Substrate Specificities and Identification of Putative Substrates of ATM Kinase Family Members*

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Ataxia telangiectasia mutated (ATM) phosphorylates p53 protein in response to ionizing radiation, but the complex phenotype of AT cells suggests that it must have other cellular substrates as well. To identify substrates for ATM and the related kinases ATR and DNA-PK, we optimized in vitro kinase assays and developed a rapid peptide screening method to determine general phosphorylation consensus sequences. ATM and ATR require Mn2+, but not DNA ends or Ku proteins, for optimal in vitro activity while DNA-PKcs requires Mg2+, DNA ends, and Ku proteins. From p53 peptide mutagenesis analysis, we found that the sequence S/TQ is a minimal essential requirement for all three kinases. In addition, hydrophobic amino acids and negatively charged amino acids immediately NH2-terminal to serine or threonine are positive determinants and positively charged amino acids in the region are negative determinants for substrate phosphorylation. We determined a general phosphorylation consensus sequence for ATM and identified putative in vitro targets by using glutathione S-transferase peptides as substrates. Putative ATM in vitro targets include p95/nibrin, Mre11, Brcal, Rad17, PTS, WRN, and ATM (S440) itself. Brca2, phosphatidylinositol 3-kinase, and DNA-5B peptides were phosphorylated specifically by ATR, and DNA Ligase IV is a specific in vitro substrate of DNA-PK.

The gene that is mutated in AT, ATM (ataxia telangiectasia, mutated), encodes a 370-kDa protein with a carboxyl-terminal sequence homologous to the catalytic domain of phosphatidylinositol 3-kinases (7). Although ATM is predominantly located in the nucleus (8–11), a fraction of ATM protein also appears in cytoplasmic vesicles in certain cell types (10, 12) where it associates with β-adaptin (13). A family of gene products with similar sizes and sequences has been identified, including the yeast proteins rad3p, Mec1p, Tel1p, Tor1p, Tor2, and the mammalian proteins mTor/FRAP, DNA-PKcs (DNA-dependent protein kinase), ATR (ataxia telangiectasia and rad3-related kinase) and TRAP (7, 14). In mammalian cells, ATR and DNA-PK also appear to be involved in DNA-damage response pathways (15–17). For example, several lines of evidence suggest primary roles of DNA-PK are in DNA-repair and V(D)J recombination (17). ATR is considered the mammalian counterpart of yeast rad3 and Mec1p, two proteins involved in DNA damage responses (18).

We had previously demonstrated that ATM deficiency results in suboptimal increases in p53 protein levels after IR and that ATM is required for optimal IR-induced (but not UV-induced) phosphorylation of serine 15 of p53 protein (4, 19, 20). The ability to express the full-length ATM cDNA in mammalian cells and construction of a kinase-dead ATM mutant led to the demonstration that ATM is a bona fide protein kinase that is capable of phosphorylating serine 15 of p53 protein (20). Interestingly, IR and the DNA strand-breaking agent, necrozinostatin, but not UV irradiation, enhances the specific activity of the ATM kinase (20, 21). These results are consistent with a model in which ATM acts as a kinase upstream of p53 in the response of mammalian cells to DNA strand breakage. However, p53 dysfunction cannot account for the majority of the phenotypic abnormalities in AT patients and AT cells (2). Indeed, radiation sensitivity and the defects in S phase and G2/M checkpoint control in response to IR in A-T appear to be independent of the p53 regulation by ATM (2, 22–24).

Given the pleiotropic features of AT and the apparent cell-type specificity in subcellular localization of ATM, we were interested in identifying additional physiologic substrates for the ATM kinase. In order to better understand the kinase, we first examined cofactor requirements for optimal ATM kinase activity and compared these requirements to those of the related mammalian protein kinases, ATR and DNA-PK. Subsequently, we compared and contrasted the in vitro substrate sequence specificity of these three kinases using GST-linked peptides and mutagenesis analysis. A general consensus motif for ATM was thus developed and several new putative substrates were identified from protein data base searches. Putative targets identified included Brcal, Rad17, PTS, and p95 (nibrin). Thus, characterization of the ATM kinase and elucidation of in vitro targets will lead to further insights into ATM function and AT biology.

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† The abbreviations used are: AT, ataxia telangiectasia; IR, ionizing irradiation; GST, glutathione S-transferase; PTS, putative tumor suppressor; PKcs, PK catalytic subunit.

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**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. GM0536 lymphoblast cells were grown in RPMI medium containing 15% fetal calf serum. Transfections were performed by the calcium phosphate method.

**Plasmids and GST Fusion Protein Production**—For GST fusion peptide expression vectors, complementary oligonucleotides encoding desired peptides (14 amino acids) were cloned into the BamHI/SmaI site of pGEX-2T (Amersham Pharmacia Biotech). The constructs were confirmed by restriction enzyme digests and DNA sequencing. The GST peptides were expressed in BL21(DE3). After isopropyl-β-D-thiogalactoside induction for 3 h (1 mM final concentration), GST peptide fusion proteins was recovered by binding to glutathione-Sepharose beads (Sigma) and eluted with 20 mM glutathione in 50 mM Tris/HCl, pH 8.0. Eluted protein pools were dialyzed against 20 mM Tris/HCl, pH 8.0, and concentrated by Centricon-10 (Amicon Co.).

**Western Blotting, Immunoprecipitation, and in Vitro Kinase Assays**—In vitro kinase assays for ATM and ATR were performed as described earlier (20). Briefly, cell extracts were prepared from 293T cells which had been transfected with 10 ng of either ATM or ATR by resuspending cells in modified TNG buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Tween 20, 0.3% Nonidet P-40, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and 1 μM protease inhibitor mixture from Roche Molecular Biochemicals). Cleared supernatants were immunoprecipitated with anti-Flag M2 antibody (Sigma) and protein A/G-agarose; the beads were washed with TGN buffer followed by TNG buffer plus 0.5 M LiCl, and two washes with kinase buffer (20 mM HEPES, pH 7.5, 50 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol, and 10 mM MgCl2). Finally, the immunoprecipitant was resuspended in 50 μl of kinase buffer containing 10 μCi of [γ-32P]ATP and 1 μg of GST fusion substrate. The kinase reaction was conducted at 30 °C for 20 min and stopped by the addition of SDS-polyacrylamide gel electrophoresis loading buffer. Proteins were separated on SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Immunoprecipitated Flag-ATM or Flag-ATR was confirmed by Western blotting with α-Flag M2 monoclonal antibody. Radiolabeled proteins were visualized and quantitated on PhosphorImager (Molecular Dynamics). In some reactions, either supercoiled (pBluescript II KS, Stratagene) or linearized DNA (pBluescript KS cut with EcoRI) was added. For endogenous ATM kinase reactions, endogenous ATM was immunoprecipitated with ATM monoclonal antibody (D16.11) in M buffer (phosphate-buffered saline, 10% glycerol, 0.2% Tween 20, 0.3% Nonidet P-40, 1 mM sodium fluoride, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1 μM protease inhibitor mixture). After washing with M buffer and kinase buffer, in vitro kinase reactions were carried out according to procedures described above. The purified DNA-PKcs and DNPKc were generous gifts from Dr. S. Yoo and Dr. W. S. Dynan, Medical College of Georgia. The DNA-PKc in vitro kinase reaction was performed as described previously (25).

**RESULTS**

**Co-factor Requirements for ATM, ATR, and DNA-PK**—In order to elucidate potentially distinctive biochemical properties of the ATM, ATR, and DNA-PK kinases, we examined the co-factor requirements for their optimal activities. It had been previously reported that ATM and ATR required exogenous Mn2+ for optimal in vitro kinase activity (20, 21). In contrast, DNA-PK has been reported to require Mg2+ as well as the presence of DNA ends and the DNA-binding proteins Ku70 and Ku80 for optimal activity (25). However, since ATM specific activity increases in cells following exposure to ionizing radiation (20) or introduction of double strand DNA breaks (21), it was possible that ATM or ATR kinase activities are enhanced by the presence of DNA ends and that this dependence was absent in vitro assays. For example, it was conceivable that the use of Mn2+ in these assays decreased the dependence of ATM and ATR on DNA ends. Conversely, it was conceivable that the requirement of the use of DNA ends and/or Ku proteins in DNA-PK activity assays abrogated a need for Mn2+ addition in these in vitro assays.

In order to test these possible scenarios, in vitro kinase assays with all three enzymes were performed using epoetin-tagged ATM and ATR immunoprecipitated from transfected cells and biochemically purified DNA-PK as the kinase sources and GST-p53 (1–101) recombinant protein as a substrate. Kinase-inactive forms of ATM and ATR were used as controls in the assay to ensure that these activities were intrinsic. ATM and ATR kinases were dependent on the addition of exogenous Mn2+ (Fig. 1A), while DNA-PK activity was not altered by the addition of Mn2+ (Fig. 1D). The addition of supercoiled or linearized DNA to these reactions did not alter the activity of ATM or ATR, but consistent with previous observations (25), the double strand DNA ends provided by linearized DNA significantly enhanced the activity of DNA-PK (Fig. 1B). The addition of either of these exogenous DNA sources did not relieve the dependence of ATM and ATR on exogenous Mn2+.

**Fig. 1. Co-factor requirements for ATM family members.** A, ATM and ATR require Mn2+ for optimal activity. Wild-type (wt) or catalytically inactive (kd) ATM or ATR were immunoprecipitated with anti-Flag antibody and were used in an in vitro kinase assay with 10 μg of [γ-32P]ATP and 1 μg of GST fusion substrate. The kinase reaction was conducted at 30 °C for 20 min and stopped by the addition of SDS-polyacrylamide gel electrophoresis loading buffer. Protein samples were separated on SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Immunoprecipitated Flag-ATM or Flag-ATR was confirmed by Western blotting with α-Flag M2 monoclonal antibody. Radiolabeled proteins were visualized and quantitated on PhosphorImager (Molecular Dynamics). In some reactions, either supercoiled (pBluescript II KS, Stratagene) or linearized DNA (pBluescript KS cut with EcoRI) was added. For endogenous ATM kinase reactions, endogenous ATM was immunoprecipitated with ATM monoclonal antibody (D16.11) in M buffer (phosphate-buffered saline, 10% glycerol, 0.2% Tween 20, 0.3% Nonidet P-40, 1 mM sodium fluoride, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1 μM protease inhibitor mixture). After washing with M buffer and kinase buffer, in vitro kinase reactions were carried out according to procedures described above. The purified DNA-PKcs and DNPKc were generous gifts from Dr. S. Yoo and Dr. W. S. Dynan, Medical College of Georgia. The DNA-PKc in vitro kinase reaction was performed as described previously (25).
It remained theoretically possible that these overexpressed ATM and ATR proteins had different cofactor requirements than would be seen for the normal endogenous proteins because of changes in molar ratios of ATM/ATR and cofactors. In addition, it was possible that immunoprecipitated ATM/ATR could be already contaminated with DNA or might have lost cofactors such as Ku proteins during immunoprecipitation. Therefore we re-examined both the Mn$^{2+}$ dependence and DNA-end dependence using endogenous ATM. In order to circumvent the concerns about potential loss of cofactors or prior DNA contamination, we also immunoprecipitated the endogenous ATM under very mild conditions without stringent washing (0.5 M LiCl) and assessed the kinase activity either in the presence of added linearized DNA or with the addition of ethidium bromide, a DNA intercalator which is known to interfere with protein-DNA interaction (26, 27). The activity of endogenous ATM was still dependent on the addition of exogenous Mn$^{2+}$ and neither the exogenous addition of DNA ends nor the presence of ethidium bromide to inactivate potential contamination from endogenous DNA had any effect on its activity (Fig. 1C).

DNA-PK activity is enhanced by the presence of DNA ends via Ku-dependent association with the DNA and Mn$^{2+}$ is not required for its activity in in vitro kinase assay (25). To further clarify potential distinctions between ATM/ATR and DNA-PK and to rule out the possibility that Mn$^{2+}$ might replace the requirement of DNA-PK activity for DNA ends or for the Ku70/80 cofactors, the in vitro activities of purified DNA-PKcs or DNA-PK holoenzyme containing Ku components were re-examined. Although DNA-PKcs alone has a basal level of activity toward GST-p53 (1–101), this activity was not enhanced by the addition of either Mn$^{2+}$ or DNA ends (Fig. 1D). In contrast, the activity of the DNA-PK holoenzyme was remarkably enhanced by DNA ends (approximately 10 times of basal level) and the addition of Mn$^{2+}$ had no effect on the DNA-PK activity either in the presence or absence of exogenous DNA. Thus, ATM and ATR kinases are distinguishable in vitro from DNA-PK in their lack of dependence on DNA ends and their requirement for Mn$^{2+}$ for optimal activity.

Consensus Sequence Elucidation via GST-p53 Peptide Mutagenesis Analysis—Studies on other kinases have suggested that the nature and sequence of the amino acids surrounding the target phosphorylation site can play a critical role in modulating recognition of a substrate by a kinase (28). We wished to clarify amino acids that affect the ability of ATM to phosphorylate a substrate and to then use this information to try to identify other physiologic substrates. Short peptide sequences containing target amino acids have commonly been used as in vitro substrates for kinases. We had previously found that linkage of a short peptide sequence to a GST tag provided a better in vitro substrate for ATM than a short peptide alone (data not shown). This approach also provided an easy way to generate and purify the peptide of interest. Thus, beginning with the known in vivo and in vitro target, serine 15 in p53, we attempted to determine which amino acids surrounding Ser-15 in p53 were important modulators of phosphorylation by ATM. Wild type and mutated GST-conjugated p53 peptides containing 14 amino acids surrounding Ser-15 were generated and used as in vitro kinase substrates for ATM, ATR, and DNA-PK.

Changes in some of the amino acids surrounding serine 15 had a dramatic effect on the ability of these kinases to phosphorylate the GST-p53 peptides, while others had little effect (Fig. 2, A and B). For example, peptides in which glutamine at position 16 was replaced with alanine, glycine, or asparagine were very poor substrates for all three of these kinases. Thus, as has previously been reported for DNA-PK (29), this result suggests that a glutamine adjacent to the target serine is also critical for substrate recognition by ATM and ATR. When the target serine at position 15 was mutated into threonine, the amount of phosphorylation was reduced to 13, 53, and 36% compared with the wild-type sequence for ATM, ATR, and DNA-PK, respectively. Thus, all three kinases appear to have a preference for phosphorylating serine over threonine. All three kinases poorly phosphorylated peptides with the substitutions Asp-12, Arg-12, Lys-12, Lys-13, Lys-14, and Arg-14, while the Glu-12, Ala-12, Ala-13, Ala-14, Gin-14, and Asp-14 peptides were efficiently phosphorylated. These results indicate that hydrophobic amino acids at positions N−3 and N−1 and negatively charged amino acids at N−2 are positive determinants for substrate recognition by these kinases. In contrast, positively charged residues (Lys-11, Lys-12, Arg-12, Lys-13, Lys-14, Arg-14, Lys-17, and Lys-19) surrounding the SQ appear to have a significant negative influence on substrate phosphorylation.

Although many of the amino acid substitutions had similar effects on all three of these kinases, some of the target manipulations resulted in differential effects. Peptides with Gln-10, Lys-17, Leu-17 substitutions remained relatively good substrates for ATM and ATR, but were very poor substrates for DNA-PK (Fig. 2). These observations suggest that the positions N−3 and N−2 are more important for substrate recognition by DNA-PK than for ATM and ATR. Most of the amino acid substitutions had similar effects on ATM and ATR, although the substitutions Ala-12, Ala-13, Gln-14, and Asp-14 appeared to result in differential quantitative effects on in vitro ATM and ATR activity (Fig. 2). One exception to this was the virtual abrogation of ATM activity by valine substitution for leucine at N−1 with no obvious affect on ATR activity (data not shown).
sequences of the GST peptides are as indicated in Table I. The sequences and accession numbers of the peptides studied are listed and the amounts of substrate phosphorylation relative to the Ser-15 Ser-94 motif of ATM for screening of putative substrates.

Identification of New Putative Substrates of the ATM Family—Using the preliminary consensus sequence generated from the p53 Ser-15 peptide mutational analysis and the Ser-94 sequence of PhasI, we searched a protein data base for potential ATM substrates. Since the occurrence of this motif is not uncommon, many potential targets were identified. We initially evaluated the ability of ATM, ATR, and DNA-PK to phosphorylate the peptide sequences of 36 proteins (Table I), many of which were chosen because of potential physiological relevance to the AT phenotype. Only a fraction of the peptides tested were highly phosphorylated in vitro (Fig. 3). These included Rad17, p95, Mre11, Brca1, PhasI, WRN, ATM (Ser-440, a potential autophosphorylation site), and a recently cloned sequence localized to an LOH site on chromosome 3p21.3 (which we call PTS for “putative tumor suppressor”). Using

Thus, ATM, but not ATR, may discriminate between valine and other hydrophobic amino acids at the position N—1. Further validation of this putative general consensus target sequence for ATM came from examination of a previously reported in vitro substrate for ATM, PhasI (21). An amino acid sequence around Ser-94 in PhasI contains hydrophobic amino acids at N—3 and N—1 as well as an SQ motif. As predicted, a GST-Ser-94 PhasI peptide proved to be an excellent in vitro substrate for ATM (Fig. 3). From the above results, we defined (P/L/I/M)(X)L/I/D/E/SQ as a preliminary phosphorylation site motif of ATM for screening of putative substrates.

Amino acid sequences and relative phosphorylation by ATM kinase family members of the GST peptides tested in Fig. 3

| Accession number | Protein name | Amino acid sequences | Serine No. | Relative phosphorylation | ATM | ATR | DNA-PK |
|------------------|--------------|----------------------|------------|-------------------------|-----|-----|--------|
| p04837           | p53          | SVEPELPSQETPSDL      | 15         | 100                     | 100 | 100 |
| p04837           | p53          | VLSPLPSQAMMDLM       | 37         | 9                       | 31  | 132 |
| NP 004086        | 4EBP1 (PhasI)| EPPMEASOQSHLRNS      | 94         | 78                      | 66  | 10  |
| NP 002476        | p95          | TPGSPSLSGVSVDE       | 343        | 19                      | 31  | 45  |
| p49569           | Mre11(SQ1)   | QREFYIQPGSSSV        | 264        | 20                      | 48  | 10  |
| p49569           | Mre11(SQ2)   | FSVLAFSKQFDRV        | 386        | <1                      | 17  | <1  |
| p49569           | Mre11(SQ3)   | RARALPSQESASAS      | 531        | <1                      | 15  | <1  |
| p49569           | Mre11(SQ4)   | SARSGSGORGAFFK       | 590        | <1                      | ND* | ND  |
| p49569           | Mre11(SQ5)   | SSSKIMSSQSVSKG       | 648        | <1                      | ND  | ND  |
| NP 001265        | Chk1(SQ1)    | VKYESQSFEPRTG        | 317        | 14                      | 58  | 2   |
| NP 001265        | Chk1(SQ2)    | VQGICESQFQTFCDH      | 345        | 11                      | 73  | 20  |
| AAC36334         | Rad17(SQ1)   | TVSGPLSGDQASSEL      | 646        | 81                      | 733 | 97  |
| AAC36334         | Rad17(SQ2)   | ASEPQPSQQPFSAS       | 656        | 91                      | 1065| 25  |
| AF091214         | Wrn (SQ1)    | TGMHLSQVKAGC         | 1292       | 10                      | 33  | 5   |
| AF091214         | Wrn (SQ2)    | EKAYSSSQVISIQ        | 1141       | 25                      | 49  | 14  |
| U76308           | ATR          | TVEFISQVTLVE         | 1333       | <1                      | 10  | <1  |
| X18416           | c-Abl        | VPGIDLSQYVELLE       | 446        | <1                      | 8   | <1  |
| NP 000042        | ATM          | PPLLMILSSLQFQR       | 440        | 33                      | 55  | 7   |
| Z4973            | PI–3K        | YKVVPILSQSGVLE       | 2761       | 3                      | 22  | <1  |
| U64105           | p115-RhoGEF  | DLMILYSQVLQAKL       | 397        | <1                      | 150 | <1  |
| L15929           | β-Adaptin    | CRAPEVSCVYQYAY       | 935        | 5                       | 22  | 2   |
| U43746           | Brca2        | VKYLPILSQQQDKQ        | 2156       | <1                      | 132 | <1  |
| M81735           | DNA PoL−δ   | LPIECISQVTFQG        | 717        | <1                      | 17  | <1  |
| U87269           | p120E4F      | APEPPVSEELQCSR       | 355        | 11                      | 30  | 2   |
| U14680           | Brca1(SQ1)   | SAFLFSSQCELED         | 1298       | 2                       | 38  | 6   |
| U14680           | Brca1(SQ2)   | DCGLSLSQSDLT         | 1387       | 42                      | 35  | 36  |
| U14680           | Brca1(SQ3)   | SSEYPSNLQEPQLS       | 1466       | 8                       | 33  | 12  |
| L07590           | PP2A         | LLHIVPSQFDKDL        | 61         | <1                      | 34  | <1  |
| D79987           | Cut1         | GASPVLQSVDPDRS       | 1615       | 9                       | 20  | 5   |
| AF040703         | PTS (123F2)  | WETPFLSQAIEQK        | 61         | 111                     | 40  | 33  |
| X63071           | DNA 5B       | QPEPFVSPQEISEP       | 72         | 3                       | 146 | 15  |
| X83441           | DNA LIG–IV  | DKLGLVSSQTFISF       | 132        | 5                       | 36  | 48  |
| U24186           | RPA34KD (SQ1)| FPAPAPSQAEKSKR       | 33         | <1                      | 5   | <1  |
| U24186           | RPA34KD (SQ2)| IVPCTISQSLSATL       | 52         | <1                      | 31  | <1  |
| U81504           | β3A adaptin  | ELKFPVLSSG           | 1095       | 6                       | 22  | 44  |
| U72066           | CtIP         | DPGLADLSSQMDVT       | 664        | 12                      | 12  | 2   |

* ND, not determined.
ATM kinase-inactive form as a negative control, we confirmed that the phosphorylations of these substrates are intrinsic (data not shown). Since some peptides containing sequences which fit the general consensus sequence were not phosphorylated, other amino acid sequence determinants must also be involved in determining the substrate specificity for the ATM kinase and continued characterization of these substrates should allow further refinement of the recognition sequence.

We also tested the ability of DNA-PK and ATR to phosphorylate these GST peptides and several distinctions between substrates recognized by these three kinases were apparent in the assays (Fig. 3). In general, ATM and ATR tended to recognize the same substrates, although quantitative differences were apparent in most cases. For example, even though ATR appears to be a much weaker kinase than ATM for most substrates tested (~10–20-fold lower activity), ATR exhibited greater activity than ATM for two sites in Rad17 and also demonstrated good activity against sites from Brc2 and DNA-5B. The latter two peptides were very poor substrates for both ATM and DNA-PK and the second SQ in Rad17 was not a good substrate for DNA-PK (Fig. 3). Conversely, peptides containing sequences from ligase IV and the region surrounding Ser-37 of p53 were highly phosphorylated by DNA-PK, but were not good substrates for ATM. From these approaches, we have developed a preliminary consensus target sequence which is recognized by ATM and we have identified a group of proteins containing good in vitro target sites for ATM which warrant further investigation as potential valid in vivo targets of ATM (Fig. 4).

**DISCUSSION**

Identification of physiologic substrates for cellular kinases is a daunting, but critically important, aspect of understanding biological processes. It had previously been demonstrated that p53 protein is a physiologic target of the ATM kinase (20, 21), but most of the physiologic abnormalities in AT patients and AT cells are not attributable to defects in signaling to p53. Thus, it is clear that there must be other physiologic targets of this kinase. Building upon our previous work (20), we clarified the optimal conditions for in vitro measurements of ATM kinase activity. These experiments demonstrated significant differences in the co-factor requirements of ATM and ATR compared with the related kinase, DNA-PK. In particular, ATM and ATR require Mn$^{2+}$, but not DNA ends or Ku proteins, for optimal in vitro activity while DNA-PKs requires Mg$^{2+}$, DNA ends, and Ku proteins. Optimization of in vitro conditions to evaluate these three kinases allows us to then use these in vitro assays for initial screening of potential physiologic targets of these enzymes.

To determine a phosphorylation consensus motif and identify new putative substrates of kinases, we developed a GST peptide screening method which allows us to easily make a lot of peptides to test. There are several advantages to the method we used. First, it appears that the GST peptides are much better than small synthetic peptides as in vitro substrates and it is easier and less expensive to make the oligonucleotides and clone them into the GST plasmid than to synthesize every peptide of interest. Second, easy alteration of the sequence of the oligonucleotides attached to the GST linker allows us to quickly and easily define a general consensus target motif and simultaneously identify the putative phosphorylation site for each target. Third, comparing the abilities of the three related kinases to phosphorylate each of these substrates in vitro can provide unexpected clues about how they differ in vivo function and provides potential distinctions between the enzymes if inhibitors of these kinases are to be developed. As examples of insights which were not predictable a priori, our in vitro data suggests that Rad17 may be a physiologic substrate for ATR and not DNA-PK and that ligase IV may be a physiologic substrate for DNA-PK and not ATM or ATR. This latter possibility is particularly intriguing because of the recently described role for ligase IV in V(D)J recombination events that also involve DNA-PK (30). Building upon the preliminary consensus target sequence for ATM generated from the p53 Ser-15 mutagenesis work, we were then able to identify some new potential substrates for ATM, ATR, and DNA-PK including Rad17, Brc2, Brc2 p95, PTS, Pha1, WRN, DNA-5B, and ligase IV. These targets represented the first screen of potential substrates and additional proteins with reasonable ATM target sites are continuing to be evaluated. It should be noted that the consensus target sequences we characterized should be considered as guidelines rather than concrete rules. However, it does appear that glutamine at position N+1 appears to be absolutely required for activity of this kinase family and that nearby hydrophobic amino acids, especially at positions N–3 and N–1, are important determinants. It is noted that since substitution of threonine for serine had only a quantitative effect on phosphorylation by all three enzymes, it is entirely conceivable that threonine could replace serine as the targeted amino acid in certain protein targets.

AT, with mutations in the ATM gene, and NBS, with mutations in the p95/nibrin gene, share many phenotypic abnormalities, including chromosomal instability, radiation sensitivity, and defects in cell cycle checkpoints in response to IR (31, 32). In this report, we showed that ATM phosphorylates a p95 peptide (Ser-343) and a Mre11 peptide (Ser-264) in vitro, suggesting that ATM may regulate the function of p95/Mre11-Rad50 repair complex in response to DNA damage. Other potential targets that we identified in our screens such as Brc2, could uncover other roles for ATM in DNA repair processes. Patients with heterozygous germine mutations in the *BRC1* gene have a markedly increased risk of developing breast cancer (33, 34) and it has been suggested that heterozygous germ-line ATM mutations also increase breast cancer risk (35–38). Additionally, it appears that Brc2 participates both in transcription as a transcription factor and DNA repair through association with Rad51/Rbrca2 (39, 40). It is noteworthy that Brc2 can form foci with the p95/Mre11-Rad50 complex after
ionizing radiation and formation of these foci is dramatically reduced in HCC1937 breast cancer cells carrying a homozygous mutation in Brca1 (41). The function of the mammalian Rad17 protein is currently unknown, but data from yeast implicate this protein in DNA damage checkpoint control (42–44). Elucidation of whether this is a physiologic target of either the ATM or ATR kinases may shed additional light on regulation of mammalian DNA repair processes. Finally, since ATM kinase activity is enhanced by DNA breakage (20, 21), whether the potential ATM autophosphorylation site (Ser-440) that we identified contributes to regulation of its activity will be of interest.

This in vitro peptide screening method will prove most useful if it results in the identification of valid in vivo targets of these kinases. Several of the peptide substrates described above have been further evaluated as large peptides (greater than 100 amino acids). A number of these larger peptides remained excellent in vitro substrates, including the sites identified in Rad17, Brca1, and p95 (data not shown). The putative target site in p95 has been further demonstrated to be a valid in vivo target of the ATM kinase. This demonstrates that the screening approach elucidated herein can identify valid kinase targets and follow-up in vivo studies of these in vitro targets may identify further bona fide targets of this kinase family. Such studies would provide invaluable insights into understanding AT and signaling pathways that potentially involve ATM and related kinases.

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