A Proteomic Analysis of Ataxia Telangiectasia-mutated (ATM)/ATM-Rad3-related (ATR) Substrates Identifies the Ubiquitin-Proteasome System as a Regulator for DNA Damage Checkpoints

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This on-line version of this article (available at http://www.jbc.org) contains supplemental Material I and Table I.

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2 The on-line version of this article (available at http://www.jbc.org) contains supplemental Material I and Table I.

3 Abbreviations used are: DDR, DNA damage response; ATM, ataxia telangiectasia-mutated; ATR, ATM-Rad3-related; HU, hydroxyurea; MS, mass spectrometry; siRNA, small interfering RNA; UPS, ubiquitin-proteasome system; Gy, gray; CldU, chlorodeoxyuridine; IdU, iododeoxyuridine.

ATM (ataxia telangiectasia-mutated) and ATR (ATM-Rad3-related) are proximal checkpoint kinases that regulate DNA damage response (DDR). Identification and characterization of ATM/ATR substrates hold the keys for the understanding of DDR. Few techniques are available to identify protein kinase substrates. Here, we screened for potential ATM/ATR substrates using phospho-specific antibodies against known ATM/ATR substrates. We identified proteins cross-reacting to phospho-specific antibodies in response to DNA damage by mass spectrometry. We validated a subset of the candidate substrates to be phosphorylated in an ATM/ATR-dependent manner in vivo. Combining with a functional checkpoint screen, we identified proteins that belong to the ubiquitin-proteasome system (UPS) to be required in mammalian DNA damage checkpoint control, particularly the G1 cell cycle checkpoint, thus revealing protein ubiquitylation as an important regulatory mechanism downstream of ATM/ATR activation for checkpoint control.

DNA damage response (DDR) coordinates cell cycle checkpoints, DNA repair and apoptosis. G1, intra-S, and G2/M checkpoints operate to block cells at different cell cycle stages (1). G1 checkpoint prevents G1 cells from entering S phase with damaged DNA (2, 3), which operates in two distinct phases in response to ionizing radiation (IR): an initial rapid response, typically within 4 h after IR (4) and a sustained response that operates from 4 to 24 h after IR (2). While the sustained G1 arrest is mediated by p53-dependent transcription, the rapid G1 arrest is p53-independent and is poorly understood. While many proteins that regulate intra-S and G2/M checkpoints have been identified, the number of proteins known to participate in G1 checkpoint is very limited.

Central to DDR are the two checkpoint kinases, ATM and ATR, that phosphorylate the effector kinases Chk1 and Chk2 and other downstream effectors (5). ATM/ATR substrates execute DDR directly or indirectly and are the keys for the understanding of this response. The identification of p53, NBS1, BRCA1, Chk1/Chk2, and SMCl as substrates of ATM/ATR has significantly advanced our understanding of DDR (6–12). A thorough understanding of DDR in mammalian cells awaits the identification of new ATM/ATR substrates. Unfortunately, few techniques are available today to systematically identify in vivo substrates of protein kinase.

ATM and ATR kinases preferentially phosphorylate the S/TQ motif as a consensus sequence (13). It has been observed that a phospho-specific antibody raised against a phosphopeptide is not strictly site or sequence specific; it also recognizes other proteins (cross-reacting proteins) with similar sequences flanking the phosphorylation site. It has been recently realized that these cross-reacting proteins could also be substrates of the same kinase (14–17). Thus, the phospho-antibodies can be used to find potential kinase substrates.

EXPERIMENTAL PROCEDURES

See supplemental material I for “Experimental Procedures” (18–20).

RESULTS AND DISCUSSION

To screen for putative ATM/ATR substrates, we used phospho-antibodies raised against known ATM/ATR substrates that recognize pSQ motifs to immunoprecipitate potential new substrates. HeLa nuclear extracts were prepared from cycling cells and from those treated with IR and hydroxyurea (HU), conditions that activate ATM and ATR, respectively. Each lane of Coomassie Blue-stained gels was divided into 12 regions and proteins in each region were identified with mass spectrometry (MS). As shown as an example in Fig. 1a, structure maintenance of chromosomes 1 and 3 (SMC1/3) migrating at 170 and 150 kDa, respectively, are the most abundant proteins recognized by the ATM-pS1981 antibody (21). Many proteins were identified from cycling cells and IR- or HU-treated cells (supplemental Table I). Similar experiments were carried out with antibodies against phospho-BRCA1 (pS1387, pS1423, and pS1457), and the results are summarized in supplemental Table I.

It is possible that some proteins identified using this approach were co-precipitated by association with phosphorylated proteins. To minimize precipitation of associated proteins, we used radioimmunoprecipitation buffer containing high detergent concentration to wash the immunoprecipitates.
extensively to disrupt the weak interactions. Thus, proteins identified by this method represent both potential ATM/ATR substrates and core components of the complex that associate with the substrates and have survived the stringent wash. For example, SMC1 was co-precipitated by its strong interaction with SMC3, the substrate recognized by ATM-pS1981 antibody (Fig. 1, A and B). Since interaction between two protein complexes is usually transient, the identification of protein complexes from different functional categories suggests that one or more proteins in each category are recognized by the phospho-antibodies as potential ATM/ATR substrates, and other core components in each protein complex come along with the substrate.

We used the following criteria to validate the identified proteins as ATM/ATR substrates. First, they must be phospho-proteins; second, they are phosphorylated in an IR/HU-inducible and ATM/ATR-dependent manner; and third, they must contain sequences similar to the SQ peptides used to raise the phospho-antibodies. As shown in Fig. 1B, when the lysate was treated with calf intestine phosphatase (CIP), no band was recognized by ATM-pS1981 antibody by Western blotting, demonstrating that all bands correspond to phospho-proteins. Furthermore, most of the proteins were phosphorylated in an IR- and HU-inducible manner (Fig. 1, B and C). To determine the identity of the protein band and to test whether the phospho-Western blot signal is ATM-dependent, we transfected siRNAs against ATM and the candidate protein that has a similar molecular weight to the protein band identified by immunoprecipitation/MS (supplemental Table 1). The cell lysate was analyzed by Western blotting of duplicate blots with either the phospho-antibody or a specific antibody against the candidate protein. As shown in Fig. 2A, the knockdown of either ATM or ARFBP1 reduced the p480 phospho-signal, suggesting that p480 is ARFBP1 and that this protein is phosphorylated in an ATM-dependent manner; this is confirmed by the reduction of the Western blotting signal of ARFBP1 on the duplicate membrane. Using this approach, we found that ARFBP1, USP34,
KIAA1794, SMC3, TAOK3, and TDP1 are phosphorylated in an IR-and HU-inducible and ATM/ATR-dependent manner. Analyses of amino acid sequences of these proteins reveal that they often contain a stretch of at least 4 amino acid residues or multiple stretches of 3 amino acid residues surrounding the SQ site that are identical to the phosphopeptide antigen, suggesting that these sequences are recognized by the phospho-antibodies employed in this study. Thus, these proteins are most likely to be ATM/ATR substrates (Table 1). The protein band p250 is identified as TRAP220, but its phosphorylation is neither dependent on ATM/ATR nor IR/HU-inducible. Thus, some proteins cross-reacting to phospho-antibodies are not ATM/ATR substrates.

Some of the candidate proteins identified from the immuno-precipitation/MS analysis are not recognized by the same phospho-antibody in Western blots. To demonstrate that they are potential ATM/ATR substrates, we immunoprecipitated these proteins with the phospho-antibody and Western blotted with antibodies specific to these proteins. Using this approach, we found that RFWD3 is phosphorylated in an ATR-dependent manner in response to HU (Fig. 2B), and RIF1 and VCP are phosphorylated in an ATM/ATR-dependent manner in response to IR (Fig. 2C). Substrates validated by this method could be associated proteins, but since they have survived the stringent wash and all contain sequences similar to the peptide antigen, thus it is equally possible that they are kinase substrates. Using both approaches demonstrated in Fig. 2, we validated nine ATM/ATR substrates among our collection of candidates. We conclude that the above approach is capable of finding ATM/ATR substrates.

The large number of potential ATM/ATR substrates identified in the proteomic screen poses a significant challenge for functional characterization. When we categorized the proteins into functional modules based on the known function or functional motifs in these proteins, we found many proteins involved in DDR, DNA repair, chromosomal cohesion, and transcriptional regulation, which are expected from ATM/ATR substrates (supplemental Table 1). We also found that proteins in the ubiquitin-proteasome system (UPS) are overrepresented (Table 1). This suggests the involvement of protein ubiquitylation in DNA damage response, a notion that is well known in yeast and begins to be appreciated in mammalian cells.

The UPS proteins include E3 ubiquitin ligases (Rad18, RNF20, RNF40, RFWD3, ARFBP1, EDD1), BTB domain proteins (ZBTB2 and BTBD12) that may recruit protein degradation targets to E3 ubiquitin ligase complexes (22–26), ubiquitin hydrolases (USP15, USP28, and USP34) (27, 28), a polyubiquitin-binding protein (VCP), and a ubiquitin-like domain containing protein (BAT3) (29, 30). Importantly, these proteins also contain stretches of amino acid sequences flanking the SQ sites that are similar to the phospho-antigens (Table 1), and the number of peptides detected in MS increases when cells are treated with IR or HU, suggesting that they are potential ATM/ATR substrates or at least tightly associated with the substrates. We thus focused our subproteome functional characterization on 10 UPS proteins for their roles in DNA damage response (Fig. 3).

If these candidates are bona fide ATM/ATR substrates, they may participate in regulation of DNA damage checkpoint, DNA repair, or apoptosis. Ideally, we would like to map the precise phosphorylation sites of these candidates and test the loss-of-function mutants for their functions. Unfortunately, it is too labor intensive for a single lab to carry out such analyses on 10 proteins. We decided to examine whether the UPS proteins are required to regulate DNA damage checkpoints.

We carried out intra-S- and G2/M checkpoint assays in HeLa cells depleted of these genes using siRNA. In response to IR, the intra-S checkpoint is activated to mainly suppress late origin firing and is measured by a temporary decrease of nucleotide incorporation following IR. Three days after siRNA knockdown, we irradiated the cells with 10-Gy IR and measured nucleotide incorporation 1 h post-irradiation. Of the 10 genes

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### Table 1

Comparison of phospho-specific antibody epitopes with sequences within the identified cross-reacting proteins (S/T, D/E, and I/L are treated as the same residue in the analysis)

| Protein | ATM, pS1981, EGSQST | BRCA1, pS1387, LSSQSD | BRCA1, pS1457, LTSQKS |
|---------|---------------------|----------------------|----------------------|
| Rad18   | HSQSK               | LSSQSS               | LSSQSS               |
| RNF20   | LSSQSS, LSSQSD     | LSSQSS               | LSSQSS               |
| RNF40   |                     | LSSQSS               | LSSQSS               |
| RFWD3   | WGSQGL, CSSQSS, ESSQSE | LSSQEM, CSSQSS, ESSQSE | LSSQEM, CSSQSS, ESSQSE |
| ZBTB2   | ENSSQF              | SSSQDV, SSSQDY, STSQEP | ESSQSG, NSSQSG YSSQGS, GSSQSK |
| BTBD12  |                     | IVSQQ, LSQSN         | IGSQ, LSQSN         |
| USP15   |                     | LSSQTS, LDSQTR       | LSSQTS, LDSQTR       |
| USP28   |                     | ESSQSG, NSSQSG, QCSQSL, DFSQST, GSSQSS | ESSQSG, NSSQSG, QCSQSL, DFSQST, GSSQSS |
| USP34   | GGSQSS, TGSQSS, QGSQKD, AGSQEF | GGSQSS, TGSQSS, QGSQKD, AGSQEF | GGSQSS, TGSQSS, QGSQKD, AGSQEF |
| VCP     |                     | ESSQSG, NSSQSG YSSQGS, GSSQSK | ESSQSG, NSSQSG YSSQGS, GSSQSK |
| BAT3    |                     | ESSQSG, NSSQSG YSSQGS, GSSQSK | ESSQSG, NSSQSG YSSQGS, GSSQSK |
| KIAA1794|                     | ESSQSG, NSSQSG YSSQGS, GSSQSK | ESSQSG, NSSQSG YSSQGS, GSSQSK |
| SMC3    |                     | ESSQSG, NSSQSG YSSQGS, GSSQSK | ESSQSG, NSSQSG YSSQGS, GSSQSK |
| TAOK3   |                     | ESSQSG, NSSQSG YSSQGS, GSSQSK | ESSQSG, NSSQSG YSSQGS, GSSQSK |
| TDP1    |                     | ESSQSG, NSSQSG YSSQGS, GSSQSK | ESSQSG, NSSQSG YSSQGS, GSSQSK |
| RIF1    |                     | ESSQSG, NSSQSG YSSQGS, GSSQSK | ESSQSG, NSSQSG YSSQGS, GSSQSK |
| TRAP220 |                     | ESSQSG, NSSQSG YSSQGS, GSSQSK | ESSQSG, NSSQSG YSSQGS, GSSQSK |

The identical residues to antibody epitopes are underlined.
tested, six exhibited radio-resistant DNA synthesis significantly above a control siRNA (siVim) transfected cells (Fig. 3A), indicating that these UPS proteins are required to regulate the intra-S phase checkpoint. The G2/M checkpoint is activated to prevent cells with damaged DNA from entering mitosis. We irradiated siRNA-transfected cells with 10-Gy IR and measured the mitotic index 1 h post irradiation. The population of histone H3-pS10 positive cells, which correspond to mitotic cells, was visualized using a microscope. Knocking down 2 out of the 10 genes leads to a profound G2/M checkpoint failure that cells accumulate more than 4 times as many mitotic cells as in control transfected cells (compare >30% with 7.5% relative mitotic index for control transfected cells). Interestingly, siRNA transfection of ZBTB2 causes a marked increase in mitotic index before IR and virtually no further decrease after IR, indicating that ZBTB2 may play an essential role in mitotic progression, which complicates the interpretation of its role in G2/M checkpoint control.

The G1 checkpoint arrests cells with damaged DNA at the G1/S boundary. G1 checkpoint is the least understood checkpoint among the DNA damage checkpoints, at least in part due to the lack of an accurate and sensitive assay. Current G1 checkpoint assay, which measures G1 accumulation by flow cytometry, is unable to accurately measure cell cycle progression immediately after DNA damage induction. We developed an imaging-based G1 checkpoint assay by pulse-labeling cells with two bromodeoxyuridine analogs at various times before and after DNA damage induction. By distinguishing cells that have entered S phase after DNA damage induction from the replicating cells at the time of damage induction, this assay allows for accurate measurement of G1-S transition with a better time resolution. We have validated this assay by measuring the G1 arrest of known checkpoint proteins and confirmed the existence of p53-independent rapid response (within 4 h post-IR) and p53-dependent sustained arrest (after 4 h).4 We used this assay to examine in details whether the UPS genes are involved in both rapid and sustained G1 arrest in U2OS cells. We first pulse-labeled the cells with CldU to mark the starting point and then labeled

4 Y. Wang and J. Qin, submitted for publication.

![Figure 3](image-url)

**FIGURE 3. Requirements of 10 UPS genes for the regulation of intra-S, G2/M and G1 checkpoints.** A, measurements of intra-S checkpoints by radio-resistant DNA synthesis (RDS) of HeLa cells transfected with siRNA against the UPS genes listed. The error bars represent standard deviation of quintuple repeated siRNA transfection experiments. Quintuple samples were measured for each experiment. B, measurements of G2/M checkpoint by reduction of mitotic index of HeLa cells transfected with siRNA. SiRNA transfected cells were irradiated with 10-Gy IR and mitotic index measured after 1-h recovery. Cells grown on coverslips were immunostained with an antibody against histone H3-pS10 to identify mitotic cells. The error bars represent standard deviation of two repeated siRNA transfection experiments. C, measurements of G1 checkpoints by relative S phase entry in U2OS cells transfected with siRNA. Cells were irradiated with 10-Gy IR, and S phase entries between 0–4 h, 4–8 h, and 8–12 h were measured by pulse labeling the cells with CldU at the beginning and IdU at the end of each time intervals. Relative S phase entry was calculated as the ratio of S phase entry after IR to that of cycling cells in 4-h interval. The error bars represent standard deviation of at least two repeated siRNA transfection experiments. D, summary of checkpoint assay results in A–C. Genes whose knockdown caused checkpoint defects are marked in red.
them with IdU 4 h later to mark the end point. Using Cl3U- and IdU-specific antibodies, we scored cells that were Cl3U-negative and IdU-positive as having entered S phase during the 4 h period after IR. We calculated the S phase entry as the percentage of Cl3U-negative and IdU-positive cells among DAPI-stained cells. By comparing relative S phase entry (ratio of S phase entry after and before IR in the 4 h period) between 0–4 h, 4–8 h, and 8–12 h following 10-Gy IR, we evaluated the G1 checkpoint in U2OS cells depleted of USP genes by siRNA transfection. In control siRNA-transfected cells, relative S phase entry is decreased to 60% during the first 4 h after IR as a result of rapid G1 checkpoint activation and is further decreased to and maintained below 20% starting from 4 h after IR. Of the 10 genes tested, 7 genes are required for sustained G1 arrest through p53-dependent transcriptional program as an important mechanism for the rapid G1 arrest. Moreover, the finding that UPS proteins are also required for sustained G1 arrest thus expands its regulators beyond p53-p21 and p53-independent pathways.

As summarized in Fig. 3D, 9 of 10 UPS genes tested are required for at least one cell cycle checkpoint, and 1 gene, ARFBP1, regulates three checkpoints. Our checkpoint assays utilize siRNA pools that are composed of four sequences to maximize knockdown, the possibility of off-target effect cannot be excluded. Nevertheless, the high success rate for identifying checkpoint genes demonstrates that a proteomic screen for ATM/ATR substrates combined with a functional screen is highly effective in selecting protein candidates for further elucidation of the signaling pathways mediated by ATM/ATR. The use of large number of phospho-SQ antibodies to screen for ATM/ATR substrates has revealed an extensive network of proteins in DNA damage response.

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