren Stoffen.
R 10	Entzündlich.
R 11	Leichtentzündlich.
R 22	Gesundheitsschädlich beim Verschlucken.
R 24	Giftig bei Berührung mit der Haut.
R 25	Giftig beim Verschlucken.
R 26	Sehr giftig beim Einatmen.
R 34	Verursacht Verätzungen.

R 35	Verursacht schwere Verätzungen.
R 36	Reizt die Augen.
R 37	Reizt die Atmungsorgane.
R 38	Reizt die Haut.
R 40	Verdacht auf krebserzeugende Wirkung.
R 43	Sensibilisierung durch Hautkontakt möglich.
R 45	Kann Krebs erzeugen.
R 46	Kann vererbbare Schäden verursachen.
R 61	Kann das Kind im Mutterleib schädigen.
R 62	Kann möglicherweise die Fortpflanzungsfähigkeit beeinträchtigen.
R 65	Gesundheitsschädlich: Kann beim Verschlucken Lungenschäden verursachen.
R 67	Dämpfe können Schläfrigkeit und Benommenheit verursachen.

8.3 Kombination der R-Sätze

R 14/15	Reagiert heftig mit Wasser unter Bildung hochentzündlicher Gase.
R 20/21	Gesundheitsschädlich beim Einatmen und bei Berührung mit der Haut.
R 20/21/22	Gesundheitsschädlich beim Einatmen, Verschlucken und Berührung mit der Haut.
R 23/24/25	Giftig beim Einatmen, Verschlucken und Berührung mit der Haut.
R 24/25	Giftig bei Berührung mit der Haut und beim Verschlucken.
R 36/38	Reizt die Augen und die Haut.
R 36/37/38	Reizt die Augen, Atmungsorgane und die Haut.
R 39/23/24/25	Giftig: ernste Gefahr irreversiblen Schadens durch Einatmen, Berührung mit der Haut und durch Verschlucken.

R 42/43	Sensibilisierung durch Einatmen und Hautkontakt möglich.
R 48/20	Gesundheitsschädlich: Gefahr ernster Gesundheitsschäden bei längerer Exposition durch Einatmen.
R 48/23/24/25	Giftig: Gefahr ernster Gesundheitsschäden bei längerer Exposition durch Einatmen. Berührung mit der Haut und durch Verschlucken.
R 51/53	Giftig für Wasserorganismen, kann in Gewässern längerfristig schädliche Wirkungen haben.

8.4 S-Sätze (Sicherheitsratschläge)

S 3	Kühl aufbewahren.
S 7	Behälter dicht geschlossen halten.
S 8	Behälter trocken halten.
S 9	Behälter an einem gut gelüfteten Ort aufbewahren.
S 13	Von Nahrungsmitteln, Getränken und Futtermitteln fernhalten.
S 16	Von Zündquellen fernhalten - Nicht rauchen.
S 22	Staub nicht einatmen.
S 23.2	Dampf nicht einatmen.
S 24	Berührung mit der Haut vermeiden.
S 26	Bei Berührung mit den Augen sofort gründlich mit Wasser abspülen und Arzt konsultieren.
S 27	Beschmutzte, getränkte Kleidung sofort ausziehen.
S 28	Bei Berührung mit der Haut sofort abwaschen mit viel ... (vom Hersteller anzugeben)
S 28.2	Bei Berührung mit der Haut sofort abwaschen mit viel Wasser und Seife.

S 28.6 Bei Berührung mit der Haut sofort abwaschen mit viel Polyethylenglycol 400 (807485) und anschließend Reinigung mit viel Wasser.

S 29 Nicht in die Kanalisation gelangen lassen.

S 33 Maßnahmen gegen elektrostatische Aufladungen treffen.

S 36 Bei der Arbeit geeignete Schutzkleidung tragen.

S 37 Geeignete Schutzhandschuhe tragen.

S 39 Schutzbrille/Gesichtsschutz tragen.

S 45 Bei Unfall oder Unwohlsein sofort Arzt hinzuziehen (wenn möglich, dieses Etikett vorzeigen).

S 46 Bei Verschlucken sofort ärztlichen Rat einholen und Verpackung oder Etikett vorzeigen.

S 53 Exposition vermeiden - vor Gebrauch besondere Anweisungen einholen. - Nur für den berufsmäßigen Verwender -.

S 61 Freisetzung in die Umwelt vermeiden. Besondere Anweisungen einholen / Sicherheitsdatenblatt zu Rate ziehen.

S 62 Bei Verschlucken kein Erbrechen herbeiführen. Sofort ärztlichen Rat einholen und Verpackung oder Etikett vorzeigen.

8.5 Kombination der S-Sätze

S 24/25 Berührung mit den Augen und der Haut vermeiden.

S 36/37 Bei der Arbeit geeignete Schutzhandschuhe und Schutzkleidung tragen.

S 36/37/39 Bei der Arbeit geeignete Schutzkleidung, Schutzhandschuhe und Schutzbrille/Gesichtsschutz tragen.

S 36/39	Bei der Arbeit geeignete Schutzkleidung und Schutzbrille/Gesichtsschutz tragen.
S 37/39	Bei der Arbeit geeignete Schutzhandschuhe und Schutzbrille/Gesichtsschutz tragen.

9 Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ausschließlich mit den angegebenen Hilfsmitteln durchgeführt habe.

Mit der Ausleihe durch die Bibliotheken der Universität Hamburg und Einsichtnahme bin ich einverstanden.

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