and anisomycin. IL-1 preferentially activates MKK7 and UV radiation and anisomycin preferentially activate MKK4. The overexpressed JIP-1 (which binds JNK and MKK7) decreased JNK activation caused by each stimulus (Fig. 4A). In contrast, JIP-1ΔJBD (which binds MKK7 and not JNK) selectively inhibited IL-1 signaling (Fig. 4A). This inhibition of JNK activation caused by overexpression of JIP-1 may reflect the sequestration of limiting JNK pathway components into separate complexes, and the amount of inhibition may reflect the selective binding of these components by JIP-1. JIP-1 therefore represents a useful pharmacological tool to selectively inhibit JNK signaling. However, the function of JIP-1 as an inhibitor may result from an artificial situation where JIP-1 (or an active JIP-1 fragment) is overexpressed.

To examine the effect of JIP-1 on JNK activation, we coexpressed JIP-1 together with upstream components of the JNK pathway. This experimental strategy was designed to favor the formation of JIP-1 complexes containing MLK3, MKK7, and JNK. These studies demonstrated that JIP-1 enhanced JNK activation by MKK7 but not by MKK4 (Fig. 4B). Deletion of the JNK binding domain eliminated the ability of JIP-1 to increase JNK activation by MKK7 (Fig. 4B). Furthermore, JIP-1 enhanced JNK activation mediated by MLK3 (Fig. 4C). This effect of JIP-1 was reduced by the separate deletion of the domains that bind JNK, MKK7, and MLK3 (Fig. 4, C and D). We conclude that JIP-1 enhances JNK activation by the MLK → MKK7 signaling pathway.

JIP-1 appears to function as a mammalian scaffold protein for the JNK signaling pathway (Fig. 4E). JIP-1 binds multiple components of a JNK signaling module and facilitates signal transduction mediated by the bound proteins. The function of JIP-1 as a scaffold protein appears to be selective for the signaling pathway that is formed by the MAP kinase module MLK → MKK7 → JNK. Different scaffold proteins may contribute to the activation of JNK mediated by other MAPK modules.

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13. An alternatively spliced JIP-1 cDNA was isolated from a murine brain library. The variant JIP-1 protein sequence deduced from the cDNA sequence (designated JIP-1b) contains an insertion of 47 amino acids after JIP-1 residue 557. The sequences of JIP-1 (*4*) and JIP-1b have been deposited in the GenBank database (accession numbers AF003115 and AF054611, respectively). A rat protein (IB1) similar to JIP-1b was recently reported [C. Bonney, P. Nicod, G. Waeber, *J. Biol. Chem.* **273**, 1843 (1998)]. IB1 was identified as a regulator of GLUT2 gene expression in cultured pancreatic β cells. Further studies are required to determine whether IB1, like JIP-1 and JIP-1b, can also act as a scaffold protein that binds components of the JNK MAP kinase signaling module.
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15. Plasmid DNA was transfected into COS-7 and 293 cells by the lipofectamine method (Life Technologies). The cells were treated with IL-1 (10 nM; 20 min), anisomycin (10 μg/ml; 30 min), or UV radiation (80 J/m^2; 60 min). The cells were collected at 48 hours and solubilized in lysis buffer [20 mM tris-HCl (pH 7.4), 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 2 mM pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 μg/ml), 10% (v/v) glycerol, 1% Triton X-100]. GST fusion proteins were isolated by incubation with glutathione-agarose (Pharmacia-LKB) beads (20 μl) for 3 hours at 4°C. Epitope-tagged proteins were immunoprecipitated by incubation for 3 hours at 4°C with the monoclonal antibodies M2-Flag (IBI-Kodak), hemagglutinin (HA) (Boehringer-Mannheim), or T7-Tag (Novagen Inc.) bound to protein G–Sepharose (Pharmacia-LKB). HPK1 was immunoprecipitated with a rabbit polyclonal antibody to HPK1. Immunoprecipitated proteins were examined by SDS–polyacrylamide gel electrophoresis and detected by immunoblot analysis. Protein kinase activity was measured by the in-gel method with substrate polymerized in the gel (0.25 mg/ml) [B. Dérijard *et al.*, *Cell* **76**, 1025 (1994)].
16. Mammalian expression vectors for JNK1, JNK2, JIP-1, p38α, MEKK1, MKK3, MKK4, MKK6, MKK7, and c-RAF1 have been described (*4*, *14*). The mammalian expression vectors pCDNA3-HA-MLK3, pCMV5-HA-MEK1, pCMV-HA-ERK2, pSRα-T7-DLK, pCEVHA-MEKK3-F, pCDNAI-MTK1/MEKK4, pSRα-HPK1, pEBG-KHS, and pJ3H-PAK3 were provided by S. Gutkind, N. Ahn, M. Weber, A. Rana, U. Siebenlist, H. Saito, T. Tan, J. Blenis, and R. Cerione, respectively. The expression plasmid pCDNA3-Flag-MLK3 was constructed by replacing the HA tag sequence in pCDNA3-HA-MLK3 with the Flag sequence. Full-length JIP-1 and JIP-1 deletion mutants were expressed as GST fusion proteins using the vector pEBG or with a T7-Tag fused to the NH_2-terminus using the vector pCDNA3.
17. Bacterial expression of GST-cJun (1 to 79), GST-JNK1, and GST-JIP-1 (127 to 282) have been described (*4*). Expression plasmids for GST-JIP-1 (1 to 127) and GST-JIP-1 (283 to 660) were constructed by subcloning polymerase chain reaction fragments of JIP-1 into pGEX-4T-1 (Pharmacia-LKB). Bacterial expression plasmids for epitope-tagged MKK7 and MLK3 were constructed by inserting Flag-MKK7 and Flag-MLK3 (encoding amino acids 1 to 204) into pRSETA (Invitrogen).

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Enhanced Phosphorylation of p53 by ATM in Response to DNA Damage

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The ATM protein, encoded by the gene responsible for the human genetic disorder ataxia telangiectasia (A-T), regulates several cellular responses to DNA breaks. ATM shares a phosphoinositide 3-kinase–related domain with several proteins, some of them protein kinases. A wortmannin-sensitive protein kinase activity was associated with endogenous or recombinant ATM and was abolished by structural ATM mutations. In vitro substrates included the translation repressor PHAS-I and the p53 protein. ATM phosphorylated p53 in vitro on a single residue, serine-15, which is phosphorylated in vivo in response to DNA damage. This activity was markedly enhanced within minutes after treatment of cells with a radiomimetic drug; the total amount of ATM remained unchanged. Various damage-induced responses may be activated by enhancement of the protein kinase activity of ATM.

Strand breaks in cellular DNA occur continuously as a consequence of normal processes such as recombination or the infliction of DNA damage. DNA damage triggers several signal transduction pathways that lead either to damage repair coupled with attenuation of cell cycle progression, or to programmed cell death (apoptosis). A junction of such path-

ways is controlled by the transcription factor p53. After DNA damage, the amount of p53 in cells is increased through posttranscriptional mechanisms and its transactivation activity is enhanced, leading to the activation of downstream genes (*1*).

The genetic disorder A-T results in genome instability, cerebellar and thymic degeneration, immunodeficiency, gonadal dysgenesis, radiation sensitivity, and predisposition to cancer. A-T cells exhibit acute sensitivity to ionizing radiation and radiomimetic chemicals, and their cell cycle checkpoints fail to be activated after treatment with these agents (*2*). The responsible gene, *ATM*, encodes a 370-kD protein with a COOH-terminal domain similar to the catalytic subunit of phosphoinositide 3-kinases (PI 3-kinases) (*3*). This similarity places ATM within a family of proteins that share the PI 3-kinase–related domain and function in maintenance of genome stability, in cell cycle control, and in cellular responses to DNA damage (*4*). These proteins appear to be protein kinases rather than lipid kinases, as evidenced by the DNA-dependent protein kinase (DNA-PK) and the mammalian target of rapamycin (mTOR) (*5*).

To study ATM's catalytic activity, we raised monoclonal antibodies (mAbs) to two ATM-derived peptides, ATM132 (spanning ATM positions 819 to 844) and ATML2 (positions 2581 to 2599). Both mAbs recognize a 370-kD protein in normal cells that is not detected in A-T cells but reappears upon ectopic expression of ATM in them. These mAbs also recognize endogenous, recombinant, and in vitro translated ATM immunoprecipitated by other antibodies to ATM (*6*). Protein kinase activity was revealed in immunoprecipitates obtained with these antibodies (*7*) from a normal lymphoblastoid cell line (L-40) when the translation repressor PHAS-I (*8*) was used as a substrate (Fig. 1A). This activity was not detected with an unrelated antibody, and was retained when ATM was eluted from the beads using an excess of the peptide to which the antibody was raised (*9*). Phosphorylation of PHAS-I was considerably more intense with immunoprecipitates obtained from G361, a melanoma cell line in which the amount of ATM is greater than that in lymphoblasts (Fig. 1A); it was not observed in immunoprecipitates obtained from an A-T cell line, L-6, that lacks the ATM protein (Fig. 1A). Immunoprecipitates containing recombinant ATM expressed in A-T cells (*10*) showed kinase activity similar to that of the endogenous protein (Fig. 1B). Unlike DNA-PK, this activity was strictly dependent on manganese ions and was not enhanced by the addition of 200 μg of sheared double-stranded DNA, which considerably stimulated DNA-PK activity (*9*).

We also measured ATM-associated kinase activity in two A-T cell lines containing structural ATM mutations that allow expression of the mutant proteins. The cell line 41RM is homozygous for a missense mutation, E2904G (*11*), that replaces a highly conserved glutamic acid residue with glycine within the PI 3-kinase–related domain that is expected to contain the catalytic site of the kinase. The cell line 9RM is homozygous for a nonsense mutation, R3047X, that removes the 10 COOH-terminal residues of ATM (*12*). Little or no kinase activity was detected in immunoprecipitates from either cell line (Fig. 1C). Our results indicate that ATM has an intrinsic protein kinase activity.

PI 3-kinases are inhibited by wortmannin, which covalently modifies conserved lysine residues at their catalytic sites (*13*). ATM-associated kinase activity was inhibited in immune complexes exposed to wortmannin for 30 min (Fig. 1D). The sensitivity of ATM to this drug (median inhibitory concentration IC_{50} = 100 nM) is comparable to that of other PI 3-kinase–related protein kinases (*5*).

A known target of DNA damage–induced phosphorylation is the p53 protein (*1*). Ser^{15} of p53 undergoes phosphorylation in cells after DNA damage (*14, 15*), and this response is decreased in A-T cells (*14*). ATM also interacts directly with p53 in vivo (*16*). Full-length recombinant p53 was phosphorylated in vitro by ATM immunoprecipitates (Fig. 2A). Phosphorylation of p53 fragments and full-length p53 in which both Ser^{15} and Ser^{37} had been substituted by alanine residues indicated that Ser^{15} was the predominant, if not the only, site phosphorylated by ATM (Fig. 2B). Analysis with antibodies raised to p53-derived phosphopeptides containing phosphorylated Ser^{15}, Ser^{33}, or Ser^{37}

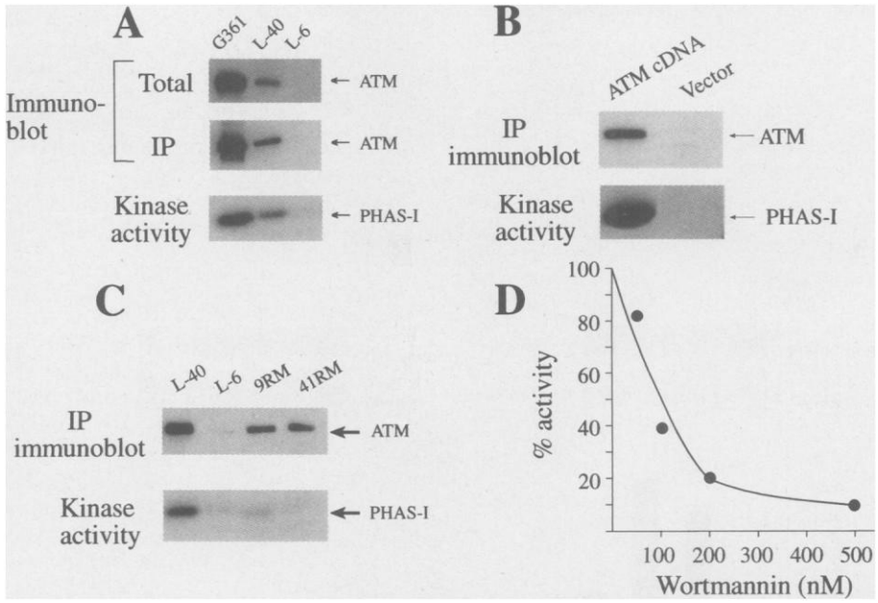

Fig. 1. Kinase activity of ATM with PHAS-I as a substrate. **(A)** Activity of ATM immunoprecipitated from the melanoma cell line G361, the normal lymphoblastoid cell line L-40, and an A-T lymphoblastoid cell line, L-6, lacking the ATM protein. Total cellular extracts (total) or ATM immunoprecipitates (IP) obtained from equal amounts of cells with ATM132 mAb were immunoblotted with ATML2 mAb. Bottom panel: autoradiography of phosphorylated PHAS-I (*7*). **(B)** Activity of recombinant ATM stably expressed in an immortalized A-T cell line, AT22IJE-T (*10*). The cells were transfected with full-length cDNA in the episomal expression vector pEBS7, or with an empty vector. Top panel: Protein immunoblotting analysis of ATM immune complexes. Bottom panel: Kinase activity of ATM (*7*). **(C)** Kinase activity of ATM immunoprecipitated from a normal cell line (L-40), an A-T cell line lacking the ATM protein (L-6), an A-T cell line homozygous for the truncation mutation R3047X (9RM), and an A-T cell line homozygous for the missense mutation E2904G (41RM). **(D)** Effect of wortmannin on ATM-associated kinase activity. ATM immune complexes were incubated with various concentrations of the drug and washed with the reaction buffer before kinase activity was measured. Activity was normalized to the amount of ATM protein obtained from immunoblots.

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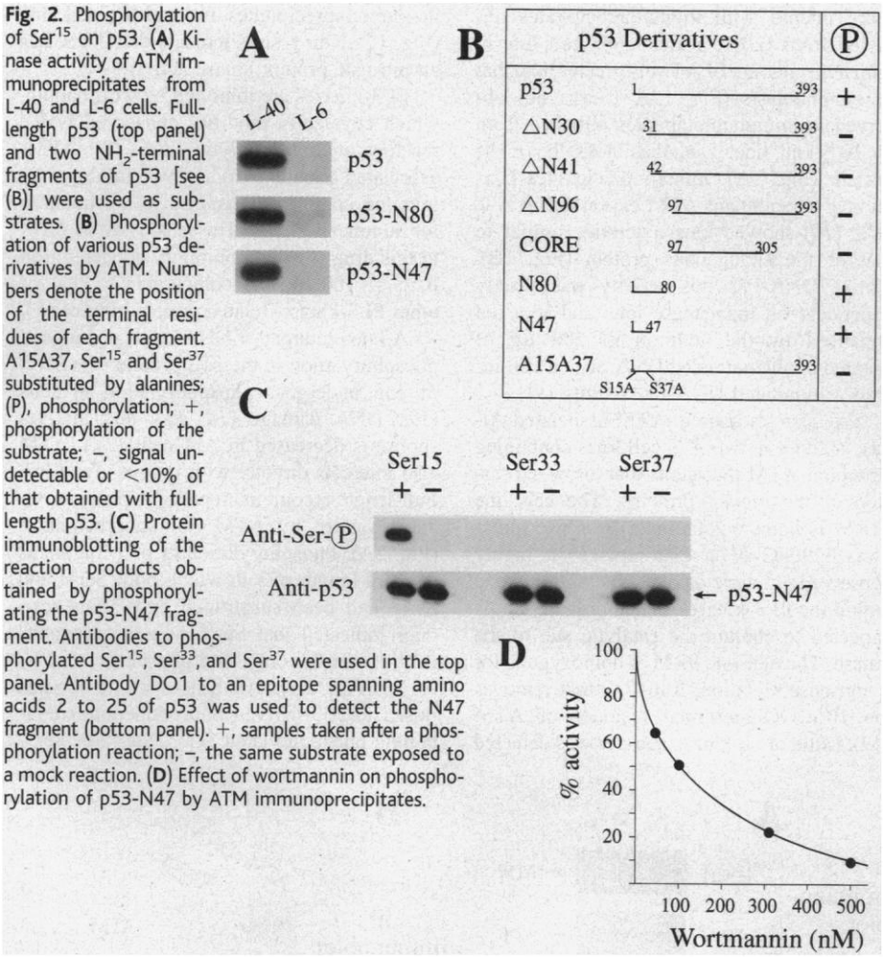

Fig. 2. Phosphorylation of Ser[15] on p53. **(A)** Kinase activity of ATM immunoprecipitates from L-40 and L-6 cells. Full-length p53 (top panel) and two NH_2-terminal fragments of p53 [see (B)] were used as substrates. **(B)** Phosphorylation of various p53 derivatives by ATM. Numbers denote the position of the terminal residues of each fragment. A15A37, Ser[15] and Ser[37] substituted by alanines; (P), phosphorylation; +, phosphorylation of the substrate; −, signal undetectable or <10% of that obtained with full-length p53. **(C)** Protein immunoblotting of the reaction products obtained by phosphorylating the p53-N47 fragment. Antibodies to phosphorylated Ser[15], Ser[33], and Ser[37] were used in the top panel. Antibody DO1 to an epitope spanning amino acids 2 to 25 of p53 was used to detect the N47 fragment (bottom panel). +, samples taken after a phosphorylation reaction; −, the same substrate exposed to a mock reaction. **(D)** Effect of wortmannin on phosphorylation of p53-N47 by ATM immunoprecipitates.

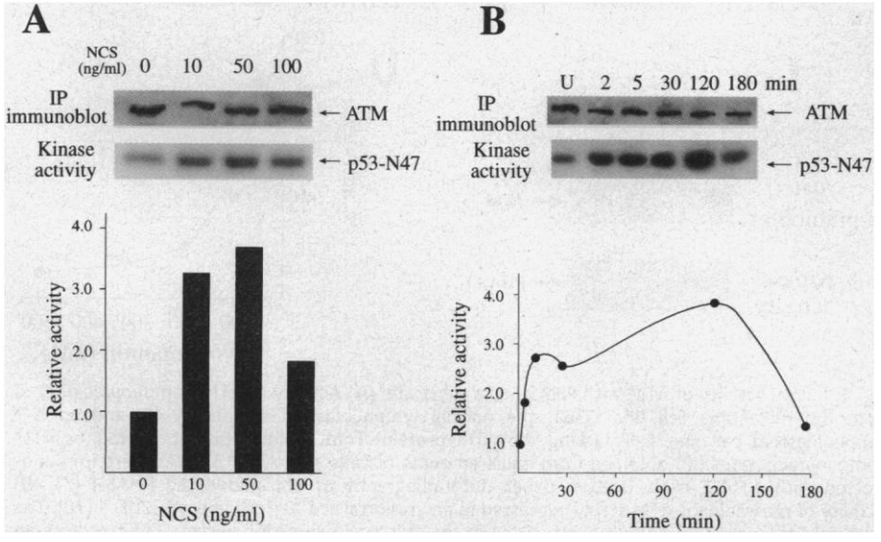

Fig. 3. Increased kinase activity of ATM immunoprecipitates from cells treated with NCS. Kinase reactions were incubated for 3 min. The concentration of nonradioactive ATP in the reaction mix (5 μM) was not rate-limiting. **(A)** ATM was immunoprecipitated from cells 20 min after treatment with various concentrations of NCS. Kinase activity was assayed using the p53-N47 substrate. The histogram presents quantitation of the data from the top panel. Phosphorylation signals were normalized against ATM amounts. **(B)** ATM's kinase activity at various time points after treatment of the cells with NCS (50 ng/ml). U, untreated cells. The curve presents quantitation of the data from the top panel.

(*14, 15, 17*) (Fig. 2C) confirmed that only Ser[15] was phosphorylated by ATM. The effect of wortmannin on this reaction was similar to that obtained with PHAS-I as substrate (Fig. 2D).

These results and the observation that damage-induced phosphorylation of Ser[15] of p53 is slower in A-T cells (*14*) suggest that this residue might be a physiological target of ATM after damage caused by ionizing radiation. This amino acid is not phosphorylated in untreated cells (*15*), so the responsible kinase must be expressed or stimulated by DNA damage. The amount of ATM remains unaltered after treatment of cells with ionizing radiation or radiomimetic drugs (*16, 18*). However, the activity of ATM immunoprecipitated from normal lymphoblastoid cells that had been treated with various doses of the radiomimetic drug neocarzinostatin (NCS) was increased (Fig. 3A). NCS belongs to a family of enediyne antibiotics that intercalate into the DNA and induce double-strand breaks by free radical attack on the deoxyribose moieties in both DNA strands. The biological effects of these compounds are similar to those of ionizing radiation (*19*). This enhancement was observed in five different cell lines (*9*). ATM was activated within minutes after treatment (Fig. 3B). In nine experiments, the maximal enhancement of ATM's activity was 4.4 ± 0.8 times its basal activity. Activated ATM still phosphorylated p53 only on Ser[15] (*9*).

Associated protein kinase activity was shown in immunoprecipitates obtained with other antibodies to ATM (*20*). Our experiments with mutant ATM indicate that the observed activity is an intrinsic property of the ATM molecule. The biological significance of Ser[15] phosphorylation on p53 is highlighted by its de novo occurrence in vivo after DNA damage, and by its delay in A-T cells (*14, 15*). Mutation of Ser[15] leads to a reduction in the ability of p53 to arrest cell growth (*21*), but p53 phosphorylated at this position maintains its ability to bind to DNA (*15*). However, the interaction of p53 with its negative regulator, MDM2, is reduced when Ser[15] and Ser[37] are phosphorylated by DNA-PK, and there is a reduced interaction of p53 with MDM2 after DNA damage in vivo (*15*). MDM2 can both inhibit p53-mediated transcription (*22*) and target p53 for proteasome-mediated degradation (*23*). Phosphorylation of p53 at Ser[15] may relieve either or both of these effects of MDM2 on p53. Our finding thus point to another mechanism by which mammalian cells maintain the stability and integrity of their genome.

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7. Cells (5×10^6) were washed in ice-cold phosphate-buffered saline and lysed in 700 μl of 10 mM tris-HCl (pH 7.5), 150 mM NaCl, 0.2% dodecyl maltoside (Sigma), 5 mM EDTA, 50 mM NaF, 2 mM phenylmethylsulfonyl fluoride, 100 μM $NaVO_4$, and aprotinin (lysis buffer) (2 μg/ml). Freshly prepared cell lysates were incubated for 2 hours at 4°C on a rotator with 10 μl of hybridoma supernatants. Magnetic beads coated with antibodies to mouse immunoglobulin G (50 μl; Dynabeads M-450, DYNAL, Oslo, Norway) were added and the mixture was further rotated at 4°C for 2 hours. Immune complexes were collected using a magnetic stand (DYNAL) and washed three times with lysis buffer. For protein immunoblotting analysis, one-third of this mixture was loaded on an SDS-polyacrylamide gel, which was later blotted onto a nitrocellulose membrane (Nitrocell MFF, Pharmacia) as described (*10*). Immune complexes were washed twice with kinase buffer [50 mM Hepes (pH 7.5), 150 mM NaCl, 4 mM $MnCl_2$, 6 mM $MgCl_2$, 10% (v/v) glycerol, 1 mM dithiothreitol, and 100 μM $NaVO_4$]. Substrate (200 ng to 1 μg), 10 μCi of γ[^{32}P]adenosine triphosphate (ATP), and ATP to a final concentration of 5 μM were added, and the reaction mixtures were incubated at 30°C for 3 to 15 min. After electrophoresis in SDS-polyacrylamide gels, the reaction products were visualized by autoradiography.
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Activation of the ATM Kinase by Ionizing Radiation and Phosphorylation of p53

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The p53 tumor suppressor protein is activated and phosphorylated on serine-15 in response to various DNA damaging agents. The gene product mutated in ataxia telangiectasia, ATM, acts upstream of p53 in a signal transduction pathway initiated by ionizing radiation. Immunoprecipitated ATM had intrinsic protein kinase activity and phosphorylated p53 on serine-15 in a manganese-dependent manner. Ionizing radiation, but not ultraviolet radiation, rapidly enhanced this p53-directed kinase activity of endogenous ATM. These observations, along with the fact that phosphorylation of p53 on serine-15 in response to ionizing radiation is reduced in ataxia telangiectasia cells, suggest that ATM is a protein kinase that phosphorylates p53 in vivo.

Ataxia telangiectasia (A-T) is a rare autosomal recessive disorder characterized by clinical manifestations that include progressive cerebellar ataxia, neuronal degeneration, hypersensitivity to ionizing radiation (IR), premature aging, hypogonadism, growth retardation, immune deficiency, and an increased risk for cancer (*1*). The gene mutated in A-T, *ATM* (ataxia telangiectasia–mutated), encodes a 370-kD protein that is a member of a family of proteins related to phosphatidylinositol 3-kinase (PI-3-K) that have either lipid or protein kinase activity. The subset of this family with the greatest identity to ATM functions in DNA repair, DNA recombination, and cell-cycle control (*2, 3*). Cell lines derived from A-T patients exhibit hypersensitivity to IR and defects in several IR-inducible cell-cycle checkpoints, including a diminished irradiation-induced arrest in the G_1 phase of the cell-cycle mediated by the p53 tumor suppressor gene product (*4, 5*). In response to DNA damage, cells with wild-type ATM accumulate p53 protein and show a subsequent increase in p53 activity, whereas cells with defective ATM show a smaller increase in the amount of p53 protein in response to IR (*4, 6*). Therefore, ATM appears to act upstream of p53 in a signal transduction pathway initiated by IR.

IR induces rapid, de novo phosphorylation of endogenous p53 at two serine residues within the first 24 amino acids of the protein, one of which was identified as Ser^{15} (*7, 8*). Phosphorylation of p53 at Ser^{15} in response to DNA damage correlates with both the accumulation of total p53 protein as well as with the ability of p53 to transactivate downstream target genes in wild-type cells (*8*). Furthermore, phosphorylation of p53 on Ser^{15} in response to IR is diminished in cell lines derived from A-T patients, suggesting that ATM participates in this response (*8*).

The PI-3-K–related protein, DNA-activated protein kinase (DNA-PK) phosphorylates p53 in vitro at two different Ser-Gln motifs, Ser^{15} and Ser^{37} (*9*). However, cells with diminished DNA-PK activity still normally accumulate p53 protein and undergo G_1 arrest in response to IR (*10*). We tested whether ATM might also phosphorylate p53 on Ser^{15} and whether the activity of ATM toward p53 as a substrate is regulated by IR.

Most naturally occurring ATM mutant proteins are unstable (*11*). Because a catalytically inactive ATM mutant is a critical control for in vitro kinase assays, we constructed such a mutant that can be stably expressed. The putative kinase domain of ATM resides in the COOH-terminus of the protein. In related proteins, three critical amino acids within this domain are necessary for phosphotransferase activity (*2, 12*). Thus, a recombinant, FLAG peptide–tagged, wild-type ATM

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