Characterization of the In Vitro Protein Binding Interactions of the Extreme C-Terminal Domains of Ataxia Telangiectasia Mutated and the DNA-Dependent Protein Kinase

by

Kelly Marie Williams

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Medical Biophysics
University of Toronto

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Characterization of the In Vitro Protein Binding Interactions of the Extreme C-Terminal Domains of Ataxia Telangiectasia Mutated and the DNA-Dependent Protein Kinase

Kelly Marie Williams, M.Sc. 1998

Graduate Department of Medical Biophysics, University of Toronto

The PI-3 related kinases are a conserved family of proteins that participate in cell cycle progression, meiotic and V(D)J recombination, and chromosomal maintenance and repair. Distinguishing structural features of this family include high molecular weight (>270 kD), a C-terminal kinase domain that is related to the PI-3 lipid kinases, and a highly conserved extreme C-terminal (Ext-C) domain. Mutations that affect residues within the Ext-C domains of both ATM and DNA-PK result in diseases with overlapping phenotypes including immunodeficiency, predisposition to lymphoid malignancies, and hypersensitivity to ionizing radiation. One protein that has been demonstrated to have functional relationships with both ATM and DNA-PK is the ssDNA binding protein, replication protein A (RPA). To determine whether the Ext-C domains of ATM and DNA-PK bind to RPA, bacterial GST fusion proteins containing each of the Ext-C domains were expressed, affinity purified, and incubated with recombinant (r) human RPA. rhRPA bound with apparent specificity to ATM and DNA-PK rExt-C proteins. Furthermore, murine RPA from cell lysates bound to ATM and DNA-PK rExt-C proteins. These results suggest that, in vitro, RPA binds to the Ext-C domains of ATM and DNA-PK. These data support a model in which PI-3 related kinases cooperate with RPA in the maintenance of genomic integrity.
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Extreme C-Terminal Domains of ATM and DNA-PK \textit{In Vitro}

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LIST OF ABBREVIATIONS

aa  amino acid
AT  ataxia telangiectasia
ATM ataxia telangiectasia mutated
ATR  ataxia telangiectasia mutated- and rad3- related
BSA  bovine serum albumin
bp  base pair
°C  degrees Celsius
cDNA  copy (coding) DNA
Ci  Curie, $2.22 \times 10^{12}$ disintegrations per minute
CO₂  carbon dioxide
C-terminal  carboxy terminal
dATP  deoxyadenosine 5'-triphosphate
dCTP  deoxycytosine 5'-triphosphate
ddH₂O  autoclaved distilled water
DEPC  diethyl pyrocarbonate
dGTP  deoxyguanine 5'-triphosphate
dH₂O  distilled water
DNA  deoxyribonucleic acid
DNA-PK  DNA-dependent protein kinase
dNTP  2'-deoxynucleoside 5'-triphosphate
dsDNA  double stranded DNA
dTTP  deoxythymidine 5'-triphosphate
DTT  dithiothreitol
ECL  enhanced chemiluminescence
EDTA  ethylene diamine tetra acetic acid
EGTA  ethylene glycol-bis-(p-aminoethylether-N,N,N',N')-tetra acetic acid
ext-C  extreme C-terminal
FCS  fetal calf serum
g  gram
xg  times the force of gravity (9.8 m/s²)
GST  glutathione S-transferase
HEPES  N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffered saline
Ig  immunoglobulin
IPTG  isopropyl β-D-thiogalactopyranoside
kD  kilodalton
kb  kilobase (1000 bp)
l  litre
mA  milliAmp
mg  milligram
ml  millilitre
M  molarity (moles per litre)
mM  millimoles
min  minute
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>MW</td>
<td>molecular weight (in kD)</td>
</tr>
<tr>
<td>ng</td>
<td>nanograms ($10^9$)</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
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<tr>
<td>OD</td>
<td>optical density</td>
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<td>O/N</td>
<td>overnight</td>
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<td>NE</td>
<td>nuclear extract</td>
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<td>NER</td>
<td>nucleotide excision repair</td>
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<tr>
<td>N-terminus</td>
<td>amino terminus</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PI-3</td>
<td>phosphatidylinositol-3</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenyl methylsulphonyl fluoride</td>
</tr>
<tr>
<td>PNE</td>
<td>post-nuclear (cytoplasmic) extract</td>
</tr>
<tr>
<td>r</td>
<td>recombinant</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPA</td>
<td>replication protein A</td>
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<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polymerase gel electrophoresis</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single stranded DNA</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>μg</td>
<td>microgram ($10^{-6}$)</td>
</tr>
<tr>
<td>μl</td>
<td>microlitre</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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INTRODUCTION

The Cellular Response to DNA Damage

The fundamental role of the cell cycle is to ensure the accurate transmission of genetic information from one generation to the next by faithfully replicating DNA during S phase and equally distributing identical chromosomes to each daughter cell during M phase (Heichman et al., 1994; Wuarin et al., 1996). The concept of a ‘checkpoint’ was proposed following the discovery of Saccharomyces cerevisiae rad9 mutants, which fail to arrest their cell cycle in response to DNA damage (Hartwell et al., 1989; Weinert et al., 1994). By definition, when the occurrence of an event requires the completion of a prior event, that dependence is due to a checkpoint if a mutation can be found that relieves that dependency (Hartwell et al., 1989). In eukaryotes, cellular checkpoints regulate cell cycle progression when prior events are incomplete and DNA damage checkpoints induce a cell cycle delay when DNA repair is necessary (reviewed in Nasmyth, 1996; Kitazono and Matsumoto, 1998). Generally, cell cycle and damage checkpoint mechanisms exist at three distinct stages during the cell cycle, at the G1/S transition, monitoring the progression of S, and at the G2/M boundary.

DNA damage can result from extrinsic sources such as chemicals and radiation, and intrinsic processes including DNA metabolism and the production of spontaneous reactive metabolites. In general, the types of DNA damage that occur can be grouped into two broad categories, modification of the nitrogenous bases and modification of the phosphodiester backbone (reviewed in Friedberg et al., 1995). Once a DNA lesion has been incurred there are three possible outcomes for a eukaryotic cell. The cell may repair the lesion such that no permanent DNA alterations are passed on to future generations.
Secondly, in a multicellular organism, the cell may undergo a process of programmed cell death (apoptosis) to prevent propagation of the damaged cell within the organism. Finally, the cell cycle can proceed with unrepaired DNA damage thus resulting in a permanent genetic alteration that is passed on to subsequent generations (Canman et al., 1994). When the appropriate combination of genetic mutations accumulate within a cell, a malignant disease can eventually result (reviewed in Paulovich et al., 1997). Several distinct DNA repair mechanisms have evolved in coordination with the cell cycle checkpoints to ensure that DNA lesions are not replicated and passed on to daughter cells.

The nitrogenous bases of DNA may become covalently joined to adjacent bases or to chemical adducts, or bases may be lost due to spontaneous depurination or depyrimidation. When these modifications occur, the structure of the DNA helix becomes distorted and mispairing and misincorporation may occur during DNA synthesis. A highly conserved mechanism that resolves this type of lesions is the nucleotide excision repair (NER) pathway. In mammals, proteins including DNA helicases, endonucleases, lesion recognition proteins, ligases, components of the transcription factor TFIH, and ssDNA binding proteins cooperate to excise the bases surrounding a lesion and replace and rejoin the missing nucleotides (reviewed in Boulikas, 1996). Xeroderma pigmentosum is a rare, inherited DNA repair disorder that results from mutations in components of the NER pathway (XPA-XPG) and is manifested by extreme sensitivity to UV light and a high incidence of skin tumors (Boulikas, 1996). Most of the mammalian NER proteins have counterparts in *S. cerevisiae* that belong to the *RAD3* epistasis group, mutants of which are primarily UV
sensitive. Homologues of some of the NER proteins have been also identified in prokaryotes underscoring the evolutionary conservation of DNA repair pathways.

In addition to base lesions, the phosphodiester backbone of the DNA helix may be nicked or broken creating single- or double-stranded ends. Left unrepaired, these DNA ends are highly recombinogenic and present a major threat to the integrity of the genome (Hartwell, 1992; Paulovich et al., 1997). Two major pathways have evolved to repair double-stranded DNA breaks (DSB) in eukaryotic cells, homologous recombination and non-homologous end-joining (reviewed in Friedberg et al., 1995). In yeast and lower eukaryotes, breaks are preferentially repaired by a high fidelity mechanism of homologous exchange between sister duplexes which uses key genes in the RAD52 epistasis group (Game et al., 1993; Haber et al., 1995). In contrast, in mammalian cells repair is achieved by direct ligation of DNA ends containing no or short stretches of homology. This non-homologous or illegitimate repair process is considered to be error-prone due to the frequent addition or loss of non-templated nucleotides (Roth et al., 1985; Roth and Wilson, 1986). One of the key enzymes in the mammalian DNA DSB repair complex is the DNA-dependent protein kinase (DNA-PK) (Jeggo et al., 1995; Hartley et al., 1995). DNA-PK, a protein mutated in the murine severe combined immunodeficiency (SCID) defect, and the protein mutated in ataxia telangiectasia (ATM) are two members of a highly conserved family of PI-3 related kinases that are discussed in the following sections and together constitute the major focus of this thesis.

**The PI-3 Related Kinase Family**

Recently, a family of high molecular weight (>250 kD) proteins have been identified that share sequence conservation in their carboxyl terminal domains with high
homology to the phosphatidylinositol-3 (PI-3) kinases (Figure 1). These proteins also contain a larger domain that is N-terminal and adjacent to the kinase motifs that has much lower homology to the S. pombe cell cycle checkpoint gene RAD3. Functionally, these proteins have been implicated in essential cellular processes including V(D)J recombination, meiosis, cell cycle progression, and DSB repair. In contrast to the conventional PI-3 proteins that phosphorylate lipids (Hunter T, 1997), PI-3 related proteins with demonstrated catalytic activity have been shown to function as serine-threonine protein kinases (Carter et al., 1990; Lees-Miller et al., 1990; Hartley et al., 1995; Bentley et al., 1996; Keegan et al., 1996).

The PI-3 related kinase family includes the S. cerevisiae genes TOR1 and TOR2 and their mammalian homologues FRAP and RAFT1, the loss of which results in a G1 arrest (Kunz et al., 1993; Brown et al., 1994; Sabatini et al., 1994; Helliwell et al., 1994; Zheng et al., 1995). RAD3, a gene that encodes a 270 kD protein in S. pombe, plays an integral role in DNA repair, mitotic stability, meiotic recombination, and in both the S&ar; G2/M phase DNA damage checkpoints (Bentley et al., 1996). Homologues of RAD3 exhibit conservation of both structure and function and include MEC1 (mitosis entry checkpoint) in S. cerevisiae, MEI41 in D. melanogaster, and the ATM- and rad3-related (ATR) protein in mammals (Hartwell et al., 1989; Weinert et al., 1993; Weinert et al., 1994; Kato et al., 1994; Paulovich et al., 1995; Hari et al., 1995; Cimprich et al., 1996). DNA-PK, the largest known protein in the PI-3 kinase related family, is a non-essential mammalian gene integral to DSB repair (Jeggo et al., 1995; Hartley et al., 1995; Shin et al., 1997). Two other non-essential proteins whose mutants result in DNA damage checkpoint defects and telomere instability include the homologues TEL1 (S. cerevisiae)
and the mammalian gene mutated in Ataxia telangiectasia (ATM) (Greenwell et al., 1995; Morrow et al., 1995; Savitsky et al., 1995). Interestingly, severe combined immunodeficient (SCID) mice and patients with ataxia telangiectasia (AT) share overlapping disease phenotypes that have been demonstrated to result from mutations in two of these PI-3 related kinase homologues, DNA-PK and ATM.

The Murine Severe Combined Immunodeficiency Disease

The murine SCID defect is an autosomal recessive disorder that arose spontaneously in an inbred strain of mice. First described in 1983, SCID mice are characterized by the absence of mature B and T lymphocytes (Bosma et al., 1983). During the maturation of lymphocytes, site-specific gene rearrangements occur in immunoglobulin and T-cell antigen receptor loci which assemble non-identical variable (V), diversity (D), and joining (J) gene segments (Lewis SM, 1994). The V(D)J recombination process is crucial in providing the adaptive immune system with the diversity of antigen receptors required to target and destroy a vast array of pathogenic organisms. During the V(D)J recombination process, the RAG1 and RAG2 endonucleases recognize specific recombination signal sequences and introduce DSB between the signal sequence and the V,D, or J coding element (McBlane et al., 1995; VanGent et al., 1996). Four DNA ends result from the RAG-mediated DSBs, two of which are joined to form the signal joint. The remaining two ends undergo further processing before eventually being joined to form the coding exon for the variable domain of the antigen receptor. In SCID mice, the lack of mature lymphocytes is due to impaired joining of the coding ends during V(D)J recombination (Schuler et al., 1986;
Lieber et al., 1988; Malynn et al., 1988). Interestingly, normal signal end joining is observed in SCID lymphocytes (Lieber et al., 1988).

In addition to defective re-joining of V(D)J coding ends during lymphocyte maturation, both lymphoid and non-lymphoid cells from SCID mice are hypersensitive to ionizing radiation and other agents that induce DSB (Fulop et al., 1990; Hendrickson et al., 1991; Biedermann et al., 1991). Somatic cell fusion analysis with rodent cell lines sensitive to ionizing radiation have identified at least 10 complementation groups which are designated x-ray cross complementing (XRCC) groups (Jeggo et al., 1991; Thacker et al., 1991; Thompson et al., 1995). Three of these groups, XRCC4, XRCC5, and XRCC7, are characterized by defects in V(D)J recombination and DSB repair which implies a functional role for ubiquitous DNA repair proteins in V(D)J recombination. Defects in SCID cells and the Chinese Hamster Ovary (CHO) V3 cell line both map to XRCC7 and share a defect in coding joint formation (Blunt et al., 1995; Kirchgessner et al., 1995; Taccioli et al., 1994a). In contrast, XRCC4 and XRCC5 mutants are unable to repair either signal or coding ends during V(D)J recombination (Pergola et al., 1993; Taccioli et al., 1993). Although the XRCC4 gene has been cloned, its function(s) remain elusive (Li et al., 1995). Interestingly, the XRCC4 gene product has been shown to coimmunoprecipitate with DNA ligase IV, which implicates XRCC4 directly with the repair machinery (Critchlow et al., 1997; Grawunder et al., 1997). The genes defective in XRCC5 and XRCC7 encode subunits of the DNA-dependent protein kinase, as will be discussed in the next section.

A relationship between V(D)J recombination, thymocyte differentiation, and DSB repair was established by the discovery that treatment of newborn SCID mice with DNA-
damaging agents partially restores V(D)J recombination in T cell progenitors (Danska et al., 1994; Murphy et al., 1994). Interestingly, 100% of the treated mice developed thymic lymphoma by 17-20 weeks post irradiation (Danska et al., 1994). The bias towards lymphomagenesis despite the global hypersensitivity to ionizing radiation phenotype of SCID mice may be attributable to the accumulation of broken V(D)J DNA ends in lymphocyte precursors, however this remains to be clearly demonstrated.

The DNA-Dependent Protein Kinase

DNA-PK is a 470 kD protein that was identified in 1990 when it was demonstrated to be the first protein kinase identified whose catalytic activity is dependent on the presence of DNA ends (Carter et al., 1990; Lees-Miller et al., 1990). To date, no other such DNA-activated protein kinases have been identified. DNA-PK DNA end-binding and kinase activity is thought to be regulated by the Ku70/86 heterodimer. DNA-PK is capable of autophosphorylation, which causes disassociation of p470 from DNA ends and the Ku subunits resulting in abrogation of catalytic activity (Chan et al., 1996). Another potential mode of regulation stems from the observation that in cells treated with apoptotic stimuli DNA-PK is rapidly cleaved by caspases (Casciola-Rosen et al., 1996; Han et al., 1996; Song et al., 1996). It is not presently understood whether the resulting two fragments retain any functional capabilities, possess novel activities, or if there is a complete ablation of function.

The Ku heterodimer was first characterized as an antigen recognized by sera of autoimmune patients (Mimori et al., 1981). It has since been demonstrated that Ku is an abundant nuclear-localized heterodimer that binds in a sequence-independent manner to distortions in the DNA helix including dsDNA ends, nicks, gaps, and hairpin structures
Baculovirus expression studies have shown that Ku DNA binding activity requires assembly of the heterodimer (Ono et al., 1994). Ku is both biochemically and genetically conserved across eukaryotic species and homologues have been described in monkeys, rodents, Caenorhabditis elegans, Drosophila melanogaster, and S. cerevisiae (Mimori et al., 1990; Porges et al., 1990; Paillard et al., 1991; Feldmann et al., 1993; Jacoby et al., 1994; Boulton et al.; 1996; Milne et al., 1996).

Ku86 has been identified as the gene mutated in XRCC5 cells, one of the three complementation groups defective in DSB repair and V(D)J recombination (Smider et al., 1994; Taccioli et al., 1994b). In contrast to SCID mice, which have TCR+ or Ig+ lymphocytes that accumulate in older animals (reviewed in Hendrickson, 1993), lymphocyte progenitors in Ku86−/− mice are arrested at an early stage and do not mature (Nussenzweig et al., 1996; Zhu et al., 1996). Also in contrast to SCID mice, Ku86−/− mice are runted and their cells enter senescence prematurely (Nussenzweig et al., 1996; Zhu et al., 1996).

Although no mutant CHO cell lines have been found which lack Ku70 activity, Ku70−/− mice have been generated by gene disruption and share the hypersensitivity to ionizing radiation and severe combined immunodeficiency phenotypes with SCID and Ku86-deficient animals (Gu et al., 1997; Ouyang et al., 1997). Ku70-deficient embryonic stem cells are also hypersensitive to γ-radiation (Gu et al., 1997b). Ku70−/− animals are similar to SCID mice in that they reproducibly develop small populations of T cells and are predisposed to developing thymic lymphoma (Gu et al., 1997; Ouyang et al., 1997).
Like Ku86-deficient mice, *Ku70/-* mice are smaller than their littermates (Gu et al., 1997; Ouyang et al., 1997).

In summary, in addition to the defective DNA repair phenotype characterized by SCID animals, Ku-deficient mice also exhibit a growth defective phenotype that suggests a possible role for Ku in DNA replication. Yeast cells deficient in the Ku70 homologue, *hdf1*, display growth defects and DNA replication anomalies as well as radiation sensitivity, which supports the evolutionary conservation of Ku activity in DNA repair and replication (Barnes and Rio, 1997). A general model for heterotrimeric DNA-PK activity has been proposed in which DNA binding by the Ku heterodimer recruits and activates the 470 kD catalytic subunit (Gottlieb et al., 1993). However, Ku-independent DNA-PK kinase and DNA binding activities have recently been demonstrated (Yaneva et al, 1997; Hammarsten et al., 1998). This, together with the different phenotypes of Ku70/-, Ku80/-, and SCID animals, would suggest that this model is oversimplified.

**The SCID Mutation in DNA-PK**

Further insight into the SCID phenotype came in 1995 when several groups independently mapped the genetic defect to the catalytic subunit of the DNA-dependent protein kinase (DNA-PK), and reported the absence of DNA-PK catalytic activity in SCID fibroblasts (Blunt et al., 1995; Kirchgessner et al., 1995; Peterson et al., 1995). Although DNA-PK was clearly demonstrated to be the SCID gene, the nature of the defect and its impact on V(D)J recombination and DSB repair were not defined. Initially, several groups reported that expression of DNA-PK in SCID fibroblast cell lines was undetectable (Blunt et al., 1995; Kirchgessner et al., 1995; Peterson et al., 1995). However, we demonstrated that DNA-PK protein is detectable in SCID lymphocyte
precursors and fibroblasts, but is reduced at least 10-fold relative to wild-type levels (Danska et al., 1996). Since steady state levels of DNA-PK mRNA are unperturbed by the SCID mutation, this suggests that the reduction in protein levels reflects a defect which leads to protein instability (Blunt et al., 1996; Danska et al., 1996). The entire coding region of the SCID gene has been sequenced (Araki et al., 1997) and an ochre mutation has been identified which results in the truncation of the C-terminal 83 amino acids (Blunt et al., 1996; Danska et al., 1996). This loss of 8 kD out of 470 kD occurs downstream from the conserved PI-3 kinase motifs but includes a highly conserved extreme C-terminal (ext-C) domain that is specific to the PI-3-related kinase family (Figure 1).

**Substrates of DNA-PK**

The 470 kD catalytic subunit of DNA-PK has been shown to possess serine/threonine protein kinase activity and a plethora of *in vitro* substrates including Ku, Sp1, replication protein A, c-jun, c-myc, c-fos, and p53 have been identified (reviewed in Lees-Miller, 1996). Despite demonstration of several *in vitro* targets, elucidation of a physiologically relevant DNA-PK-mediated signaling pathway has remained elusive. In 1995, two independent groups suggested a role for DNA-PK in down regulating ribosomal gene transcription however, since the identification of DNA-PK as the SCID protein no further investigations into RNA polymerase I repression have been reported (Labhart, 1995; Kuhn et al., 1995).

With the discovery that the SCID protein belongs to a family of highly conserved proteins that coordinate cellular responses to genotoxic stress, expectations were high that DNA-PK defective cells might be deficient in DNA damage checkpoint controls. The
p53 tumor suppressor gene is a mammalian tetrameric transcription factor that plays a central role in the checkpoint response to DNA damage. Levels of p53 increase following UV or ionizing radiation, which induces a cell-cycle arrest in G₁ and G₂ (reviewed in Levine, 1997). In some cells, treatment with radiation and other DNA damaging agents induces p53-mediated apoptosis (programmed cell death) to prevent propagation of mutated cells (reviewed in White, 1996). Since in vitro phosphorylation of p53 by DNA-PK had been previously established, several studies have investigated whether the p53 checkpoint is intact in SCID cells. No differences in p53 upregulation in wild-type versus SCID cells have been observed following irradiation (Hunag et al., 1996; Fried et al., 1996; Guidos et al., 1996) suggesting that DNA-PK either functions downstream or independently of p53, or that redundant signaling pathways exist in mammalian cells. Moreover, p53 has been shown to accumulate in response to the presence of unrepaired V(D)J coding ends in SCID lymphocyte precursors, thereby activating a DNA damage checkpoint and restricting the oncogenic potential of DNA ends (Guidos et al., 1996). In addition, both Ku- and DNA-PK-deficient cells have functional G₁/S and G₂/M DNA damage checkpoints following irradiation and show prolonged delay of DNA synthesis in the presence of unrepaired DNA damage (Jeggo, 1985; Weibezahn et al., 1985; Nussenzweig et al., 1996). All of these data would suggest therefore that DNA-PK and Ku70/80 are not required for the p53-mediated cell cycle or DNA damage checkpoint pathways. Recently, it has been proposed that DNA-PK acts upstream of p53 in the response to DNA damage and is required but not sufficient for p53 activation and DNA binding (Woo et al., 1998). In these studies, p53 sequence-specific DNA binding was assayed in vitro following exposure to genotoxic agents in wild-type DNA-PK cells,
BALB/c 3T3, versus a SCID-derived cell line with no detectable DNA-PK activity, SCGR11. In contrast to the wild-type cells, p53 DNA binding was not detected in the SCGR11 cells following DNA damage. The authors of this study propose that the DNA-damage induced upregulation of p53 previously described in SCID cells (Hunag et al., 1996; Fried et al., 1996; Guidos et al., 1996) was due to low level of DNA-PK activity in primary SCID cells, as will be discussed later.

Another protein that has been identified in vitro as a substrate of DNA-PK is one of the highly conserved components of NER, replication protein A (RPA, also called replication factor A and human single stranded binding protein) (Brush et al., 1994; Pan et al., 1994; Hendrickson et al., 1996). Homologues of RPA have been identified in virtually every eukaryotic organism examined and are all heterotrimeric proteins with subunits of approximately 70, 32, and 12 kD (RPA-1, RPA-2, RPA-3, respectively) (reviewed in Wold, 1997). Separate genes encode the three subunits of RPA and each of these genes has been determined to be essential for cell survival in S. cerevisiae (Heyer et al., 1990; Brill and Stillman, 1991). RPA has been implicated in multiple diverse processes including replication (Wobbe et al., 1987; Wold et al., 1988; Fairman et al., 1988), meiotic recombination (Heyer et al., 1990; Moore et al., 1991), and DNA repair (Coverley et al., 1991; Coverley et al., 1992). RPA binds in a sequence-independent manner to ssDNA and stabilizes the molecule by eliminating secondary structures and facilitating the formation of nucleoprotein complexes (reviewed in Wold, 1997). In both yeast and mammalian cells, the 32 kD subunit of RPA is phosphorylated at multiple serine residues (Dutta and Stillman, 1992) during the S and G2 phases of the cell cycle (Din et al., 1990) and in response to UV and ionizing radiation (Liu et al.,
1993; Carty et al., 1994). DNA-PK was purified from HeLa cell extracts as the ssDNA-dependent RPA protein kinase, and immunodepletion of DNA-PK from in vitro SV40 replication assays inhibits phosphorylation of RPA-2 (Brush et al., 1994). Irradiation of wild type murine cells results in two hyperphosphorylated states of RPA-2 of approximately 35 and 36 kD. In contrast, radiation-induced phosphorylation of RPA-2 in SCID cells generates only the 35 kD hyperphosphorylated form and is therefore incomplete (Boubnov et al., 1995). The detection of some DNA damage-induced RPA phosphorylation in DNA-PK-deficient SCID cells suggests that DNA-PK is not the only kinase capable of phosphorylating RPA (Boubnov et al., 1995; Fried et al., 1996).

Finally, a physical interaction between DNA-PK and the tyrosine kinase c-Abl has been reported (Kharbanda et al., 1997). c-Abl is hypothesized to be involved in the growth-arrest response to DNA damage and acts upstream of the stress activated protein kinase (SAPK/JNK) in signaling pathways that respond to DNA damage (Kharbanda et al., 1995). c-Abl also modulates the expression of target genes via phosphorylation of RNA polymerase II (Baskaran et al., 1996), and interacts with the key regulator of the G1/S cell cycle transition, the retinoblastoma (Rb) protein (Welch et al., 1993). DNA-PK is reported to interact constitutively with c-Abl and irradiation of cells increases this interaction and results in the binding of Ku to c-Abl (Kharbanda et al., 1997). DNA-PK is also capable of phosphorylating and activating c-Abl in vitro, and SCID cells lack activation of c-Abl post-irradiation (Kharbanda et al., 1997). These observations suggest that DNA-PK may contribute to the response to DNA damage through functional interactions with c-Abl.
Ataxia Telangiectasia

Ataxia telangiectasia (AT) is a multisystem disease characterized by progressive cerebellar degeneration, oculocutaneous telangiectasia, radiosensitivity, immunodeficiency, infertility, premature aging of the skin and hair, and a profound predisposition to lymphoid and other malignancies (reviewed in Jorgensen and Shiloh, 1996; Lavin and Shiloh, 1997; Shiloh, 1997). In addition to the neurological and immunological AT manifestations, a number of variable clinical features characterize the disease including hypogonadism, an unusual type of diabetes mellitus with resistance to insulin treatment, growth retardation, and metabolic abnormalities consistent with developmental defects in the liver (reviewed in Lavin and Shiloh, 1997).

Ataxia is the presenting feature of AT, progressively worsening until affected children are confined to a wheelchair by the end of their first decade of life. Neuromotor dysfunction is due to cerebellar degeneration and/or dysgenesis primarily involving Purkinje and granular cells, but also affecting basket cells and potentially all other cells in the CNS (reviewed in Lavin and Shiloh, 1997). Telangiectasias (red, spider-like lines) are typically observed in the eyes, face, and ears of children beginning between 2-8 years of age (reviewed in Lavin and Shiloh, 1997). In non-AT individuals, telangiectasias may be observed in elderly persons and in patients undergoing exposure to radiotherapy, which suggests that their appearance in AT children could be a manifestation of the premature aging phenotype and/or the radiation hypersensitivity (reviewed in Lavin and Shiloh, 1997).

The immunodeficiencies associated with AT are highly variable and predispose patients to recurrent sinopulmonary infections (reviewed in Lavin and Shiloh, 1997). AT
individuals may have defects in one or both arms of their humoral and cell-mediated immunity, which accounts for the observed variability in immune failure. Responses to bacterial and viral antigens are significantly reduced in AT patients, concomitant with a defect in maturation of specific classes of B cells and abnormally low levels of IgA, and in some cases IgE and/or IgG2 (reviewed in Lavin and Shiloh, 1997). The thymus of AT individuals is usually absent or underdeveloped, in association with a reduction in the proportion of lymphocytes bearing T cell markers (Boder, 1985). In contrast to the SCID defect, extrachromosomal recombination assay results suggest that AT cells do not have an obvious defect in the DSB repair machinery (Hsieh et al., 1993). One model proposes that ATM may be required to temporarily halt the cell cycle thereby allowing immunoglobulin and T cell receptor gene rearrangements to occur in G1. Loss of this ATM-regulated cell cycle pause may cause chromosomal instability due to the presence of unresolved DNA ends during replication and/or mitosis and eventually lead to aberrant immune gene recombination and apoptosis (Meyn, 1995).

Another clinical hallmark of AT patients is their striking cancer predisposition. The incidence of cancer in AT patients is increased several hundred-fold over the normal population. Acute lymphoblastic leukemia (ALL) and lymphomas of primarily T cell origins are the most frequent malignancies, affecting 10-20% of patients by early childhood (reviewed in Swift et al., 1991). Various solid tumors have also been detected in older AT patients.

**Cellular Abnormalities in AT**

Early attempts to treat cancers in AT patients with radiation therapy revealed another key feature of the disease, a profound hypersensitivity to radiation and
radiomimetic drugs (reviewed in Shiloh, 1997). AT cells have a sensitivity to radiation that is approximately four-fold over normal cells, which results in chromosomal instability due to an increased frequency of chromosomal breakage (reviewed in Shiloh, 1997). Unlike other chromosomal instability syndromes such as xeroderma pigmentosum that is caused by a defect in NER (reviewed in Boulikas, 1996), there is no evidence for a gross DNA repair defect in AT (Vincent et al., 1975; Taylor et al., 1976; Fornace et al., 1980; Lavin et al., 1981). The pronounced increase of chromosomal aberrations found in AT cells is likely due, rather, to a defect in the ability of AT cells to detect DNA damage (reviewed in Meyn, 1995).

As mentioned earlier, one of the key features of the cell cycle is the ability to arrest in the presence of DNA damage. In contrast to normal cells, AT cells fail to delay DNA synthesis post-irradiation (Houldsworth et al., 1980; Painter et al., 1980; DeWit et al., 1981; Edwards et al., 1981). In addition to radioresistant DNA synthesis, AT cells fail to activate either the G1-S or G2-M phase checkpoints in response to DNA damage (Zampetti-Bosseler et al., 1981; Scott et al., 1982; Imray et al., 1983; Nagasawa et al., 1983; Beamish et al., 1994). Unchecked progression of the cell cycle in AT cells in the presence of DNA damage results in propagation and establishment of chromosomal abnormalities, which may contribute to the development of cancer.

**The Ataxia Telangiectasia Mutated (ATM) Gene**

Considerable insight into the mechanistic basis of the complex phenotype of AT ensued from the cloning of the gene mutated in AT patients on human chromosome 11q22-23 (Savitsky et al., 1995). The 360 kD product, which is denoted Ataxia Telangiectasia Mutated (ATM), was determined to be a member of the PI-3 kinase
related family which regulates DNA damage checkpoints in yeast, flies, and mammals (Savitsky et al., 1995). Murine models of AT resulting from targeted disruption of the ATM gene have been generated and exhibit most of the symptoms found in human AT patients including radiosensitivity, cell cycle defects, immunodeficiency, growth retardation, and increased risk of malignant lymphomas (Barlow et al., 1996; Xu et al., 1996; Elson et al., 1996).

Most AT patients are compound heterozygotes, underscoring the presence of many variant ATM mutations in the human population. Molecular characterization of AT cell lines has demonstrated that although mutations occur throughout the entire coding region of ATM, several AT patients have C-terminal truncations of only 10 to 115 residues (Figure 2) (McConville et al., 1996; Telatar et al., 1996; Wright et al., 1996; Vorechovsky et al., 1997; Stilgenbauer et al., 1997; Gilad et al., 1998). Similar to the SCID truncation in DNA-PK, these minor truncations occur downstream of the conserved PI-3 kinase catalytic motifs, do not cause significant instability of the transcript, and result in severely reduced protein abundance with classical AT disease progression (Gilad et al., 1998).

**ATM Functions and Substrates**

As described earlier, the p53 tumor suppressor protein is induced by radiation and activates a G1-S phase checkpoint (Kastan et al., 1991; Levine, 1997). Induction of p53 and its target proteins GADD45 and p21 (WAF1, Cip1) is delayed and/or reduced in AT cells in response to ionizing radiation (Kastan et al., 1992; Khanna and Lavin, 1993; Lu and Lane, 1993; Canman et al., 1994; Khanna et al., 1995). This defect in can be corrected by constitutive overexpression of wild type p53 (Khanna et al., 1995).
Immunoprecipitated and recombinant ATM have been demonstrated to phosphorylate serine residues on p53 following exposure to ionizing radiation (Banin, et al., 1998; Canman et al., 1998). Unlike DNA-PK, the kinase activity of ATM does not require the presence of DNA ends but is dependent on the presence of Mn$^{2+}$ (Banin et al., 1998; Canman et al., 1998). Phosphorylation of Ser15 by ATM may result in p53 protein stabilization by preventing mdm2-mediated p53 proteolytic degradation (Shieh et al., 1997). p53 levels are upregulated in AT cells following exposure to UV radiation, which suggests that ATM is not required for the immediate response to UV damage. However, activation of ATM kinase activity at more than one hour following exposure to UV has been detected in wild-type cells, suggesting that ATM may be involved downstream in the cellular response to UV lesions (Canman et al., 1998). In addition to defective stabilization of p53, lack of inhibition of members of the cyclin-dependent kinases following irradiation of AT cells has also been described (Beamish et al., 1996). Since cyclin-dependent kinases control progression throughout the cell cycle (Nasmyth, 1996), this suggests involvement of ATM at multiple cell cycle checkpoints.

Radiation-induced phosphorylation of RPA is also defective in AT cells (Liu et al., 1993). Although the physiological role for RPA phosphorylation is not known, phosphorylation of RPA may reduce its binding affinity for DNA which could inhibit replication since RPA is necessary for both initiation and elongation of DNA synthesis (reviewed in Wold, 1997; Lavin and Shiloh, 1997). In addition to having defective IR-induced phosphorylation in SCID and AT cells, yeast RPA is phosphorylated by the PI-3 related homologue MEC1 (Brush et al., 1996). This suggests that phosphorylation of
RPA by PI-3 kinases may be an evolutionarily conserved response to DNA damage (Brush et al., 1996).

Like DNA-PK, ATM has also been shown to bind constitutively to the c-Abl proto-oncogene (Baskaran et al., 1997; Kharbanda et al., 1997; Shafman et al., 1997). As reviewed earlier, c-Abl is activated in response to ionizing radiation and interacts with the stress activated protein kinase, RNA polymerase II, and the G1/S cell cycle transition regulator Rb (Welch et al., 1993; Baskaran et al., 1996; Kharbanda et al., 1997). In the absence of this constitutive interaction in AT cells, c-Abl is not activated following irradiation (Baskaran et al., 1997; Shafman et al., 1997). These findings suggest that ATM contributes to the DNA damage-induced activation of c-Abl and that the absence of this interaction may explain some of the DNA damage checkpoint defects observed in AT cells. Since a defect in the post-IR activation of c-Abl has also been described in SCID cells (Kharbanda et al., 1997), which do not exhibit DNA damage checkpoint defects, the physiological implications of c-Abl activation have clearly yet to be elucidated.

**ATM in Meiosis**

In addition to the DNA lesions generated by somatic cells during V(D)J recombination, DSBs also initiate the process of meiotic recombination in gametes. Some of the proteins involved in the response to DNA damage in mitotic cells including MEC1, MEI41, RPA, CHK1, ATM, and ATR, also function in meiotic nuclei by safeguarding against chromosomal fragmentation (Kato et al., 1994; Hari et al., 1995; Keegan et al., 1996; Xu et al., 1996; Flaggs et al., 1997; Plug et al., 1997).

Immunodetection studies have demonstrated that RPA binds to synapsed meiotic chromosomes at sites of interaction between homologous chromosomes (Plug et al.,
ATM has also been shown to co-localize with RPA at the synaptonemal complex, a specialized structure at which meiotic recombination occurs (Keegan et al., 1996). In contrast, the mammalian PI-3 kinase-like homologue ATR associates at sites of unpaired or unsynapsed chromosomes (Keegan et al., 1996). Identification of PI-3-kinase related homologues and a NER repair protein in meiotic recombination suggests that some aspects of the somatic response to DNA damage is shared by meiotic cells.

In ATM/- mice, development of oocytes and spermatocytes is disrupted by chromosomal fragmentation, which results in meiotic arrest (Xu et al., 1996). One possible explanation for this defect is that in ATM/- cells, neither CHK1 nor ATM associates with meiotic chromosomes (Flaggs et al., 1997). In S. pombe, the CHK1 protein kinase is activated by RAD3 in response to DNA damage and is responsible for the DNA damage checkpoint arrest (Walworth et al., 1993; Al-Khodairy et al., 1994; Walworth et al., 1996; O'Connell et al., 1997). In wild-type mammalian meiotic nuclei, CHK1 localizes along both synapsed and unsynapsed chromosomes (Flaggs et al., 1997). It has therefore been proposed that mammalian CHK1 and ATM may cooperate during meiosis to monitor the progress of meiotic recombination (Flaggs et al., 1997). All of these data contribute to establishing the importance of ATM and some of its substrates in meiosis and therefore help to elucidate the diverse functions of these proteins.

**Summary, Research Rationale, and Hypothesis**

In both SCID mice and patients with AT, mutations that cause extremely short truncations in PI-3-kinase related homologues result in complex, multi-faceted diseases with overlapping phenotypes that include immunodeficiency, an increased incidence of
lymphoma, and hypersensitivity to ionizing radiation. Alignment of the C-terminal domains of PI-3 kinase related proteins including TOR1, TOR2, FRAP, RAD3, MEC1, TEL1, mei-41, ATM, and DNA-PK reveals a highly conserved extreme C-terminal (ext-C) domain that is downstream from the conserved PI-3 lipid kinase catalytic motifs (Figure 1). The conservation of these ext-C residues and the similarities in the diseases that result from the loss of these residues from the ATM and DNA-PK proteins strongly suggests that the ext-C domain fulfills extremely important function(s), which may be distinct from the catalytic activity of the kinase domain. These functions could involve stability of the PI-3 related proteins and/or interactions with other proteins. The focus of this thesis has been to identify proteins that specifically interact with the ext-C domains of ATM and DNA-PK in an attempt to begin to elucidate the role of this region.
MATERIALS AND METHODS

Cloning of the ATM and DNA-PK Extreme C-Terminal Domains

(i) cDNA synthesis and RT-PCR

Fragments of human ATM and murine DNA-PK genes containing the ext-C domains defined in Figure 3 were generated by RT-PCR. cDNA was synthesized from total cellular RNA from human and murine thymus obtained from I. Grandal and C. Williams, respectively, using established methods (Gubler et al., 1983). Briefly, heat-denatured RNA (2.5 μg) was incubated (1 hr at 42°C, 15 minutes at 70°C) in a reaction mixture containing 1.25 μM each of oligo (dT)15 primer (Promega, Madison, WI) and ATM-SC-EcoRI or DPK-SC-EcoRI (Table 1), 10 mM DTT, 0.5 mM each dATP, dCTP, dGTP, dTTP, 1X First Strand buffer (GibcoBRL, Gaithersburg, MD), and 200 U SUPERSCRIPT™ II RNase H⁻ reverse transcriptase from Moloney Murine Leukemia Virus (GibcoBRL, Gaithersburg, MD). Mock reactions that did not contain reverse transcriptase were included with each series of reactions to provide negative controls.

RT-PCR reaction mixtures (25 μl) were prepared containing cDNA (0.25 μg input RNA), 40 mM Tricine-KOH pH 9.2, 15 mM KOAc, 3.5 mM Mg(OAc)₂, 3.75 μg/ml bovine serum albumin (BSA), 50 μM each of dATP, dCTP, dGTP, dTTP, 0.5 μl KlenTaq LA polymerase mix (Clonetech, Palo Alto, CA) and 0.5 μM of the appropriate 3’ and 5’ primers. Since high dNTP concentrations induce mutations due to deleterious effects on DNA polymerase fidelity (reviewed in Kunz et al., 1994), a titration was performed and 50 μM was determined to be the lowest concentration of dNTP that did not adversely affect PCR product yields. All RT-PCR reactions occurred in a DNA 480 thermal cycler.
The quality of each cDNA was assessed by RT-PCR amplification of β-actin. As negative controls, mock RT-PCR reactions were prepared by substitution of ddH2O for the template. PCR conditions for amplification of the ATM ext-C region (5' ATM-SC-BamHI, 3'ATM-SC-EcoRI) were 25 cycles at 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute. PCR conditions for amplification of the DNA-PK ext-C region (5' DPK-SC-BamHI, 3' DPK-SC-EcoRI) were identical except the annealing temperature was 68°C. A 10 minute extension at 72°C was performed following the final cycle. PCR products were resolved in 1% agarose containing ethidium bromide, visualized under longwave UV light, and bands containing the RT-PCR product were excised. The DNA was extracted as per manufacturers' instructions with the QIAquick gel extraction kit (QIAGEN GmbH, Hilden, Germany).

(ii) Restriction Enzyme Digestions

The ext-C RT-PCR fragments were cloned in frame with a 5’ glutathione S-transferase (GST) gene from Schistosoma japonicum in the pGEX-2TK bacterial expression vector (Amersham Pharmacia Biotech, Uppsala, Sweden). RT-PCR fragments (1-2 μg) were digested in 20 μl containing enzyme (BamHI 10 U/μl, EcoRI 12 U/μl) and 1X of the appropriate 10X buffer (Promega, Madison, WI). Sequential digestions were performed with BamHI (1 hr at 37°C) followed by EcoRI (1 hr at 37°C). To optimize reaction salt concentrations, the DNA was chloroform extracted, ethanol precipitated, and resuspended ddH2O before proceeding with the EcoRI digestion. DNA was resolved and visualized in 1% agarose, as described earlier. BamHI and EcoRI digested PCR and pGEX-2TK plasmid fragments were identified by comparison to a known DNA size
ladder (GibcoBRL, Gaithersburg, MD) and to uncut DNA controls. BamHI and EcoRI digested fragments were extracted from the agarose, as described as above.

(iii) Ligations

Ligation reactions (10 μl) containing 1X ligation buffer (Boehringer Mannheim GmbH, Mannheim, Germany), 4 U T4 DNA ligase (Boehringer Mannheim GmbH, Mannheim, Germany), 10 ng BamHI and EcoRI digested pGEX-2TK, 0-175 ng of BamHI and EcoRI digested insert DNA, and ddH2O were incubated (16 hours at 16°C). Mock ligations that either lacked insert DNA or T4 DNA ligase were also performed. Ligation reactions were ethanol precipitated and resuspended in 10 μl of ddH2O.

(iv) Preparation of Competent Cells

Two strains of E. coli bacteria were used in the course of these experiments and were made competent by different protocols (Maniatis et al., 1982). Briefly, electroporation-competent XL1-blue cells (Stratagene, La Jolla, CA) for cloning purposes were generated from a single colony that inoculated 5 ml sterile SOC (Maniatis et al., 1982) and was grown O/N (37°C at 250 rpm) in a shaking incubator (VWR Canlab, Mississauga, ON). Cultures were diluted into 400 ml SOC, grown (37°C at 300 rpm) until O.D.600 = 0.5-0.6 (1.6-1.9x10^8 cells/ml), cooled to 0°C, and pelleted (20 min at 2540 x g). Cells were sequentially washed and pelleted with 400 ml, 200 ml, and 10 ml of sterile, ice-cold 10% glycerol. The cells were resuspended in 1 ml of 10% glycerol, dispensed into 50 μl aliquots at 2-4 x10^10 cells/ml, and stored at -70°C until used for electroporation.

Protease-deficient ToPP3 E. coli (Stratagene, La Jolla, CA) for protein expression were made competent using calcium chloride (CaCl2). Briefly, a single colony of ToPP3
cells inoculated 100 ml of SOC, was incubated (37°C at 250 rpm) until O.D.₆₀₀ = 0.5-0.6 (1.6-1.9x10⁸ cells/ml), cooled to 0°C, and the cells were pelleted (2540 x g for 10 min). The cells were washed with 10 ml ice-cold 0.1 M CaCl₂, each pellet was resuspended in 2 ml of ice-cold 0.1 M CaCl₂ with 10% glycerol, dispensed into 200 µl aliquots, and stored at -70°C.

(v) Transformations

Transformation of ligation products from (iii) into XL1-blue competent cells was achieved by electroporation with a Gene-Pulser at 2.5 kV, 25 µF, and 400 Ω (Bio-Rad, Hercules, CA). Briefly, 50% (5 µl) of the ligation product was added to 50 µl of thawed XL1-blue competent cells on ice. The DNA/cell mixture was pulsed in a 0.2 cm Gene-Pulser cuvette (Bio-Rad, Hercules, CA), incubated in 1 ml SOC (1 hr at 37°C at 250 rpm), plated on LB/agar (Maniatis et al., 1982) containing 100 µg/ml ampicillin, and grown overnight at 37°C. Thawed competent cells without ligation products were plated on ampicillin-containing LB/agar to control for spontaneous antibiotic resistant revertants.

CaCl₂-prepared ToPP3 E.coli were transformed with pGEX-2TK plasmids containing the ATM or DNA-PK ext-C fragments. Briefly, plasmid DNA (50 ng) was added to 200 µl of freshly thawed ToPP3 cells. The DNA/cell mixture was stored on ice for 30 minutes, incubated (42°C for 90 seconds), placed in ice (2 min), and incubated in 800 µl SOC (37°C for 45 min at 250 rpm). Transformation reactions (150 µl) were streaked on LB/agar containing 100 µg/ml ampicillin and grown O/N at 37°C. Non-transformed competent ToPP3 cells were also streaked on LB/agar with ampicillin to control for spontaneous antibiotic resistance and contamination.
(vi) Identification of Positive Colonies by Hybridization

XL1-blue colonies were screened for vectors containing ATM or DNA-PK ext-C fragments using DNA hybridization (Maniatis et al., 1982). Briefly, ampicillin-resistant colonies were grown on LB/agar, transferred to nitrocellulose (Millipore, Bedford, MA), lysed, plasmid DNA was bound to the nitrocellulose, and plasmids containing the ATM or DNA-PK ext-C fragments were identified by hybridizing radiolabeled ATM or DNA-PK RT-PCR fragments to the nitrocellulose. As a negative control, colonies containing unmodified pGEX-2TK vector were spotted on each nitrocellulose filter. Bacterial colonies transformed with a plasmid containing the C-terminal kinase domain of murine DNA-PK were spotted on the DNA-PK ext-C cloning filters as a positive control.

To generate radiolabeled probes, 500 ng of ATM or DNA-PK PCR products were denatured and added to 11.4 μl of labeling solution [250 mM Hepes pH 6.6, 25 μM each dATP, dGTP, and dTTP, 62.5 mM Tris pH 8.0, 12.5 mM β-mercaptoethanol, 6.25 mM MgCl₂, 70 μM Tris pH 7.5, 70 μM EDTA, 7x10⁻² units random hexamer oligonucleotides (p(dN)₆; Amersham Pharmacia Biotech, Uppsala, Sweden)], 1 μl BSA (10 mg/ml), 2 μl ddH₂O, 50 μCi ³²P-dCTP (3000 Ci/mmoll; Amersham Pharmacia Biotech, Uppsala, Sweden), and 5 U Klenow fragment (Promega, Madison, WI). Probes were labeled for 1 hour at RT, unincorporated radionucleotides were removed in a NucTrap pushcolumn (Stratagene, La Jolla, CA), and purified probes were stored at -20°C.

(vii) Plasmid DNA Preparation

Bacteria containing vectors that hybridized to the radiolabeled ATM or DNA-PK RT-PCR fragments were used to inoculate LB (5 ml containing 100 μg/ml ampicillin) (Maniatis et al., 1982) and were incubated overnight (37°C at 250 rpm). Cells were
pelleted (2540 x g at 4°C), resuspended (5 min at RT) in 100 μl of buffer A [50 mM glucose, 10 mM EDTA, 25 mM Tris-Cl (pH 8.0)], buffer B (200 μl) [0.2 M NaOH, 1% SDS], and sodium acetate (150 μl) [3M NaOAc (pH 4.8)]. The cell suspensions were centrifuged (16000 x g for 10 min), the supernatants transferred to 1:1 neutral phenol/chloroform (450 μl), vortexed (15 seconds), and centrifuged (10 minutes at 16000 x g). The aqueous phase was transferred chloroform (450 μl), vortexed (15 seconds), centrifuged (16000 x g for 10 min), and the aqueous phase was added to 1 ml of ice-cold 100% ethanol. Following a 10 minute centrifugation, the pellet was washed (450 μl of 70% ethanol), dried, and resuspended in ddH2O (50 μl). To confirm the presence of the ATM or DNA-PK ext-C fragments in the vectors, 10% of the plasmid DNA (5 μl) was digested with EcoRI and BamHI, separated by electrophoresis on 1% agarose, and UV-visualized.

(viii) DNA Sequencing

The accuracy of cloning was confirmed by sequencing plasmid DNA in a 6% polyacrylamide-urea gel using the T7 sequencing kit (Amersham Life Science, Cleaveland, OH) according to the Sanger dideoxy chain termination method (Sanger et al., 1977). Forward and reverse sequencing primers (Table 1) were designed to anneal to regions 5’ and 3’ of the multiple cloning site of pGEX-2TK. Sequences were analyzed using MacDNASIS Pro V3.5 software (Hitachi Software Engineering Corp.) and compared published human ATM (U33841) and murine DNA-PK (U1944422) sequences in the GenBank database http://www.ncbi.nlm.nih.gov. The sequences of the ATM, DNA-PK, and control pre-T alpha receptor cytoplasmic domain (construct provided by Dr. Trang Duong) are provided in Figure 3.
**Production and Purification of GST-Fusion Proteins**

(i) **Expression of Bacterial GST-Fusion Proteins**

ToPP3 *E.coli* bacterial cells containing pGEX-2TK plasmids without insert DNA, with ATM Ext-C, with DNA-PK Ext-C, and with pre-T alpha-cyt were grown overnight (37°C at 250 rpm) in LB containing 100 μg/ml ampicillin. The temperature was decreased to 30°C, the cultures diluted 1:10 with LB, and grown for 2 hours. Expression of the pGEX-2TK vector was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG; GibcoBRL, Gaithersburg, MD) for 2-4 hours at 30°C. Cultures were cooled to 4°C, the cells pelleted (2540 x g at 4°C), washed with ice-cold phosphate buffered saline (PBS; (pH 7.4), 137 mM NaCl, 2.7 mM KCl, 5.4 mM Na₂HPO₄, 1.8 mM KH₂PO₄], and stored at -20°C.

(ii) **Affinity Chromatography Purification of GST-Fusion Proteins**

To purify GST proteins, the pellets were thawed and resuspended in lysis buffer (50 μl per ml of original culture) [pH 8.0; 1X PBS, 1 mM EDTA, 1 mM ethylene glycol-bis-(β-aminoethylether-N,N',N')-tetra acetic acid (EGTA), 1% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 0.1 mM phenyl phosphate disodium salt (PMSF), 1 mg/ml chicken egg white lysozyme (Sigma, St. Louis, MO)]. After a 30 minute incubation (4°C), the cells were sonicated and pelleted (2540 x g at 4°C for 15 min). The supernatants were incubated (1 hr at 4°C) with glutathione-sepharose 4B resin (Pharmacia Biotech, Uppsala, Sweden). The resin was pelleted (45 x g) and washed with ice-cold PBS (4 x 1 volume). The GST proteins were eluted from the matrix (2 hr at 4°C) with glutathione elution buffer [10 mM reduced glutathione, 50 mM Tris-Cl (pH
dialyzed against PBS (O/N at 4°C), diluted 1:10 in PBS, and bound to fresh glutathione-sepharose matrix. Matrix-bound GST proteins were suspended (75% slurry) with 1X PBS, aliquoted, and stored at −20°C. Estimation of protein concentration was achieved by comparing the Coomassie stained intensity of a given volume of fusion protein against serial dilutions of BSA standards (0.1-5 μg) resolved on SDS-PAGE (see Table 3 for the description of fusion proteins).

(iii) General Protein Biochemistry Techniques

**SDS-PAGE Gels**

Proteins were separated on the basis of size using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Before electrophoresis, proteins were boiled for 5 min with a 1:6 dilution of SDS sample buffer [370 mM 1 M Tris-Cl (pH 6.8), 60% glycerol, 416 mM SDS, 600 mM dithiothreitol (DTT), 0.3% Bromophenol blue], then briefly centrifuged (16000 x g for 5 s). All gels were 1.5 mm thick with a 4% polyacrylamide stacking gel and a separating gel composed of varying concentrations of acrylamide. The gels were subjected to electrophoresis in a running buffer [25 mM Tris base, 192 mM glycine, 3.5 mM SDS] at constant current until the dye front reached the bottom of the gel. Molecular weight estimations were made by visual comparison to known protein standards (Amersham Life Science, Cleveland, OH).

**Coomassie and Silver Staining of Proteins**

Gels were gently agitated in Coomassie stain [0.5% coomassie blue, 10% acetic acid, 30% isopropanol] for 10-30 minutes and then washed repetitively in destaining solution [5% acetic acid, 16.5% methanol] until the background staining was reduced to visualize protein bands (2-12 hr). Silver staining reagents and methodologies were
obtained from a Silver Stain Plus kit (Bio-Rad, Hercules, CA). Following detection of proteins, gels to be preserved were laid flat on Whatman paper (Whatman Corp., Maidstone, England), covered with plastic wrap, and dried in a gel dryer at 65°C under vacuum (Bio-Rad, Hercules, CA).

**Western Transfer of SDS-PAGE Gels**

Following electrophoresis, proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane by first soaking the gel in transfer solution [pH8.6, 20 mM Tris base, 120 mM glycine, 20% methanol] for 15 minutes. A nitrocellulose membrane was wetted in transfer buffer, placed on the gel, and the gel and membrane were sandwiched between transfer buffer pre-soaked Whatman paper and sponges. The entire sandwich was emerged in transfer buffer in a vertical transfer apparatus (Bio-Rad, Hercules, CA) in an ice-filled container and transferred for 2-3 hours at 300mA.

**Western Blotting of Proteins**

Upon completion of the electroblotting, the membrane was gently washed in ddH₂O and then blocked (2-24 hr) in Tris-buffered saline [TBS-T; 50 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20, 0.001% Thiomersal] containing 5% milk powder. Optimal concentrations of primary and secondary antibodies were determined by titration and/or provided by the manufacturer and are described in Table 2. The blocked membrane was agitated with 5% milk/TBS-T containing the primary antibody for 1-2 hours, washed (4 x 10 min with TBS-T), and agitated with 5% milk/TBS-T containing the secondary antibody for 1 hour. The membrane was washed (4 x 10 min with TBS-T), rinsed in ddH₂O, and exposed to enhanced chemiluminescence (ECL) solution (Amersham Life Science, Buckinghamshire, England) for 1 minute. The blotted
membrane was covered in plastic wrap, inserted in an autoradiography cassette, and exposed to X-ray film.

Membranes to be reassessed with different antibodies were washed (20 min in TBS-T), agitated (30 min at 42°C) in membrane stripping solution [100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-Cl (pH 6.7)], rinsed (4 x 10 min in TBS-T), and blocked for 1 hour (TBS-T with 5% milk).

**Protein Quantification**

Protein concentrations were determined using a detergent compatible (DC) protein assay kit (Bio-Rad, Hercules, CA), as per manufacturers’ instructions. Briefly, a standard absorption curve at 750 nm of 0-30 µg/ml of BSA was generated using a spectrophotometer (Beckman, Fullerton, CA). Two dilutions of the sample protein were prepared such that the absorbance would fall in the linear range of the standard curve. The sample protein concentration was determined by linear regression.

**Protein Binding Experiments**

(i) Metabolic Labeling of Cells

VL3-3M2 murine thymic lymphoma cells (Groves, 1994) were plated at 0.5 x 10^6 cells per ml in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% fetal calf serum (FCS; GibcoBRL, Gaithersburg, MD), 55 µM β-mercaptoethanol, 10 mM L-glutamine, and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffered saline (HEPES, pH 7.2). After growth at 37°C in a 5% CO₂ incubator for 24 hours, cells were pelleted (500 x g at RT for 10 min), washed with RT PBS (3 x 5 ml), and resuspended at 2 x 10^6 cells/ml in RPMI 1640 without methionine or cysteine (Mediatech,
Herndon, VA) supplemented with 10% FCS (GibcoBRL, Gaithersburg, MD), 55 μM β-mercaptoethanol, 10 mM L-glutamine, and 10 mM HEPES (pH 7.2). Following a 30 minute incubation, 1 mCi /6 x 10^6 cells of L-[35S]-methionine and L-[35S]-cysteine protein labeling mix (1175 Ci/mmol; DuPont, Markham, ON) was added. After 5 hours at 37°C in a 5% CO₂ incubator, the cells were pelleted (500 x g at RT for 10 min), washed with ice-cold PBS (3 x 5 ml), and counted.

(ii) Post-Nuclear and Nuclear Lysate Extraction Protocol

Following the final wash above, the cells were resuspended in ice-cold hypotonic buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 0.1 mM PMSF] to obtain a final concentration of 3 x 10^7 cells/ml. After 15 minutes, 6.25 μl of Nonidet P40 per ml of hypotonic buffer was added, the cells vortexed (10 seconds), and centrifuged (16000 x g at 4°C for 30 min). The supernatants (called the post-nuclear extracts, PNE) were removed, aliquoted, and stored at -70°C. Before use, the salt concentration of the post-nuclear lysates was increased from 10 mM to 100 mM with 5M NaCl. The nuclear pellet was agitated (15 min at 4°C) in 0.1 volumes of hypertonic buffer [20 mM HEPES (pH 7.9), 400 mM KCl, 1 mM EDTA, 0.5 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 0.1 mM PMSF]. The nuclear pellets were then centrifuged (16000 x g at 4°C for 10 min), the supernatant (called the nuclear extract, NE) was removed, and 0.23 volumes of no-salt buffer [20 mM HEPES (pH 7.9), 1 mM EDTA, 14.3% glycerol, 0.5 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 0.1 mM PMSF] were added. Diluted (final salt concentration of 120 mM) nuclear extracts were aliquoted and stored at -70°C.
(iii) Quantitative Assessment of Incorporation of $^{35}$S

To assess the amount of radioactive incorporation, 2 µl of either PNE or NE were precipitated (−20°C) with 25 µl of BSA (10 mg/ml), 100 µl of 10% trichloroacetic acid (TCA), and 75 µl of ddH$_2$O. The mixtures were centrifuged (16000 x g for 10 min), the pellet washed (2 x 500 µl 5% TCA), and resuspended in 500 µl of Ready Safe scintillation fluid (Beckman, Fullerton, CA). The radioactive counts were determined in a scintillation counter (Beckman, Fullerton, CA).

(iv) Pre-Clearing of Lysates

Proteins that bound non-specifically to either the GST moiety or to the glutathione-sepharose resin were depleted from the $^{35}$S-labeled lysates by incubating the lysates (30 min at 4°C) with resin-bound GST (25-50 µg GST/100 µg lysates) and fresh glutathione-sepharose resin (5-10 µl/100 µg lysates). The resin was then pelleted (45 x g for 5 min) and the supernatant containing unbound $^{35}$S-labeled proteins was collected.

(v) Binding and Detection of $^{35}$S-Labeled Proteins to GST Affinity Matrices

Pre-cleared NE or PNE (100 µg) were added to 3-5 µg of each purified resin-bound fusion protein (as estimated by Coomassie staining) suspended in 800 µl of binding buffer [120 mM NaCl, 50 mM Tris-Cl (pH 8.0), 0.5% Nonidet P-40, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 µg/ml pepstatin, 0.1 mM PMSF]. The radiolabeled lysates and matrix-bound proteins were incubated (2-3 hours at 4°C) on a rotating platform, the affinity matrix was pelleted (45 x g for 5 min), and the resin was washed (5 x 0.5 ml binding buffer). The $^{35}$S-labeled proteins were eluted from the affinity matrix either by incubation of the matrix in binding buffer with increasing concentrations of NaCl (0.5 M – 1 M) or by boiling the resin in SDS sample buffer for 5 minutes. Eluates
were then resolved by SDS-PAGE (4-20% gradient) and the proteins were either silver stained, fixed in 1 M salicylic acid to enhance the 35S signal (1 hour), and dried on Whatman paper, or the proteins were electroblotted onto nitrocellulose membrane. Radiolabeled eluates were then detected by autoradiography (1-4 week exposure).

(vi) Binding and Detection of Recombinant Human RPA to GST Affinity Matrices

For all of the following experiments, the concentration of the full-length (non-degraded) proteins in each GST affinity-matrices was visually approximated by Coomassie staining to be ~2 mg/ml for GST and pre-T alpha and ~0.1 mg/ml for ATM ext-C and DNA-PK ext-C. Resin-bound ATM ext-C (3 µg), DNA-PK ext-C (3 µg), pre-T alpha (60 µg), and GST (60 µg) were washed (2 x 100 µl) and resuspended in binding buffer (final volume 100 µl). Purified recombinant human RPA (rhRPA) was provided by Dr. C.J. Ingles from the Best Institute, University of Toronto. Briefly, all three subunits of hRPA were co-expressed in E.coli from a modified pET-3a vector containing a single T7 RNA polymerase promoter followed by the coding sequences for RPA70, RPA14, and RPA32, each preceded by a Shine-Dalgarno ribosome-binding site. rhRPA was purified from induced bacterial lysates by affinity chromatography on Affi-Gel blue (Bio-Rad, Hercules, CA) followed by ion-exchange chromatography on a Mono-Q column (Pharmacia Biotech, Baie d’Urfé, QU) (Hendricksen et al., 1994). rhRPA (250 ng) was incubated (2 hr at 4°C) with each of the affinity matrices, the resin was pelleted (45 x g for 5 min), washed (5 x 0.5 ml binding buffer), and resuspended in elution buffer (100 µl) [1 M NaCl, 50 mM Tris-Cl (pH 8.0), and 0.5% Nonidet P-40] for 10 minutes at 4°C. Eluates were resolved by SDS-PAGE (5-20% gradient), electroblotted, and assessed with anti-RPA rabbit serum (1:300) (Dr C.J. Ingles, Best Institute, University of
Toronto, ON) followed by HRP-protein A (1:7500). As a positive control for RPA, 50 ng of rhRPA were also resolved by SDS-PAGE.

(vi) Binding of Murine VL3-3M2 PN Lysates to the GST Affinity Matrices and Detection of RPA

To determine which fraction of VL3-3M2 cells contained the majority of murine RPA, 75 μg of N and PN lysates were resolved by SDS-PAGE, electroblotted, and assessed with anti-RPA sera (1:300) followed by HRP-protein A (1:7500). PN lysates (400 μg) were added to affinity matrices containing ATM ext-C (3 μg), DNA-PK ext-C (3 μg), pre-T alpha (60 μg), and GST (60 μg) in binding buffer to a final volume of 400 μl. Following a 2 hour incubation (4°C), the resin was pelleted (45 x g for 5 min), washed (5 x 0.5 ml binding buffer), and incubated in elution buffer (10 min at 4°C). The eluates were resolved by SDS-PAGE, electroblotted, and evaluated with anti-RPA rabbit serum (1:300) followed by HRP-protein A (1:7500).
Table 1. Description of Oligonucleotides. All primer sequences are written in the 5' to 3' direction. Internal restriction enzyme sites are indicated within the name of the oligonucleotide. In addition to being employed as reverse primers during PCR reactions, the ATM-SC-EcoRI and DPK-SC-EcoRI oligonucleotides were also used to prime the synthesis of cDNA from RNA, as described in the Materials and Methods. The pGEX multiple cloning site (MCS) sequencing oligonucleotides are commercially available from Pharmacia Biotech, Baie d'Urfe, Quebec.
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'-3')</th>
<th>Synthesis</th>
<th>Applications</th>
</tr>
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<td>ATM-SC-BamHI</td>
<td>GCG GGA TCC ACT CTG AAT GCA GAT GAC CAA GAA</td>
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<tr>
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<tr>
<td>DPK-SC-EcoRI</td>
<td>GCG GAA TTC TCA CAT CCA GGG CTC CCA</td>
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<td>RT, reverse PCR</td>
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<tr>
<td>β-actin 5'</td>
<td>TGG GTC AGA AGG ACT CCT ATG</td>
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<tr>
<td>β-actin 3'</td>
<td>CAG GCA GCT CAT AGC TCT TCT</td>
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<td>reverse PCR</td>
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<tr>
<td>pGEX 3'</td>
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<td>Dalton Chemicals, North York, ON</td>
<td>sequencing pGEX-2TK MCS</td>
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Table 2. Description of Antibodies. The three different primary antibodies utilized in the experiments contained within this thesis are listed in the first column. Where applicable, the source of the reagents is indicated in parentheses. Proteins A and G were conjugated with horseradish peroxidase (HRP) and the antibody complex was detected by ECL. Optimal concentrations were determined by titration. The RPA antisera was produced by Dr. C.J. Ingles at the University of Toronto. Specificity denotes the specific polypeptide or protein used to immunize the source species, which is recognized by the antibody. Where applicable, the mode of sera affinity purification is indicated.
Table 2.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Specificity</th>
<th>Source</th>
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<th>Concentration</th>
<th>Detection Reagent</th>
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<td>column affinity</td>
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<td>(Amersham Life Sciences, Baie d'Urfe, Que)</td>
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<td>H. sapiens</td>
<td>column</td>
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<td>rabbit polyclonal</td>
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<tr>
<td></td>
<td>(Dr. C.J. Ingles, Univ. of Toronto, ON)</td>
<td>H. sapiens</td>
<td></td>
<td></td>
<td>(Amersham Life Sciences, Baie d'Urfe, Que)</td>
</tr>
</tbody>
</table>
Table 3. Description of GST Fusion Proteins. All fusion proteins used in these studies were expressed with an amino terminal glutathione S-transferase (GST) moiety for the purpose of column affinity purification. Insert DNA sequences were determined by sequencing and are given in Figure 3. Where applicable, changes from the published GenBank submissions (human ATM U33841, murine DNA-PK 1944422, and murine pre-TCR alpha U16958) have been indicated. The ATM Ext-C 3003D→N mutation was observed in all clones generated by separate PCR reactions from a non-AT human thymus sample and may represent a polymorphism.

*Please note that the pre-TCR alpha cytoplasmic domain (pre-T alpha) was cloned by Dr. Trang Duong and was employed within this thesis as a control GST fusion protein.
Table 3.

<table>
<thead>
<tr>
<th>Name</th>
<th>Source of Insert DNA</th>
<th>Insert Length</th>
<th>Insert Size</th>
<th>Vector</th>
<th>Expression Strain</th>
<th>Calculated Total MW</th>
<th>Mutations</th>
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<tbody>
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<td>GST</td>
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<td>n/a</td>
<td>n/a</td>
<td>pGEX-2TK</td>
<td>ToPP3</td>
<td>26kD</td>
<td>n/a</td>
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<td>ToPP3</td>
<td>34.4 kD</td>
<td>3003D-N</td>
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<td>ToPP3</td>
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<td>pre-T alpha-cyt*</td>
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<td>110bp/32AA</td>
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<td>pGEX-30X</td>
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</table>
RESULTS

1. Production of Fusion Proteins

To identify proteins that interact with the residues of murine DNA-PK that are truncated by the SCID mutation, and with a homologous region on human ATM, RT-PCR amplified fragments containing these residues were cloned and expressed as C-terminal fusion proteins with the glutathione S-transferase (GST) gene in the pGEX-2TK vector. These recombinant GST proteins are denoted rExt-C or GST-ATM/DNA-PK ext-C. The identity and accuracy of the DNA inserts were confirmed by sequencing. A pGEX-30X vector containing the cytoplasmic region of an immature murine T-cell receptor complex (pre-T alpha) was generously provided by Dr. Trang Duong. The pre-T alpha cytoplasmic domain was chosen as a specificity control for protein binding experiments because it is not related to the PI-3 related kinase family and because of its similarity in size to the ext-C domains. The GST moiety was also expressed from an unmodified pGEX-2TK vector and used as a control for proteins that bound to either GST or to the glutathione-sepharose resin. The sequences of all three of the fusion proteins are shown in Figure 3.

To confirm the purity and predicted molecular weight (MW) of each of the four GST-containing proteins, each protein was affinity purified by glutathione-sepharose affinity chromatography (Materials and Methods), resolved by SDS-PAGE, and detected by Coomassie blue staining (Figure 4A), GST antibodies (Santa Cruz Biotech., Santa Cruz, CA)(Figure 4B), and by an antibody raised against residues 3032-3051 of human ATM (Santa Cruz Biotech., Santa Cruz, CA)(Figure 4C). Based on primary sequence, the calculated MW of GST-containing proteins are as follows; 26 kD (GST), 29.7 kD (pre-T
alpha), 34.4 kD (ATM ext-C), and 35.3 kD (DNA-PK ext-C). The relative MW of the largest band in each of the purified protein preparations (Figure 4 A&B) was determined using linear regression from known protein standards and was 28 kD (GST), 32.5 kD (pre-T alpha), 32 kD (ATM ext-C), and 34 kD (DNA-PK ext-C). These values correspond well with the calculated MW for each fusion protein. The 32 kD protein observed by Coomassie staining and by GST antibodies in the GST-ATM ext-C preparation was also detected by anti-ATM sera (Figure 4C) which provides further support for the identity of this protein.

In addition to observing the largest MW bands described above, smaller proteins were also detected by Coomassie staining and by GST antibodies (Figure 4A&B). In the three fusion protein preparations, a 28 kD band was observed and likely resulted from aborted translation and/or degradation of the fusion proteins that resulted in cleavage of the GST moiety from the rExt-C and pre-T alpha proteins. In both the ATM and DNA-PK rExt-C preparations, several other bands were observed below the full-length protein and were likely the result of degradation of the fusion proteins.

Prior to using the affinity purified fusion proteins in protein binding experiments, their concentrations were estimated by comparing the intensity of a known volume of the Coomassie stained full-length protein to a BSA standard. Yields of bacterially expressed fusion proteins normally range from 1-10 mg/l and vary with the protein, the host strain, and the conditions of the expression (Smith et al., 1990). In TOPP E. coli, both the GST and the GST-pre-T alpha proteins produced 2.5-5 mg/l after a two hour induction at 30°C. In contrast, yields of the full-length fusion proteins containing the rExt-C domains ranged from 10-20 µg/l. These low yields were possibly the result of in vivo degradation.
of the rExt-C fusion proteins as suggested by the relative abundance of degradation products in these preparations versus either of the GST or GST-pre-T alpha preparations.

2. Binding of $^{35}$S-Labeled Murine Proteins to Recombinant Extreme C-Terminal Domains of ATM and DNA-PK

In an attempt to begin to identify proteins that interact specifically with the ext-C domains of ATM and DNA-PK, affinity matrices containing equal amounts (3-5 μg) of ATM rExt-C, DNA-PK rExt-C, and pre-T alpha protein were incubated with radiolabeled lysates from a murine lymphoma cell line (VL3-3M2). Following the incubation, the unbound radiolabeled lysates were removed, the affinity matrices washed repetitively, bound lysates were eluted by boiling the matrices in SDS sample buffer, and the eluates were resolved by SDS-PAGE and detected by autoradiography. To identify proteins that bound specifically to the rExt-C domains, the MW of the proteins that bound to these domains were compared to the proteins that bound to the GST-pre-T alpha affinity matrix. The $^{35}$S-labeled murine lysates were separated into nuclear (N) and post-nuclear (PN) pools, as described in the Materials and Methods, to assist in identification of specifically bound proteins.

As shown in Figure 5, many nuclear and post-nuclear proteins ranging in size from >220 kD to <14 kD bound to all three of the affinity matrices. No specific binding to either ATM or DNA-PK rExt-C was detected. Specific proteins were potentially obscured by major distortions in the mobility of the proteins in the region of the gel surrounding the MW of the fusion protein (Figure 5). The location of these distortions suggests that they may have been caused by elution of the fusion proteins in addition to
the $^{35}$S-labeled lysates when the matrices were boiled in SDS sample buffer. The large number of proteins that bound non-specifically to the fusion proteins in addition to the poor resolution of the radiolabeled proteins between 27-35 kD made interpretation of these results extremely difficult.

To ameliorate the problems described above several attempts were made to reduce the non-specific binding of radiolabeled proteins to either the GST moiety or to the glutathione-sepharose resin. Prior to incubation with the affinity matrices, the radiolabeled lysates were pre-cleared with a mixture of resin-bound GST and glutathione-sepharose resin to deplete proteins that bound to either of these entities. No reduction in the amount of proteins that bound to the fusion proteins was observed (data not shown). The stringency of the wash buffer following incubation of the lysates with the fusion proteins was increased through the addition of 0.1% SDS and 0.5% deoxycholate (DOC), however this did not notably reduce the non-specific binding (data not shown). Finally, the bound proteins were sequentially eluted from the affinity matrices with 0.5 M NaCl, 1.0 M NaCl, and by boiling the remaining proteins in SDS buffer. As demonstrated in Figure 6, the sequential elutions did not circumvent the difficulties described above as similar proteins were eluted from the GST, pre-T alpha, and rExt-C affinity matrices. It was concluded that the experimental approach described above to identify proteins that interact specifically with the ATM and DNA-PK ext-C domains was not adequately discriminating under the conditions employed here, and an alternative method was pursued.

3. Recombinant Human Replication Protein A Binds to Recombinant Extreme C-Terminal Domains of ATM and DNA-PK In Vitro
Because of the high degree of conservation among the C-terminal residues of DNA-PK and ATM, it was hypothesized that these domains may interact with the same protein(s). One protein that has been demonstrated to have a functional relationship to both DNA-PK and ATM is the ssDNA binding protein, RPA. In cells from SCID mice and AT patients, DNA damage-induced phosphorylation of RPA is defective (Liu et al., 1993; Boubnov and Weaver, 1995; Fried et al., 1996). In S. cerevisiae, phosphorylation of RPA has been shown to be dependent on another PI-3 related kinase homologue, MEC1 (Brush et al., 1996). It was hypothesized, therefore, that RPA might bind to the ext-C domains of ATM and DNA-PK.

To determine whether the ext-C domains of ATM and DNA-PK bind to RPA, ATM rExt-C, DNA-PK rExt-C, and pre-T alpha affinity matrices were incubated with recombinant human RPA (rhRPA). All three subunits of hRPA were coexpressed in E. coli and were affinity purified as a stable heterotrimer, as described in the Materials and Methods section. Following a two hour incubation, the affinity matrices were washed repetitively and the bound proteins were eluted in 1.0 M NaCl. The eluates were resolved by SDS-PAGE and the presence of RPA was detected with rabbit sera raised against rhRPA (antisera described in He et al., 1995). Fifty nanograms of the rhRPA were also resolved as a positive control.

Proteins migrating at 70, 32, and 12 kD were observed in the rhRPA control lane, and these sizes correspond to RPA-1, RPA-2, and RPA-3 of human RPA (Figure 7A, lane 1). Proteins that co-migrated with rhRPA-1 and rhRPA-2 were eluted from the ATM and DNA-PK rExt-C affinity matrices (Figure 7A, lanes 3&4). Neither of these bands was observed in the eluates from the pre-T alpha affinity matrix (Figure 7A, lane 2) or in any
of the eluates from incubations that lacked input rhRPA (Figure 7A, lanes 5-7). To confirm that equal amounts of fusion protein were bound to the affinity matrices, the membrane from Figure 7A was re-assessed with GST antibodies (Figure 7B). The intensity of the full-length protein in each of the affinity matrices appears to be approximately equal.

A protein migrating at approximately 72 kD was detected in eluates from the ATM and DNA-PK rExt-C domains in both the presence and absence of added rhRPA (Figure 7A, lanes 3,4,6,7), which suggest that this 72 kD protein may have originated from the affinity matrices. One candidate for the 72 kD protein is DnaK, a bacterial protein chaperone that may bind preferentially to abnormally folded or fusion proteins thereby enhancing their susceptibility to cellular proteases (Craig and Gross, 1991; Leustek et al., 1992; Yu-Sherman and Goldberg, 1992). Since a bacterially expressed protein was used to generate the polyclonal anti-RPA rabbit serum, the 72 kD protein contaminant in the ATM and DNA-PK affinity matrices may be recognized by DnaK-specific antibodies due to contamination in the original immunogen. Nonetheless, the presence of rhRPA in the eluates from the ATM and DNA-PK rExt-C affinity matrices, and not in the eluate from the pre-T alpha affinity matrix, demonstrates that in vitro the ext-C domains of both ATM and DNA-PK bind to RPA.

4. The Recombinant Extreme C-Terminal Domains of ATM and DNA-PK Bind to Murine Replication Protein A In Vitro

To identify which fraction of VL3-3M2 lysates contained the majority of murine RPA, 75 μg each of N and PN extracts were resolved by SDS-PAGE, transferred to
nitrocellulose, and assessed with anti-RPA sera. As reported in the literature (reviewed in Wold, 1997), the abundance of RPA in lysates fractionated by osmotic lysis was determined to reside in the PNE (Figure 8A). To determine whether the rExt-C domains could bind to RPA in a mixture of cellular proteins, lysates from murine VL3-3M2 cells were incubated with glutathione-sepharose affinity matrices containing GST, pre-T alpha, and the ATM and DNA-PK rExt-C domains. Following a two hour incubation with 400 µg of PNE, the affinity matrices were washed repetitively, the bound proteins were eluted in 1 M NaCl, and anti-RPA serum was used to detect murine RPA.

Proteins with a similar MW as the 70 and 32 kD subunits of rhRPA were bound by the ATM and DNA-PK rExt-C affinity matrices (Figure 8B). In contrast, no RPA was detected in the eluates from either the GST or pre-T alpha affinity matrices. A band at approximately 72 kD was observed in both of the GST-ext-C domain lanes and, as discussed earlier, is likely due to contamination of the rExt-C preparations by bacterial DnaK. Binding of murine RPA by the ATM and DNA-PK rExt-C domains and not by either GST or the pre-T alpha fusion protein suggests that the ext-C domains bind specifically to RPA.

5. Binding of Murine RPA by ATM and DNA-PK rExt-C Domains Increases Proportionally to the Amount of Fusion Protein

If cellular RPA binds specifically to the ext-C domains of ATM and DNA-PK, then increasing the amount of matrix-bound fusion protein that is incubated with cellular lysates should increase the recovery of RPA. Varying quantities (1, 2, and 5 µg) of ATM and DNA-PK rExt-C proteins were incubated with VL3-3M2 PN lysates. The protein
fraction that was bound to and eluted from each affinity matrix (bound) and the fraction that did not bind to the affinity matrix (unbound) were resolved by SDS-PAGE and evaluated with anti-RPA sera.

A protein co-migrating with rhRPA-2 was detected in the VL3-3M2 PNE, in all of the unbound fractions, and in proteins that were eluted from the ATM and DNA-PK rExt-C affinity matrices (Figure 9A). In both the ATM and DNA-PK binding experiments, more RPA-2 was detected in the eluates from the highest concentration of rExt-C protein than from either of two lesser concentrations (Figure 9A, lanes 7, 9, 11 and 13, 15, 17).

Small amounts of RPA-2 were also detected in the proteins that were eluted from the GST and pre-T alpha affinity matrices, however the intensity of the RPA p32 bands was considerably less than the RPA-2 detected in either of the 5 μg rExt-C titrations. Because the proportion of the full-length rExt-C protein in the affinity matrices was relatively small versus the amount of degradation products (Figure 4A&B), the maximum concentration of the full-length species of either of the rExt-C affinity matrices, as estimated by Coomassie staining, was 0.1 mg/ml. In contrast, 20-fold higher concentrations of the GST and GST-pre-T alpha proteins were bound to the affinity matrices (Figure 4A&B). Since the volume of the GST and GST-pre-T alpha affinity matrices incubated with PNE in this experiment was equal to the volume (and not amount) of rExt-C affinity matrices used in the highest titration, RPA may have bound non-specifically to the GST and GST-pre-T alpha matrices due to the excess amount of protein.
A 72 kD protein was observed in both the bound and unbound protein fractions from each of the four affinity matrices (Figure 9A). Since this protein was not detected in the murine PN lysates it may have originated from the bacterial fusion proteins. The anti-RPA sera has previously detected a 72 kD protein in the GST fusion protein affinity matrices and this signal is likely caused by contamination of the fusion proteins by DnaK, as discussed above. Several bands of varying sizes that did not include the MW of any of the RPA subunits were detected in both the bound and unbound fractions from the rExt-C proteins. Since these bands were not detected in the PNE, they also may have originated from the bacterial proteins.

A 70 kD protein that co-migrated with RPA-1 was detected in the PNE, in all of the unbound protein fractions, and in the protein eluted from the 5 µg titration of DNA-PK rExt-C (Figure 9A). RPA-1 was not observed in the eluates from GST or GST-pre-T alpha affinity matrices. Due to the broad smear caused by a 72 kD protein in the rATM ext-C lanes (Figure 9A, lanes 7-12), it was not possible to determine whether the 70 kD RPA-1 subunit was present. RPA-3 was not detected in any murine lysates. One possible explanation for the variable presence of the subunits of the RPA heterotrimer is that the anti-RPA sera appears to detect the 32 kD subunit more readily than the 70 or 14 kD proteins, as demonstrated by the reaction of the anti-sera to both rhRPA and murine PNE. In addition, it has been reported that despite the high level of conservation, RPA homologues are generally not antigenically related (reviewed in Wold, 1997). Therefore, the rhRPA anti-sera used in these experiments may react weakly to murine RPA. Since RPA exists both in vitro and in vivo as a relatively stable heterotrimer (reviewed in Wold,
1997), it is possible that all three subunits may be present in the eluates in which only RPA-2 is detected by anti-RPA sera.

Due to the small quantity of RPA that bound to the rExt-C domains, it was not possible to detect depletion of RPA from the lysates. This lack of binding may be due to spatial interference caused by the large amount of proteins that bind to the GST moiety and to the glutathione-sepharose resin (Figures 5&6). The relative scarcity of full-length rExt-C protein in the ATM and DNA-PK affinity matrices (Figure 4A&B) may mean that only a small fraction of the protein bound to the affinity matrix has the extreme C-terminal residues necessary for the RPA interaction. Finally, it is possible that cellular RPA is already complexed with DNA-PK and/or ATM or has a conformation that does not permit binding to the rExt-C proteins.

To confirm that varying amounts of rExt-C proteins were used in each of the titrations, the membrane from Figure 9A was re-assessed with anti-GST antibodies (Figure 9B). Although GST fusion proteins were detected in the bound and unbound fractions from each step of the titration, no significant difference in the amount of total protein could be observed. Since a considerable fraction of the GST fusion proteins remain bound to glutathione-sepharose resin following treatment with 1 M NaCl (see Figure 6A&B, ‘residual bound’), the majority of the fusion protein may have been discarded with the resin. This lack of observable difference may also be due to incomplete transfer of the fusion proteins from the polyacrylamide gel to the nitrocellulose. Collectively, these results are consistent with the idea that RPA binds with apparent specificity to ATM and DNA-PK.
Figure 1. Alignment of the PI-3 Related Kinase Domains. Carboxy terminal amino acid sequences of PI-3 related kinases from several species were aligned based on the conserved PI-3 kinase motifs (DRXXN and DFG). Sequences were obtained from GenBank with the following accession numbers: *Saccharomyces cerevisiae* TOR1 (X74857), TOR2 (X71416), MEC1 (626949), and TEL1 (626948), *Schizosaccharomyces pombe* RAD3 (Y09076), *Drosophila melanogaster* mei-41 (1583574), *Homo sapiens* ATM (U33841), and FRAP (L34075), and *Mus musculus* DNA-PK (1944422). A conserved lysine residue that is involved in ATP-binding and kinase function in PI-3 lipid kinases was arbitrarily chosen for the beginning of this alignment. The PI-3 kinase motifs are in solid boxes and the extreme C-terminal domain (ext-C) is enclosed in a hatched box. The extreme C-terminal domains of the PI-3 related kinases were defined based solely on sequence conservation and do not imply any information on their structure, previously defined structural motifs, or biophysical data. Of the 31 residues within the ext-C domain, 6 are conserved across every member of the PI-3 related proteins. These amino acids are indicated by an asterisk (*). There is 45% identity and 58% similarity between the human ATM and murine DNA-PK ext-C domains.
Figure 1.

scid mutation
Figure 2. ATM Mutations Found in AT Patients. ATM mutations have been identified throughout the length of the gene including several distinct pathological mutations localized to exon 65, the coding region that contains the ATM extreme C-terminal domain. Copied with permission from the on-line Virginia Mason Research Center ATM database (www.vmmc.org/vmrc/atm.htm).
Figure 2.

Mutations

Exons

Protein

+ Founder effect for one or more mutations in this exon

ATM

Rad3 homology

PI-3 kinase homology

AT Mutation Database

http://www.vmmc.org/vmrc/atm.htm
Figure 3. Sequences of Fusion Protein Constructs. Fusion proteins were generated using the pGEX-2TK cloning vector, which encodes a glutathione S-transferase from *Schistosoma japonicum* N-terminal and in frame with a multiple cloning site (GenBank accession U13851). To generate each specific fusion protein, the insert DNA sequences described in A, B and C of this figure were cloned into the plasmid vector. The numbers beside the sequences correspond to the position assigned to each residue in the following GenBank submissions, human ATM (U33841), murine DNA-PK (1944422), and murine pre-TCR alpha (U16958). As illustrated in Table 3, the highlighted asparagine (N) residue at 3003 in the ATM fusion protein is a change from the aspartate (D) residue reported in the published sequence and may represent a polymorphism. The ATM and DNA-PK ext-C domains illustrated in this figure and described throughout the thesis were defined based on sequence conservation and not by any previously characterized protein motifs.
Figure 3.

A. Human ATM Extreme C-Terminal Domain

5' ACT CTG AAT GCA GAT GAC CAA GGA TGC AAA CCA ACT CTC AGT GAT ATG GAC CAG

2982 T L N A D D Q E C K R N L S D I D Q

3001 AGT TTC AAC CAA CTA GCT GAA GTG TTC TTA ATG ACA CCA GAG AAA CIG AAA

3019 S F X K V A E R V L M R L Q E K L K

3036 GCA GTG GAA GAA GCC ACT GTG CTC AGT GCT GCC GAA CTA GTG AAT TTG CTC ACT ATT

3054 Q Q A I D P K N L S R L F P G W K A

TGG GTG TGA 3'

3055 W V *

3056

B. Murine DNA-PK Extreme C-Terminal Domain

5' TAT GCT AAG AGA AAG TTA GCA GGC GCC AAC CCA GCT GCT AAT ACT TAT GAT GAG

4046 Y A K R K L A G A N P A V I T C D E

4064 L Y L G H E A S S A F S R Y T A V A

4081 CGA GCC AAC AGA GCA TAC AAC ATT CCT CCA CAA GAG CCA GAG ACT GGG CTT TCA

4099 R G N R D Y N I R A Q E P E S G L S

4100 E E T Q V K C L V D Q A T D P N I L

4117 GCC AGG ACT TGG GAA GGA TGG GGC CCC TGG ATG TTA 3'

4118 G R T W E G W E P W M *

4128

C. Pre-TCR Alpha Cytoplasmic Domain

5' ACC TGC AGG CAC CTC CTC CTC CAT GTG GTC GCC GCC CAG CAC CTC CAG CCA CCA

175 T C S H L R L H V L A G Q H L Q P P

192 CCC TCA CCC AGG TCC CIG CCT CCC ACC CAC AGA ATG TGG ACA TAG 3'

193 P S R K S L P P T H R I W T *

206
Figure 4. Expression and Purification of GST Fusion Proteins. The constructs described in Table 3 were expressed in a protease-deficient strain of *Escherichia coli* and purified on glutathione sepharose resin. The proteins were competitively eluted from the resin using 10 mM free reduced glutathione, dialyzed against PBS, and rebound to fresh glutathione sepharose. The purified proteins were eluted by boiling in SDS sample buffer, resolved by SDS-PAGE in 12% polyacrylamide, and stained by Coomassie blue (A). Purified proteins from the same preparation as in (A) were then resolved by SDS-PAGE, transferred to nitrocellulose, and detected by anti-GST (1:5000) followed by HRP-protein A (1:7500) (B). The membrane from (B) was stripped and reassessed with 1 μg/ml of goat serum raised against the terminal 20 residues of human ATM followed by 1:1000 anti-goat IgG (C). The arrows and letters indicate the highest MW protein detected by Coomassie blue staining and GST antibodies in each fusion protein preparation, and were interpreted to be the full-length species of each fusion protein.
Figure 4.

A. GST (a)
   GST-pre-T alpha (b)
   GST-ATM Ext-C (c)
   GST-DNA-PK Ext-C (d)

B. GST (a)
   GST-pre-T alpha (b)
   GST-ATM Ext-C (c)
   GST-DNA-PK Ext-C (d)

C. GST (a)
   GST-pre-T alpha (b)
   GST-ATM Ext-C (c)
   GST-DNA-PK Ext-C (d)
Figure 5. Binding of Radiolabeled Murine Proteins to the Extreme C-Terminal Domains of ATM and DNA-PK. One hundred micrograms of $^{35}$S-labeled VL3-3M2 nuclear (N) or post-nuclear (PN) lysates were incubated for 3 hours with 5 µg of each affinity purified GST fusion protein bound to glutathione sepharose resin. The unbound proteins were removed and the resin washed repetitively. The bound proteins were eluted by boiling the resin in SDS sample buffer, resolved by SDS-PAGE (4-20% gradient), and detected by autoradiography. A ~12 kD band (*) from both the N and PN fractions was detected in the eluates from the Ext-C domains and not from the pre-T alpha control, however this result was not reproducible in subsequent experiments.
Figure 6. Salt Elution of $^{35}$S-Labeled Proteins Bound to rExt-C Affinity Matrices.

One hundred micrograms of $^{35}$S-labeled VL3-3M2 nuclear (A) or post-nuclear (B) lysates were incubated with glutathione sepharose affinity matrices prepared with 5 µg of GST (1), GST-pre-T alpha (2), GST-ATM-ext-C (3), or GST-DNA-PK-ext-C (4). Following a 2 hr incubation, unbound proteins were removed and the resin washed repetitively. Bound proteins were sequentially eluted for 15 min in 0.5 M NaCl followed by 1.0 M NaCl and the eluates were collected. Residual bound proteins were eluted by boiling the matrices in SDS sample buffer. All of the eluates were resolved by SDS-PAGE (4-20% gradient) and detected by autoradiography.
Figure 6.

A. Nuclear Lysates

B. Post-Nuclear Lysates
Figure 7. Recombinant hRPA Binds to the Extreme C-Terminal Domains of ATM and DNA-PK. Two hundred and fifty nanograms of affinity purified recombinant human RPA (rhRPA) were incubated with 5 μg each of affinity matrices containing the three purified GST fusion proteins (+RPA). After two hours, the supernatant was removed and the resin washed repetitively. The bound proteins were eluted in 1.0 M NaCl, resolved by SDS-PAGE (5-20% gradient), transferred to nitrocellulose, and detected by anti-RPA rabbit serum (1:300) followed by HRP-protein A (1:7500) (A). Fifty nanograms of rhRPA were also resolved as a positive control. Five micrograms of the same preparation of the purified fusion proteins were also resolved (-RPA). To control to equal loading of fusion proteins, the membrane was stripped and reassessed with anti-GST (1:5000) followed by HRP-protein A (1:7500) (B).
Figure 7.
Figure 8. RPA in Murine Lysates Binds to the Extreme C-Terminal Domains of ATM and DNA-PK. Seventy-five micrograms of murine VL3-3M2 nuclear (N) and post-nuclear (PN) lysates and 50 ng of rhRPA were resolved by SDS-PAGE in 15% polyacrylamide, transferred to nitrocellulose, and assessed with anti-RPA rabbit serum (1:300) followed by HRP-protein A (1:7500) (A). Murine RPA was determined to be more abundant in the PN fraction of VL3-3M2 lysates. Affinity purified GST proteins bound to glutathione sepharose were incubated with 400 µg of murine VL3-3M2 PN lysates in 0.1 M NaCl. After two hours, the unbound proteins were removed and the glutathione sepharose resin washed repetitively. The bound proteins were eluted in 1.0 M NaCl, resolved by SDS-PAGE (5-20% gradient), transferred to nitrocellulose, and detected with anti-RPA sera (1:300) followed by HRP-protein A (1:7500) (B). rhRPA (50 ng) was co-resolved with the eluates as a positive control. Note that the recombinant human RPA and the murine RPA subunits migrate at slightly different MW due to sequence differences between the two species. Despite a high level of conservation, RPA molecules from different species are generally not antigenically related (reviewed in Wold, 1997), which may explain the poor detection of the different subunits of murine RPA by the antisera raised against rhRPA.
Figure 8.

A.

B.
Figure 9. Recovery of RPA from Murine Lysates by the Extreme C-Terminal Domains of ATM and DNA-PK Increases with the Amount of Fusion Protein.

Varying amounts (1, 2, and 5 µg) of purified resin-bound GST-ATM and GST-DNA-PK ext-C domains were incubated with 500 µg of murine VL3-3M2 PN lysates in 0.1 M NaCl. Two hundred micrograms of bound GST and GST-pre-T alpha proteins were also incubated with 500 µg of PN lysates. After two hours, 125 µg of the unbound proteins were collected (UB), the resin washed repetitively, and the bound proteins (B) eluted in 1.0 M NaCl. Both fractions were resolved by SDS-PAGE (12% polyacrylamide), transferred to nitrocellulose, and RPA was detected with anti-RPA sera (1:300) followed by HRP-protein A (1:7500) (A). Fifty nanograms of rhRPA and 100 µg of PNE were resolved in lanes 1 and 2, respectively. The membrane from (A) was stripped and reassessed with anti-GST (1:5000) followed by HRP-protein A (1:7500) (B).
Figure 9.

rhRPA
PNE (100 ug)
GST (200 ug)
GST-pre-T alpha (200 ug)

GST-ATM Ext-C
GST-DNA PK Ext-C
Figure 9.

- GST-DNA-PK-EXC
- GST-ATM-EX-C
- GST-pre-T-alpha (200 ug)
- GST (200 ug)
- PNE (100 ug)
DISCUSSION

The focus of this thesis was to identify proteins that interact with the extreme C-terminal domains (ext-C) of ATM and DNA-PK, two members of a conserved family of proteins that are involved in cell cycle progression, DNA damage checkpoints, meiotic recombination, and DNA repair. The rationale to study the carboxy-terminal domains of DNA-PK and ATM was supported by identification of an ochre mutation that truncates SCID DNA-PK 83 residues upstream of the normal C-terminus and by reports that mutations affecting the ext-C region of ATM result in a disease with phenotypic similarities to SCID mice (Blunt et al., 1996; Danska et al., 1996; McConville et al., 1996; Telatar et al., 1996; Wright et al., 1996; Vorechovsky et al., 1997; Stilgenbauer et al., 1997; Gilad et al., 1998). The results of the experiments described in this thesis suggest that replication protein A (RPA) binds to the ext-C domains of ATM and DNA-PK. Similar to the PI-3 related kinases, RPA has been determined to function in multiple processes that serve to maintain the integrity of the genome.

When the sequences of PI-3 related kinase homologues in yeast, flies, and mammals were aligned, a highly conserved region at the extreme carboxy terminus of the proteins was defined (Keith and Schreiber, 1995). Within this domain, there is 19.4% amino acid identity among all members of the PI-3 related kinases and 45% identity between ATM and DNA-PK. No previously characterized protein motifs were identified within this ext-C domain (http://www.expasy.ch). The conservation of the amino acids within the ext-C region of the PI-3 related kinase homologues suggests an important role for these residues, which could involve protein-protein interactions and/or protein stability.
Mutations in both ATM and DNA-PK have been identified that assist in further defining the essential residues within the ext-C domains. In one AT homozygote, deletion of 10 amino acids from the C-terminus of both alleles of ATM was sufficient to cause the disease (Gilad et al., 1998). Within the truncated sequence, there is a glycine-tryptophan pair (GW) that is conserved in every homologue of the PI-3 related kinases (Figure 1). The C-terminal region of DNA-PK in irs-20, a DNA-PK-defective CHO cell line with a mild γ-sensitivity phenotype, has been sequenced and determined to have a point mutation at 4124 that results in the substitution of a lysine residue for glutamic acid (Priestley et al, 1998). The glutamic acid residue is the fourth residue from the C-terminus and is not conserved in any of the other PI-3 related kinases, which could account for the mild radiation sensitivity phenotype of irs-20 cells. Further speculation regarding the impact of this substitution on DNA-PK activity will require verification of the N-terminal sequence of the gene in irs-20 cells.

In my studies, the 31 C-terminal residues of both murine DNA-PK and human ATM were used to perform a blast search of GenBank (July 1998, http://www.ncbi.nlm.nih.gov/entrez using default settings). In addition to recognizing the known PI-3 related kinases, a C. elegans sequence (accession U97016) with 40% and 32% identity to ATM and DNA-PK, respectively, was identified. The C. elegans cDNA encodes a ~300 kD protein that contains both the DRXXXN and DFG PI-3 catalytic motifs and the conserved PI-3 related kinase ext-C domain. Because both the sequence and C-terminal location of these motifs are conserved within this C. elegans sequence, this cDNA likely encodes a novel PI-3 related kinase homologue. Elucidation of the
functional significance of this protein in *C. elegans* will contribute to the global understanding of the impact of the PI-3 related kinase homologues within the cell.

A 434 kD mammalian protein, TRRAP (transformation/transcription domain-associated protein), was recently identified as a cofactor for the c-Myc oncoprotein (McMahon et al., 1998). C-myc is a sequence-specific transcription factor with functions that include the ability to transform cells, induce apoptosis, control the cell cycle, block differentiation, and regulate transcription of certain genes (reviewed in Facchini and Penn, 1998). TRRAP is highly conserved in eukaryotic organisms and its C-terminal sequence exhibits significant homology to the kinase domain of the PI-3 related kinases (McMahon et al., 1998). Interestingly, this homology includes conservation of 4 of the 6 identical residues within the PI-3 kinase related ext-C domain but excludes all of the residues that were determined by site-specific mutagenesis to be essential for PI-3 catalytic activity (Dhand et al., 1994). Within the ext-C domain, there is 32% identity between human TRRAP and either human ATM or murine DNA-PK (Figure 10). The presence of the ext-C domain in a protein that lacks the PI-3 kinase catalytic motifs supports the hypothesis that the function of the ext-C residues is distinct from the kinase domain. The conservation of the ext-C domain both within and beyond of the PI-3 related kinase homologues clearly suggests that these residues participate in an essential function, which may involve the mediation of protein-protein interactions. The ext-C domain provides an effective means of identifying novel PI-3 related kinase homologues, and may aid in the elucidation of biological function(s) of the proteins that contain this motif.
Figure 10. Alignment of the extreme C-terminal domains of human TRRAP, ATM and murine DNA-PK. Four of the 6 identical PI-3 related kinase ext-C residues (arrows) are also present in the ext-C domain of TRRAP. Within the ext-C domains, there is 32% identity between TRRAP and ATM as well as TRRAP and DNA-PK (italicized residues).

The objective of the research described in this thesis was to identify protein(s) that interact with the ext-C domains of ATM and DNA-PK. The results of the multiple \textit{in vitro} experiments suggest that RPA binds to these ext-C domains. RPA has been directly implicated in diverse cellular functions including DNA replication, repair, and meiotic recombination. Regulation of these activities is achieved through RPA p70 (RPA-1) mediated binding to DNA, changes in RPA p32 (RPA-2) phosphorylation status, and interactions with other proteins (Figure 11). The central region of RPA p70 contains a DNA binding domain (Gomes and Wold, 1995; Kim et al., 1996; Lin et al., 1996; Gomes and Wold, 1996; Gomes et al., 1996b). RPA binds tightly to ssDNA, sites of transcriptional regulation on dsDNA, and to single and double strand DNA lesions (reviewed in Wold, 1997). RPA binding to DNA induces a conformational change that enhances RPA p32 as a substrate for phosphorylation (Blackwell et al., 1996; Gomes et al., 1996). Phosphorylation of RPA occurs within a small N-terminal region on RPA-2 (Lee et al., 1995; Hendricksen et al., 1996), however the biological consequence of RPA phosphorylation has yet to be identified. DNA binding by RPA is essential for replication initiation and elongation and is not impaired by mutations that delete the N-
terminal phosphorylation domain on RPA-2 (reviewed in Wold, 1997). Another RPA function that is not affected by deletion of the phosphorylation sites is its role in the nucleotide excision repair (NER) pathway (Pan et al., 1995). RPA functions at multiple stages in NER repair by binding to the damaged region, recruiting and interacting with other repair proteins, and by stabilizing the single-stranded gap during synthesis of the corrected strand (reviewed in Wold, 1997).

**Figure 11. Functional domains on RPA.** RPA is a heterotrimer with subunits of approximately 70, 32, and 14 kD. The DNA binding domain of RPA is located in the central portion of the largest subunit (RPA-1) (checkered pattern) (Gomes and Wold, 1995; Kim et al., 1996; Lin et al., 1996; Gomes and Wold, 1996; Gomes et al., 1998). The N-terminal region of RPA-1 is not required for DNA binding, formation of the heterotrimer, or DNA replication, however may be involved in protein interactions (Gomes and Wold, 1995; Kim et al., 1996; Lin et al., 1996; Gomes and Wold, 1996). The C-terminal region of RPA-1 is both necessary and sufficient for RPA complex formation. In this illustration, the regions that have been determined to be necessary for heterotrimerization are depicted with polka dots. Phosphorylation of RPA occurs within the N-terminal domain of p32 (RPA-2) (diagonal lines) (Lee et al., 1995; Hendrickson et al., 1996). The C-terminal region of RPA-2 is required for complex formation and RPA activity (Lee et al., 1995; Hendrickson et al., 1996). No known function(s) has been
mapped to RPA-3, however this subunit is essential for formation of the heterotrimer (Lin et al., 1996).

RPA is phosphorylated in a cell cycle dependent manner and in response to both UV- and IR- induced DNA lesions (reviewed in Wold, 1997). Although multiple kinases phosphorylate RPA \textit{in vitro}, some evidence suggests that RPA phosphorylation \textit{in vivo} involves DNA-PK activity (Brush et al., 1994; Boubnov and Weaver, 1995). Interestingly, IR-induced phosphorylation of RPA is delayed in AT cells (Liu et al., 1993) and is incomplete in SCID cells (Boubnov and Weaver, 1995). Since RPA mutants that lack phosphorylation sites on RPA-2 support both replication and NER, the biological impact of phosphorylation must involve other functions of the RPA complex.

Another potential phosphorylation-dependent RPA function may be association with p53 \textit{in vitro} and \textit{in vivo} (Dutta et al., 1993). It has been proposed that RPA may sequester p53 until DNA damage-induced phosphorylation of RPA results disassociation of the two proteins thereby allowing p53 to interact with downstream targets (Abramova et al., 1997). UV-induced phosphorylation of RPA p32 inhibits the RPA-p53 association which increases the ability of damaged cells to perform DNA repair (Abramova et al., 1997; Zernik-Kobak et al., 1997). However, UV-induced upregulation of p53 and phosphorylation of RPA is normal in AT and SCID cells, which suggests that another kinase may be responsible for RPA phosphorylation and the p53 response to UV damage (reviewed in Lavin and Shiloh, 1997). Interestingly, a delayed activation of ATM kinase activity has been detected following UV-irradiation, which suggests that ATM may be involved in the downstream processing of UV-induced lesions and/or repair intermediates (Canman et al., 1998). Since the repair of UV lesions in AT cells appears to be grossly
normal, this argues that any involvement by ATM in this repair process is likely to be minimal and/or redundant. AT cells are deficient in the IR-induced upregulation of p53, and undergo DNA replication in the presence of DNA damage (Kastan et al., 1992; Khanna and Lavin, 1993; Lu and Lane, 1993; Canman et al., 1994; Khanna et al., 1995).

One possible explanation for the p53-dependent S-phase checkpoint defect in AT cells is that phosphorylation of RPA may be required for inhibition of DNA replication. However, radiation-induced phosphorylation of RPA is not associated with the S-phase checkpoint (Morgan and Kastan, 1997) which is consistent with the known integrity of the p53 checkpoint in SCID cells (Fried et al., 1996; Guidos et al., 1996; Huang et al., 1996; Nacht et al., 1996; Lee et al., 1997). Collectively, the data suggest that while RPA and p53 appear to be integral to multiple DNA damage response pathways, ATM and DNA-PK are primarily involved in the response to IR-induced lesions. Thus, other kinases likely phosphorylate RPA and activate the p53-dependent DNA damage checkpoint in response to UV irradiation.

One function of RPA that may have implications for the AT and SCID phenotypes is its involvement in DNA repair. RPA has been demonstrated to participate at multiple steps in the NER pathway, which repairs DNA lesions generated by UV light, cis-diamminedichloroplatinum-II (cisplatin), and methyl methanesulphonate (MMS) (reviewed in Wold, 1997). Although DNA-PK serves an important role in the repair of DSB (reviewed in Jeggo, 1997), there are conflicting reports concerning the role of DNA-PK in the repair of UV-induced DNA lesions (Jeggo et al., 1983; Whitmore et al., 1989; Biederman et al., 1991; Hendrickson et al., 1991). To evaluate the possible role of DNA-PK in these repair processes, two different DNA-PK-deficient rodent cell lines,
SCID and V-3, and a Ku80-deficient hamster cell line, xrs6, were assessed for sensitivity to UV-induced lesions using multiple in vivo and in vitro assays (Muller et al., 1998). Following exposure to UV-C, a modest 2-2.5-fold decrease in survival of DNA-PK-defective cells versus their wild type parental cell types was observed. When the experiments were performed in the presence of wortmannin, a PI-3-kinase and DNA-PK inhibitor, survival of wild type cells decreased 2-3-fold whereas survival of the DNA-PK- and Ku80-defective cells remained unchanged. In addition, DNA-PK-defective cell lines were deficient in NER-coupled DNA synthesis in vivo. Although the physical presence of DNA-PK in the NER multiprotein complex was not observed, these data suggest that DNA-PK activity contributes to the resolution of DNA lesions by NER.

In eukaryotic cells, DSBs may be repaired by homologous recombination or by direct joining broken dsDNA ends (non-homologous end joining). The latter is the predominant DSB repair mechanism in mammalian cells (reviewed in Ivanov and Haber, 1997). In yeast, proteins in the Rad52 epistasis group orchestrate homologous recombination and mutants are hypersensitive to ionizing and high dose UV radiation. A genetic screen to identify S. cerevisiae mutants in homologous recombination identified a mutant RPA allele, rfa1-44 (Firmenich et al., 1995). Rfa1-44 has a point mutation in the DNA binding domain of RPA-1. The inability of rfa1-44 to perform DSB by homologous recombination was complemented by overexpression of Rad52, which suggests that RPA is upstream of Rad52 in the homologous repair pathway (Firmenich et al., 1995). Furthermore, a physical interaction between RPA-2 and Rad52 has been demonstrated in vivo and in vitro (Park et al., 1996), and a model proposed in which RPA and Rad52 coordinate recruitment of DNA repair proteins at the site of DSBs (Firmenich...
et al., 1995). RPA may also participate in the resolution of DSBs by stabilizing DNA strands during the repair process, a function of RPA that has been well-established during the gap-filling process in NER (Mu et al., 1995; Aboussekhra et al., 1995; Guzder et al., 1995; Shivji et al., 1995). In addition to the well-established role of RPA in NER, these data suggest that RPA also participates in DSB repair.

One of the most well defined non-homologous DSB repair reactions is the rearrangement of multiple variable (V), diversity (D), and joining (J) coding segments at immunoglobulin and T-cell receptor loci. During V(D)J recombination, a cleavage reaction generates free dsDNA ends that are eventually resolved into signal and coding joins by two end-joining reactions (reviewed in Lewis, 1994). The signal ends are joined precisely through a blunt ligation reaction, whereas non-templated nucleotide additions and deletions occur at the coding ends before they are resolved through the general non-homologous DSB repair pathway (reviewed in Smider and Chu, 1997). The relationship between the end-joining reactions in V(D)J recombination and general DSB repair was first recognized when cells from SCID mice were shown to be defective in both reactions (Fulop et al., 1990; Biedermann et al., 1991; Hendrickson et al., 1991). In addition to the severe defect in coding joint formation in DNA-PK-deficient XRCC7 cells, the IR-hypersensitive hamster cell lines XRCC4 and XRCC5 are defective in both signal and coding end resolution (reviewed in Smider and Chu, 1997). Ku80, the protein mutated in XRCC5, and XRCC4 are therefore necessary for both the blunt ligation of signal ends and the non-homologous end-joining process. Ku86/-/- and Ku70/-/- mice are hypersensitive to ionizing radiation and profoundly immunodeficient due to an inability to resolve either signal or coding V(D)J ends, which provides further evidence that the
Ku heterodimer is involved in DSB repair (Nussenzweig et al., 1996; Zhu et al., 1996; Gu et al, 1997; Ouyang et al., 1997). Although AT patients are profoundly hypersensitive to ionizing radiation and exhibit immunodeficiencies (reviewed in Shiloh, 1997), there is no evidence for a gross DNA repair defect in AT (Vincent et al., 1975; Taylor et al, 1976; Fornace et al., 1980; Lavin et al, 1981) and fibroblasts from multiple AT cell lines recombine extrachromosomal V(D)J substrates with normal frequency and precision (Hsieh et al., 1993). In summary, using V(D)J recombination as a model for studying non-homologous DSB repair, DNA-PK, Ku, and XRCC4 have been identified as protein components in this repair pathway.

Recombination between homologous chromosomes occurs during meiosis, and is facilitated by the presence of large nucleoprotein complexes along the synaptonemal complex (Plug et al., 1997). Immunodetection assays have revealed that during normal gametogenesis, RPA and ATM co-localize on meiotic chromosomes at sites of homologous recombination and at DSB sites (Plug et al., 1997). In atm-/- spermatocytes, RPA is still detected at these sites (Plug et al., 1997). Since gametogenesis in atm-/- mice is disrupted due to chromosomal fragmentation and AT patients and atm-/- mice exhibit gonadal dysgenesis, ATM co-localization at the sites of recombination with RPA may be necessary for successful meiosis. The physical and functional associations between RPA and Rad52, and the detection of RPA at sites of homologous recombination on meiotic chromosomes support a role for RPA in the repair of DSBs.

In S. cerevisiae, some data suggest that RPA may cooperate with non-homologous end-joining proteins Ku70, Mre11, and Rad50 by monitoring the extent of
ssDNA degradation at DSBs (Lee et al., 1998). The Rad50/Mre11/Xrs2 complex was identified in *S. cerevisiae* homologous repair-deficient Rad52 mutants and functions in the non-homologous DSB repair pathway in yeast (reviewed in Petrini et al., 1997). In human cells, Rad50/Mre11 from discrete nuclear foci that are induced by IR- and not UV-radiation, which suggests that Rad50/Mre11 participates in DSB repair (Nelms et al., 1998). One hypothesis proposes that the 3’ to 5’ exonuclease activity of Mre11/Rad50 (Paul and Gellert, 1998) facilitates the repair of DSB by exposing short homologies near the break points, a process that may be inhibited by binding of Ku proteins to the DSB (Lee et al., 1998). Coordination of repair activities with the G2-M checkpoint could result from RPA binding to the ssDNA generated by 3’ to 5’ degradation. This hypothesis is supported by the observations that RPA is phosphorylated in the presence of DNA damage and ssDNA (Liu and Weaver, 1993; Carty et al., 1994; Boubnov and Weaver, 1995) and that in *rad52* yeast cells, DSBs activate a lengthy G2-M checkpoint that is suppressed by a mutation in RPA (Lee et al., 1998). The participation of RPA in NER, homologous recombination, and potentially during the non-homologous end-joining process suggests that RPA may function ubiquitously in DNA repair by stabilizing damaged DNA and/or recruiting additional repair proteins.

Both SCID mice and AT patients display immunodeficiency. The lack of mature B or T cells in SCID mice is due to inefficient coding end resolution during V(D)J recombination, which results in the absence of immunoglobulin or T-cell receptor complexes and the developmental arrest of B and T cell progenitors (von Boehmer, 1994). This defect in SCID mice is caused by an 83 amino acid deletion from the C-terminus of DNA-PK, a protein that has been identified as a component of mammalian
non-homologous DSB repair (Blunt et al., 1996; Danska et al., 1996). p53 is not detectable in normal lymphocyte progenitors undergoing V(D)J recombination, which suggests that the G₁ cell cycle arrest that occurs in these cells is p53-independent (Guidos et al., 1996). In contrast, the accumulation of V(D)J-specific DSB in SCID lymphocytes activates a p53-mediated DNA damage checkpoint (Guidos et al., 1996; Nacht et al., 1996). All p53-/-SCID mice develop disseminated pro-B or immature T cell lymphoma/leukemia by 7-12 weeks of age, which strongly implies that the p53-dependent DNA damage checkpoint minimizes the oncogenic potential of these DSB (Guidos et al., 1996; Nacht et al., 1996). In contrast to SCID mice, the underlying dysfunction that results in the immune defects observed in AT patients has not been thoroughly elucidated. In atm-/- mice, the total number of mature thymocytes is significantly reduced versus wild type animals despite having similar amounts of B and T-cell progenitors. These data suggest that ATM may function in the maturation and/or expansion of lymphocytes (Barlow et al., 1996; Elson et al., 1996; Xu et al., 1996). There is also a reduction in the total number of mature B cells in atm-/- mice. However, B cells isolated from atm-/- mice appear to function normally whereas T-cell-dependent immune responses are defective (Xu et al., 1996). In summary, the lack of mature lymphocytes in SCID mice results from a developmental arrest in B and T cell precursors due to a defect in a key enzyme in the DSB repair process, DNA-PK. In AT patients and atm-/- mice, the reduction in the absolute number of lymphocytes and in the function of T-cell-dependent immunity suggests that one role of ATM may be to monitor the precision and productivity of DNA rearrangements of V(D)J gene segments through the
p53 checkpoint, therefore functioning as a component of the DNA damage checkpoint mechanism.

The mutations in both SCID mice and patients with AT predispose to developing lymphoma and acute lymphoblastic leukemia. An estimated 10% of all AT patients develop a malignancy and the predisposition to lymphocytic leukemia and lymphoma is 70-fold and 250-fold, respectively, excess over the non-AT incidence of these types of cancer (Morrell et al., 1986). In addition, although the proportion of T- to B- cell lymphoma in AT patients is not fully defined, there appears to be a predominance of T-cell tumors whereas B lineage tumors are more common in the general population (Taylor et al., 1996). Chromosomal translocations are a major feature in AT lymphoma and the majority involves antigen receptor loci, specifically T-cell receptor (TCR) genes (Taylor et al., 1996). Although translocations of these types do occur in non-AT individuals, the frequency and proportion of specific translocations differ between AT and non-AT patients (Taylor et al., 1996). The incidence of other cancers including carcinomas of the stomach, liver, ovary, and uterus are also increased in AT patients (Morrell et al., 1986; Filipovich et al., 1992), which suggests that ATM contributes ubiquitously to DNA damage surveillance and/or checkpoint control in all cell types.

The loss of the ext-C domain from DNA-PK predisposes SCID lymphocytes to transformation. T-cell derived lymphoma resulted in 100% of irradiated SCID mice (Danska et al., 1994) and when the SCID mutation in DNA-PK was transferred onto the diabetes-susceptible non-obese (NOD) background, 67% of the mice developed spontaneous thymic lymphoma by 40 weeks of age (Prochazka et al., 1992). Despite participating in all cell types in DNA surveillance and repair, the preponderance of
malignancies in AT patients and SCID mice involve cells originating from the lymphoid lineage, which suggests some lymphoid-specific increase in susceptibility to genetic alterations. In contrast to SCID mice, thymic lymphoma is not observed in irradiated RAG-2-/-/SCID double mutant mice, which cannot introduce DSBs during lymphogenesis (Williams, Vesprini, Danska and Guidos, unpublished data). These data support a model in which the accumulation of recombinogenic dsDNA ends in SCID lymphocyte precursors cause chromosomal instability, which results in translocations that could lead to de-regulated expression of proto-oncogenes. A similar mechanism has also been proposed for AT-associated leukemia and lymphoma, in which cells the accumulation of DSB does not appear to result from a gross DNA repair defect but rather an inability to properly detect and respond to DNA damage.

Mutations that result in the loss of C-terminal residues from two PI-3 related protein kinases, ATM and DNA-PK, result in diseases that have some similar features including immunodeficiency, cancer predisposition, and radiation-sensitivity. Despite firmly establishing the role of PI-3 related kinases in the maintenance of genomic integrity, relatively little is understood about the mechanisms by which these proteins function. In both SCID and AT cells, there is a significant reduction in the abundance of DNA-PK and ATM, respectively, which suggests that mutations in these proteins may decrease protein stability. It is possible that ATM and DNA-PK stability is mediated through interactions with other proteins, potentially within the highly conserved PI-3 related kinase ext-C domain. Targeted disruption of the conserved ext-C residues in different PI-3 related kinase homologues might help to reveal the significance of this domain. An in vitro physical interaction between RPA and the ATM and DNA-PK ext-C
domains was described in this thesis. Possible functional consequences of these interactions are suggested by previous observations including the participation of RPA in both NER and DSB repair, that damage-induced phosphorylation of RPA is defective in SCID and AT cells, and that in *S. cerevisiae* MEC1 is necessary for RPA phosphorylation (reviewed in Wold, 1997). In addition, it has been recently demonstrated that ATM co-immunoprecipitates with phosphorylated RPA from irradiated HeLa cells (Gately et al., 1998). These data are suggestive that a cooperation exists between PI-3 related kinases and RPA homologues in the maintenance of genomic integrity, however further investigations are required to elucidate the roles of the highly conserved ext-C domains and of RPA within the cell.
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