

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

(Received for publication, September 17, 1999)

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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 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. 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 NH_2 -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, Brca1, 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.

Ataxia telangiectasia (AT)¹ is a complex human autosomal recessive disease characterized by a pleiotropic phenotype including neuronal degeneration, oculocutaneous telangiectasias, immune dysfunction, cancer predisposition, and premature aging (1). Cells derived from AT patients exhibit a variety of abnormal responses to ionizing irradiation (IR), such as checkpoint alterations in the G_1 , S, and G_2 phases of the cell cycle and radiosensitivity with increased chromosomal breakage and telomere fusions (2, 3). The G_1 checkpoint abnormality results from a quantitative defect in p53 induction (4–6), but the molecular mechanisms underlying the radiosensitivity and the S-phase and G_2 phase checkpoint defects have not yet been elucidated.

* This work was supported in part by National Institutes Health Grants CA71387, ES0577, and CA21765, Glaxo-Wellcome, and the American Lebanese Syrian Associated Charities (ALSAC) of the St. Jude Children's Research Hospital. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: AT, ataxia telangiectasia; IR, ionizing irradiation; GST, glutathione S-transferase; PTS, putative tumor suppressor; PKcs, PK catalytic subunit.

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, neocarzinostatin, 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 G_2 /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 Brca1, 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.

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 *Bam*HI/*Sma*I site of pGEX-2T (Amersham Pharmacia Biotech). The constructs were confirmed by restriction enzyme digests and DNA sequencing. The GST peptides were expressed in BL2/(CDE3). 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 μ g of either ATM or ATR by resuspending cells in modified TGN buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Tween 20, 0.3% Nonidet P-40, 1 mM sodium fluoride, 1 mM Na_3VO_4 , 1 mM phenylmethylsulfonyl fluoride, and 1 $\times$ 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 TGN buffer plus 0.5 M LiCl, and two washes with kinase buffer (20 mM HEPES, pH 7.5, 50 mM NaCl, 10 mM MgCl_2 , 1 mM dithiothreitol, and 10 mM MnCl_2). Finally, the immunoprecipitant was resuspended in 50 μ l of kinase buffer containing 10 μ Ci of [γ - ^{32} P]ATP and 1 μ g of GST fusion substrate. The kinase reaction was conducted at 30 $^\circ\text{C}$ 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 II KS cut with *Eco*RI) 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 Na_3VO_4 , 1 mM phenylmethylsulfonyl fluoride, 1 $\times$ 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-PKs and Ku70/80 were generous gifts from Dr. S. Yoo and Dr. W. S. Dynan, Medical College of Georgia. The DNA-PK *in vitro* kinase reaction was performed as described previously (25).

RESULTS

Cofactor 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 cofactor requirements for their optimal activities. It had been previously reported that ATM and ATR required exogenous Mn^{2+} for optimal *in vitro* kinase activity (20, 21). In contrast, DNA-PK has been reported to require Mg^{2+} 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 our *in vitro* assays. For example, it was conceivable that the use of Mn^{2+} 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 Mn^{2+} addition in these *in vitro* assays.

In order to test these possible scenarios, *in vitro* kinase assays with all three enzymes were performed using epitope-tagged ATM and ATR immunoprecipitated from transfected

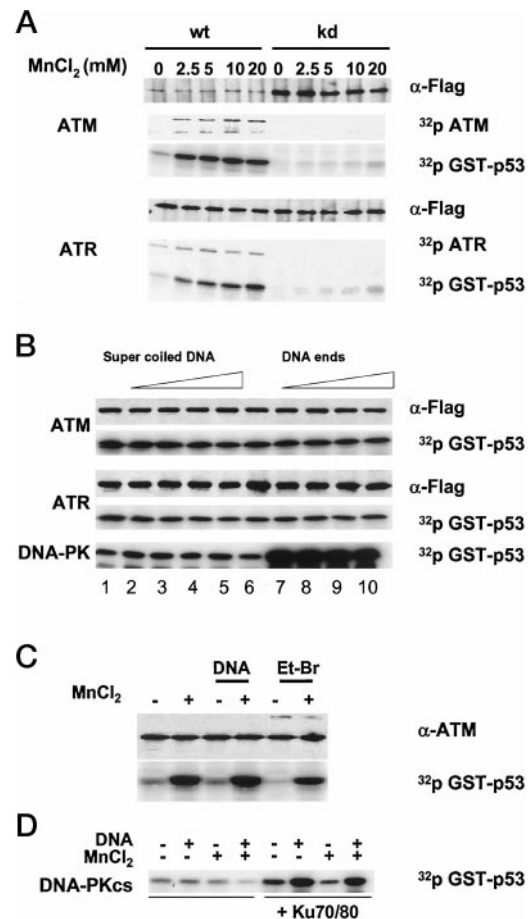


FIG. 1. Co-factor requirements for ATM family members. A, ATM and ATR require Mn^{2+} for optimal activity. Wild-type (*wt*) or catalytically inactive (*kd*) ATM or ATR were immunoprecipitated with anti-Flag antibody and were used in an *in vitro* kinase assay with 10 μ Ci of [γ - ^{32} P]ATP and 1 μ g of GST-p53 (1–101) in the presence of 10 mM MgCl_2 and various concentration of Mn^{2+} as indicated. Use of equivalent amounts of Flag-tagged ATM or ATR in the different lanes was confirmed by immunoblotting with anti-Flag antibody (*top panel*). The amount of radiolabeled ATM or ATR (*middle panel*) and substrate (*lower panel*) in the reaction was assessed. B, DNA ends do not enhance the activity of ATM and ATR kinases. Kinase reactions for ATM, ATR, and DNA-PK were performed with addition of varying amount of supercoiled or linear DNA: no DNA (*lanes 1 and 6*); 0.2 μ g of DNA (*lanes 2 and 7*); 0.5 μ g of DNA (*lanes 3 and 8*); 1 μ g of DNA (*lanes 4 and 9*); and 2 μ g of DNA (*lanes 5 and 10*). C, endogenous ATM depends on Mn^{2+} , but not on DNA ends, for its activity. ATM was immunoprecipitated from GM0536 lymphoblasts and preincubated on ice for 15 min with either 1 μ g of linearized DNA or 100 μ g of ethidium bromide prior to addition of substrate and [γ - ^{32} P]ATP. The reaction contained 10 mM MgCl_2 and 0 (–) or 10 (+) mM MnCl_2 as indicated. D, Mn^{2+} does not affect DNA-PKs or DNA-PK activity. *In vitro* kinase assays were performed with 20 ng each of purified DNA-PKs or DNA-PK (20 ng of DNA-PKs premixed with 20 ng of Ku70/80). 10 mM MnCl_2 or 1 μ g of linearized DNA was added as indicated.

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 Mn^{2+} (Fig. 1A), while DNA-PK activity was not altered by the addition of Mn^{2+} (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 Mn^{2+}

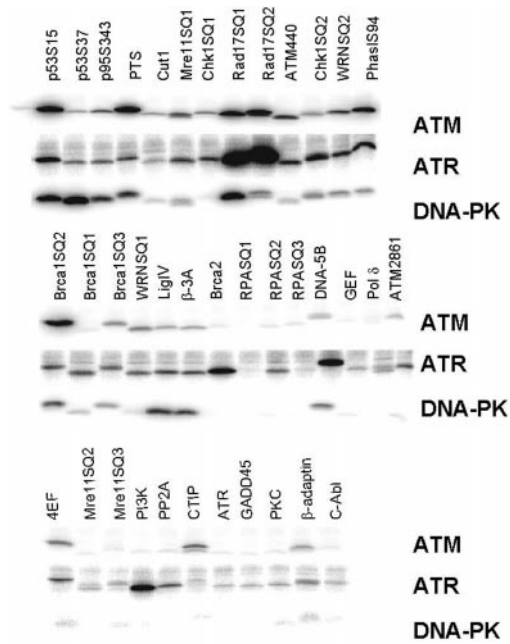


FIG. 3. Phosphorylation of candidate targets of by ATM, ATR, and DNA-PK *in vitro*. GST peptides containing the putative target sequences were used as substrates for the kinase assay. The amino acid sequences of the GST peptides are as indicated in Table I.

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.

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

TABLE I

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

The sequences and accession numbers of the peptides studied are listed and the amounts of substrate phosphorylation relative to the Ser-15 phosphorylation of p53 by each kinase are shown.

Accession number	Protein name	Amino acid sequences	Serine No.	Relative phosphorylation		
				ATM	ATR	DNA-PK
p04637	p53	SVEPPLSQETFSDL	15	100	100	100
	p53	VLSPPLSQAMDDL	37	9	31	132
NP_004086	4EBP1 (PhasI)	EPPMEASQSHLRNS	94	78	66	10
NP_002476	p95	TPGPSLSQGVSVDE	343	19	31	45
p49959	Mre11(SQ1)	QQLFYISQPGSSVV	264	20	48	10
	Mre11(SQ2)	FSVLRFSQKFVDRV	386	<1	17	<1
	Mre11(SQ3)	RARALRSQSEESAS	531	<1	13	<1
	Mre11(SQ4)	SASRGGSQRGRAFK	590	<1	ND ^a	ND
	Mre11(SQ5)	SSSKIMSQSQVSKG	648	<1	ND	ND
NP_001265	Chk1(SQ1)	NVKYSSSQPEPRTG	317	14	58	2
	Chk1(SQ2)	VQGIFSFSQPTCPDH	345	11	73	20
AAC36334	Rad17(SQ1)	TWSLPLSQDSASEL	646	81	733	97
	Rad17(SQ2)	ASELPASQPQPFSA	656	91	1065	25
AF091214	Wrrn (SQ1)	TIGMHLAQAVKAGC	1292	10	33	5
AF091214	Wrrn (SQ2)	EKAYSSSQPVISAQ	1141	25	49	14
U76308	ATR	TVEPIISQLVTVLL	1333	<1	10	<1
X16416	c-Abl	YPGIDLSQVYELLE	446	<1	8	<1
NP_000042	ATM	PLLMLLSQLLPQQR	440	33	55	7
	ATM	YKVVPLSQRSQVLE	2761	3	22	<1
Z46973	PI-3K	DLLMYLSQLVQALK	397	<1	150	<1
U64105	p115-RhoGEF	RLRPLLSQLGGNSV	899	<1	15	<1
L13939	β-Adaptin	CRAPEVSHVYQAY	935	5	22	2
U43746	Brca2	KVSPYLSQFQQDKQ	2156	<1	132	<1
M81735	DNA Pol-δ	LPCLEISQSVTGFG	717	<1	17	<1
U87269	p120E4F	APEPPVSQELPCSR	355	11	30	2
U14680	Brca1(SQ1)	SASLFSSQCSELED	1298	2	38	6
	Brca1(SQ2)	DCSGLSSQSDILTT	1387	42	35	36
	Brca1(SQ3)	SSEYPISQNPGLS	1466	8	33	12
L07590	PP2A	LLHIPVSQFKDADL	61	<1	34	<1
D79987	Cut1	GASPVLSQGVDPDRS	1615	9	20	5
AF040703	PTS (123F2)	WETPDLSQAEIEQK	61	111	40	33
X63071	DNA 5B	QPEPPVSQSEISEP	72	3	145	15
X83441	DNA LIG-IV	DLKLGVSQQTIFSV	132	5	36	48
U24186	RPA34kd (SQ1)	FPAPAPSQAEKKSR	33	<1	5	<1
	RPA34kd (SQ2)	IVPCTISQLLSATL	52	<1	31	<1
	RPA34kd (SQ3)	TGNVEISQVTIVGI	72	<1	8	<1
U81504	β3A adaptin	ELKPVLSQ	1092	6	22	44
U72066	CtIP	DPGADLSQYKMDVT	664	12	12	2

^a ND, not determined.

P53	S15	SVEPPLSQETFSDL
PTS	S61	WETPDLSQAEIEQK
P95	S343	TPGPSLSQGVSVDE
Rad17	S646	TWSLPLSQDSASEL
Rad17	S656	ASELPASQPQPFSA
Phas-I	S94	EPPMEASQSHLRNS
ATM	S440	PLLMILSQLLPQQR
Brca1	S1387	DCSGLSSQSDILT
WRN	S1141	EKAYSSSQPVISAQ
Mre11	S264	QQLFYISQPGSSVV

ATM consensus motif

B^a (P/M/Y/F/G) -x-B (S/A/D/E) -S/TQ

FIG. 4. Candidate ATM target sequences and a general phosphorylation site motif for ATM kinase. Sequences of selected proteins that were highly phosphorylated by ATM *in vitro* kinase reaction are shown. Based on the *in vitro* kinase assays with the mutant GST-p53 peptides and the candidate target GST peptides, it appears that hydrophobic amino acids at N-1, N-3 are preferred, although some exceptions (P/M/Y/G/F and S/A/D/E at N-3 and N-1, respectively) were found as indicated. The glutamine at N+1 is an essential amino acid for substrate recognition by ATM kinase. Positively charged residues (K, lysine; R, arginine) around the target SQ negatively inhibit substrate recognition by ATM. B^a indicates hydrophobic amino acids.

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 Brca2 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 com-

pared with the related kinase, DNA-PK. In particular, ATM and ATR require Mn²⁺, but not DNA ends or Ku proteins, for optimal *in vitro* activity while DNA-PKs requires Mg²⁺, 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 *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, Brca1, Brca2 p95, PTS, PhasI, 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 is 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 Brca1, could uncover other roles for ATM in DNA repair processes. Patients with heterozygous germline mutations in the *BRCA1* gene have a markedly increased risk of developing breast cancer (33, 34) and it has been suggested that heterozygous germline ATM mutations also increase breast cancer risk (35–38). Additionally, it appears that Brca1 participates both in transcription as a transcription factor and DNA repair through association with Rad51/Brca2 (39, 40). It is noteworthy that Brca1 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.² 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.

Acknowledgments—We thank Dr. T. M. Gilmer and Kathy Kilpatrick (Glaxo-Wellcome) for mouse ATM monoclonal antibodies and S. Yoo and W. S. Dynan (Medical College of Georgia) for purified DNA-PKcs and Ku70/80. We are also grateful to Dr. C. Leonard (NCBI) for help and advice with ATM target screening from protein sequence data base and C. Bakkenist for helpful comments on the manuscript.

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² D.-S. Lim, Kim, S. T., Xu, B., Maser, R. S., Lin, J., Petrini, J. H. J., and Kastan, M. B., submitted for publication.